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Preparation and characterization of MAP kinase transgenic alfalfa lines

Ph.D. Thesis

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I hereby declare that solely I have written this thesis. All the sources used in this thesis are cited and included in the References part. All published results included in this work are approved by co-authors.

In Olomouc …… ……

Miroslava Hrbáčková

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Abstrakt

Strukoviny sú schopné vytvárať symbiotické interakcie s rhizobaktériami, ktoré sú schopné premieňať atmosférický dusík na amoniak, ktorý je následne asimilovaný hostiteľskou rastlinou. Signalizácia pomocou mitogen-aktivovaných protein kináz (MAPK) môže byť do tejto symbiózy zapojená. MAPK kaskády sú jedny z najviac konzervovaných a najlepšie charakterizovaných proteín kinázových signalizačných dráh. U Lucerny siatej (*Medicago sativa* L.) bola identifikovaná SIMK ako MAPK indukovaná soľným stresom a elicitormi. SIMKK je nadradeným aktivátorom SIMK a bolo dokázané, že aktivuje SIMK predovšetkým pri soľnom strese. Jedným z hlavných cieľov tejto dizertačnej práce bola príprava a transformácia konštruktov pre fluorescenčne značené SIMK a SIMKK do rastlinných buniek. Boli využité pokročilé mikroskopické metódy na vizualizáciu a imunolokalizáciu SIMK v živých a fixovaných pletivách a bunkách *M. sativa*. Ďalej bola študovaná nadexpresia SIMK a jej úloha pri raste koreňových vláskov, vytváraní infekčných vlákien, klastrovaní koreňových hľúzok a tvorbe zelenej biomasy.

Prvá časť dizertačnej práce je zameraná na plodinu *M. sativa*. Sumarizuje základné poznatky a popisuje jej biotechnologický potenciál. Táto kapitola sa tiež

zaoberá interakciami strukovín s pôdnymi rhizobaktériami a MAPK identifikovanými v *M. sativa* a v modelovej rastline *Arabidopsis thaliana.*

Nasledujúca kapitola je venovaná príprave a testovaniu fúznych proteínov GFP-SIMK a tagRFP-SIMKK. Expresia fluorescenčne značených SIMK a SIMKK bola overená pomocou tranzientnej transformácie listov *Nicotiana benthamiana*. Na prípravu nových stabilne transformovaných línií lucerny bol použitý vylepšený a účinný transformačný protokol pomocou *Agrobacterium tumefaciens* a somatickej embrogenézy.

Posledná časť dizertačnej práce je zameraná na charakterizáciu a parametre produkcie u transgénnych línií lucerny s geneticky upravenou SIMK po inokulácii *Sinorhizobium meliloti*. U transgénnych línií *SIMKK RNAi* bolo zaznamenané výrazné potlačenie expresie *SIMKK* a *SIMK* génov. Tieto línie sa vyznačujú zníženým rastom koreňových vláskov a nižšou schopnosťou tvoriť infekčné vlákna, a následne tiež koreňové hľúzky. Naopak, konštitutívna nadexpresia GFP-značeného SIMK vyvolala u transgénnej línie rast dlhších koreňových vláskov a vytváranie klastrov infekčných vlákien a hľúzok. Zníženie hladiny *SIMK* a *SIMKK* transkriptov viedlo k redukcii, zatiaľ čo nadexpresia GFP-SIMK znamenala zvýšenie produkcie zelenej biomasy u stabilne transformovaných rastlín *M. sativa*. Tieto výsledky poukazujú na to, že génové úpravy *SIMK* ovplyvňujú rast koreňových vláskov, klastrovanie nodulov a produkciu zelenej biomasy. To poukazuje na významný biotechnologický potenciál tejto proteín kinázy.

Klíčová slova:	*Medicago sativa,* Lucerna siata, SIMK, SIMKK, koreňový vlások, *Sinorhizobium meliloti,* infekčné vlákno, expresia, koreňová hľúzka, CLSM, imunoflourescenčné značenie
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Abstract

Legumes are able to perform symbiotic interactions with rhizobia that are able to convert atmospheric nitrogen into ammonia, which is assimilated by the host plant. Signaling by mitogen-activated protein kinases (MAPKs) seems to be involved in this symbiotic interaction. MAPK cascades are one of the most conserved and best characterized protein kinase signaling pathways. In alfalfa (*Medicago sativa* L.), SIMK was identified as a salt stress- and elicitor-induced MAPK. SIMKK is an upstream activator of SIMK during alfalfa response to the salt stress. One of the main aims of this Ph.D. thesis was to prepare and transform constructs with fluorescently-tagged SIMK and SIMKK to plant cells. Advanced microscopy techniques were used for live-cell imaging and immunolabeling of SIMK in alfalfa tissues. Next, role of overexpressed SIMK was studied in root hair growth, infection thread formation, nodule clustering and shoot biomass production.

The first part of the thesis is devoted to the crop *M. sativa*. It summarizes the current knowledge on this plant and its biotechnological potential. This chapter of thesis also describes legume-rhizobia interactions and MAPKs identified in alfalfa and model plant *Arabidopsis thaliana*.

The following chapter deals with the cloning and characterization of fusion proteins, GFP-tagged SIMK and tagRFP-tagged SIMKK. Expression of fluorescently-

tagged SIMK and SIMKK was checked using transient transformation of *Nicotiana benthamiana* leaves. New stably transformed alfalfa lines have been prepared by the improved and efficient transformation protocol using *Agrobacterium tumefaciens* and somatic embryogenesis.

The last part of the thesis is dedicated to the characterization of production parameters of transgenic alfalfa plants with genetically engineered SIMK after infection with *Sinorhizobium meliloti*. *SIMKK RNAi* lines, showing strong downregulation of both *SIMKK* and *SIMK* genes, revealed reduced root hair growth and lower capacity to form infection threads and nodules. In contrast, constitutive overexpression of GFP-tagged SIMK promoted root hair growth as well as infection thread and nodule clustering. Moreover, *SIMKK* and *SIMK* downregulation led to decrease, while overexpression of GFP-tagged SIMK promoted shoot biomass production. These data suggest that gene engineering of SIMK expression levels affects root hair, nodule and shoot formation patterns in alfalfa. It highlights new biotechnological potential of this protein kinase.

Key words:	*Medicago sativa,* alfalfa, SIMK, SIMKK, root hair, *Sinorhizobium meliloti*, infection thread, expression, nodule, CLSM, immunofluorescent labelling
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CONTENT

1 Aims of the thesis

1. Summary of the recent knowledge and biotechnological potential of alfalfa, legume-rhizobia interactions, and MAPKs.
2. Cloning and transformation of fluorescently tagged SIMK and SIMKK of alfalfa. Live-cell imaging and immunolabeling of SIMK in alfalfa tissues.
3. Role of overexpressed SIMK in root hair growth, nodule clustering and shoot biomass production.

2 Part I – General introduction

Hrbáčková, M.

2.1 Basic characteristics of *Medicago sativa*

Medicago sativa L., also known as alfalfa, "Queen of Forage", or "lucerne", belongs to Fabaceae. Its first cultivated form most likely originates from western Persia. Alfalfa then spread into many regions in Europe, Asia and America (Rashmi *et al.,* 1997; Samac and Temple, 2004). The genus *Medicago* includes both perennial and annual species. Alfalfa is a highly valuable perennial deep-rooted forage legume helping to prevent soil erosion and reduce contamination of surface and ground water (Radović *et al.*, 2009).

Generally, legumes are important forage crops worldwide because they are capable of producing high yields of high quality forage (Albrecht and Beauchemin, 2003). It can be argued that no other family of plants provides a better balance of protein, energy, and minerals in the form of forage for high-producing livestock than the legumes (Beever and Thorp, 1996; Conrad and Klopfenstein, 1988). Perennial legumes have been used historically as a hay or pasture. They provide continuous groundcover for several years, reducing opportunities for water runoff from hillsides and potential loss of soil, N, and P from fields and into the surface water (Albrecht and Beauchemin, 2003; Zemenchik *et al.,* 1996, 1997). Perennial forage legumes also play a role in reducing loss of soil and nutrients from erosion-prone landscapes (Zemenchik *et al.,* 1996, 2002). Moreover, perennial legumes have higher crude protein concentrations than either perennial or annual grasses. They contain relatively high concentrations of organic acids (Albrecht and Beauchemin, 2003; Playne and McDonald, 1966).

Alfalfa is one the most important legume forage crops in the world, mostly because of its high biomass yield, good forage quality, and palatability for ruminants (Lei *et al.,* 2017; Radović *et al.,* 2009). It has relatively vigorous and deep rooting systems helping to prevent soil erosion and reduce contamination of surface and ground water (Radović *et al.,* 2009). *M. sativa* shows high content of proteins, antioxidants, minerals, enzymes (peroxidase, amylase, coagulase, erepsin, invertase and pectinase), and vitamins

A, C, K and E (Bora and Sharma, 2011). Therefore, it serves as favourite animal and livestock feed. In addition, it is used also for biofuel, soil conservation, natural bioremediation, and for the production of pharmaceutical compounds and industrial enzymes (Kineman *et al.*, 2010; Kumar *et al.*, 2018). Importantly, alfalfa is able to fix a large amount of atmospheric nitrogen due to the symbiosis with rhizobia (Doyle and Luckow, 2003; Putnam *et al.*, 2001). This ability allows alfalfa to grow in a wide range of soil types. However, changing climate and microbial pathogens can severely limit these benefits.

Alfalfa has the obligate outcrossing and autotetraploid character (2n = 4X = 32) contributing to the large genetic diversity. So far, it has been quite intensively studied, including abiotic and biotic stress responses employing modern genomic, proteomic, and metabolomic approaches (reviewed by Hrbáčková *et al.*, 2020).

2.1.1 Somatic embryogenesis of alfalfa

Somatic embryogenesis is a regeneration method in which somatic cells are induced under *in vitro* conditions to obtain embryogenic potential. It is the main pathway of regeneration for many plant species (Sangra *et al.*, 2019) and provides an important tool for genetic modifications of plants by *Agrobacterium*-mediated transfer of foreign genes (Rai *et al.*, 2010). Steward *et al.* (1958) and Reinert (1958) first described somatic embryogenesis independently, both on *Daucus carota* suspension cells. Propagation and regeneration of plants via somatic embryogenesis is not suitable for all species (Ammirato, 1983; Bingham *et al.*, 1975; Vasil, 1988), although it can likely occur in all plant species under suitable conditions (von Arnold *et al.*, 2002). It is dependent on genotype and may differ between cultivars (Atanassov and Brown, 1984; Bingham *et al.*, 1975) as well as between genotypes of a cultivar (Kao and Michayluk, 1981). Besides genetic background, also epigenetic factors, such as the pattern of chromatin condensation, DNA methylation, histone post-translational modifications and micro RNAs (miRNAs) are important for somatic embryogenesis (Henderson and Jacobsen, 2007).

It is well known, that alfalfa and other *Medicago* species can be simply regenerated *via* somatic embryogenesis. Several successful *in vitro* regeneration protocols for alfalfa somatic embryogenesis were developed (Bingham *et al.*, 1975; Mitten *et al.*, 1984; Saunders and Bingham, 1972; Shetty and McKersie, 1993). Cultivation of explants

isolated from several organs, such as leaves, stems, hypocotyls, petioles or cotyledons on media supplemented with plant growth regulators can initiate the production of embryos directly from explant (direct somatic embryogenesis) or trigger formation of embryogenic callus (indirect somatic embryogenesis; Dijak and Brown, 1987). Despite the well-studied somatic embryogenesis of alfalfa, the major problems in the efficiency of somatic embryogenesis are connected with lack of fully developed mature somatic embryos and in their subsequent conversion ability to vital plantlets (Lai and McKersie, 1994).

2.1.1.1 Indirect somatic embryogenesis

Alfalfa plants can be regenerated from callus tissue (Saunders and Bingham, 1975) or from cells grown in a suspension culture (McCoy and Bingham, 1977). In general, the whole process of alfalfa somatic embryogenesis consists of several steps. It begins with an explant, such as leaves from well-developed plant nodes, cut into half after gentle surface sterilization. Indirect somatic embryogenesis requires callus formation, which is followed by formation of somatic embryos, embryo maturation, desiccation and subsequent development of plant. Each embryo is created from a single cell. Somatic cells contain the entire set of information to create and complete regeneration of whole functional plant. Secondary embryos can be induced and formed as well.

Endosperm contains storage carbohydrates (starch) and supplies zygotic embryo with natural plant regulators (Lai and McKersie, 1994; Vahdati *et al.*, 2008). However, somatic embryos are devoid of endosperm, therefore addition of plant growth regulators and nutrients to the culture medium is used to help development of these embryos (Amini *et al.*, 2016; Merkle *et al.*, 1995; Sangra *et al.*, 2019; Tichá *et al.*, 2020b). In general, slower growth and regeneration of somatic embryogenesis-originated plantlets is linked to the lower levels of released storage proteins and amino acids in somatic embryos (Lai and McKersie, 1994).

Optimized protocol published by Samac and Austin-Phillips (2006) contains several culture media. The first step is callus induction. A combination of growth regulators, such as auxins and cytokinins, is added into media. Response of legumes to auxins and cytokinins varies significantly depending on the species (Taji *et al.*, 2004). Typical growth regulators using in alfalfa somatic embryogenesis are 2,4-dichlorophenoxyacetic acid (2,4-D; Baker *et al.*, 1994) and kinetin (Blaydes, 1996). Callus is formed when levels of auxin and cytokinin are equal. Callus induction starts

with curling of leaf layer explants. Typical medium used for callus induction is called B5H medium (Gamborg *et al.*, 1968) with Gamborg's vitamins, growth regulators and amino acids. After some weeks, the callus is transferred to the medium for induction and development of somatic embryos, called B50 medium (B5 medium without growth regulators, only with Gamborg's vitamins and amino acids). When embryos are formed, they are transferred to rooting and plant development medium, called MMS medium (medium of Murashige and Skoog, 1962 with Nitsch & Nitsch vitamins). Finally, MS medium (Murashige and Skoog, 1962) without growth regulators, vitamins and amino acids is used for maintenance of *in vitro* regenerated plants. Recently, Sangra *et al.* (2019) developed long-term maintainable somatic embryogenesis system. Traditional protocol always starts from new leaf explants, which is time and money consuming. Moreover, it is associated with contamination by endogenous microorganisms. Protocol for continuous somatic embryogenesis system with enhanced yield of embryos and embryogenic sustainability was developed for Regen-SY hybrid cultivar (Sangra *et al.*, 2019). This hybrid alfalfa Regen-SY cultivar was produced using first generation self-parents from Regen-S (*M. sativa*) and Regen-Y (*Medicago falcata*) research cultivars and released to introduce improved regeneration traits (Bingham, 1991; Sangra *et al.*, 2019).

2.1.1.2 Direct somatic embryogenesis

Direct somatic embryogenesis was induced from leaves, cotyledons or protoplasts of alfalfa (Denchev *et al.*, 1991; Dijak and Brown, 1987; Kao and Michayluk, 1980; Lu *et al.*, 1983). This type of somatic embryogenesis leads to the plant regeneration without callus formation. First, Kao and Michayluk (1980) established plant regeneration from mesophyll protoplasts of alfalfa. They used leaves from fast growing plants under low light intensity for protoplast isolation. The ability of these protoplasts to form embryos and regenerate plants varied considerably from plant to plant within the same cultivar. Lu *et al.* (1983) have used protoplasts from leaves, cotyledons and roots for direct somatic embryogenesis. Dijak and Brown (1987) used mesophyll protoplasts of three alfalfa cultivars (Rambler, Regen-S and Rangelander). Only protoplasts from cultivar Rangelander were suitable for direct embryogenesis while those from cultivars Rambler and Regen-S formed calli. Finally, Denchev *et al.* (1991) described a highly efficient system for direct somatic embryogenesis. They have used leaf sections originating from young trifoliate leaves of *M. falcata* and alfalfa. The medium containing polyethylene glycol was used for shortening of the process. Based on this protocol, Shao *et al.* (2000)

have developed efficient transformation methods for tetraploid lines of *M. falcata* using either direct or indirect somatic embryogenesis on MS medium (first supplemented with 2,4-D and kinetin, then without phytohormones) or B5h medium.

2.1.2 Genetic transformation of alfalfa

The term transformation is used to describe the insertion of foreign molecules (usually DNA) into bacteria, plant cells and fungi (Rivera *et al.,* 2012). F. Griffith discovered transformation in 1928 (Griffith, 1928). He found out that pneumococcal cells could convert from a harmless form to a disease-causing one (Avery *et al.,* 1944; Griffith, 1928). Production of transgenic plants is a routine process in many legume crop species. Transgenes are introduced into plants to bring improved nutritional quality, tolerance to pollutants, resistance to pathogens or abiotic stress. Today, it is possible to transform plants by many transformation techniques (Rivera *et al.,* 2012). Common laboratory methods for genetic transformation are divided into indirect and direct transformation. Indirect methods can be named as biological methods using bacteria and direct methods are physical methods based on the penetration of the cell wall (Rivera *et al.,* 2012). The most popular method for indirect genetic transformation of plants is *Agrobacterium tumefaciens*-mediated transformation. The most popular methods for direct genetic transformations are vacuum infiltration, microinjection, electroporation or biolistic approach (Rivera *et al.,* 2012; Tichá *et al.*, 2020b).

2.1.2.1 *Agrobacterium tumefaciens*-mediated transformation

A. tumefaciens is a gram-negative soil bacterium exhibiting predominantly saprophytic lifestyle. The core of the transformation strategy is a genetic transformation into the plant host genome through DNA fragment called T-DNA (Bourras *et al.,* 2015). *Agrobacterium* strains transfer a single-strand form of T-DNA and virulence (Vir) effector proteins to the plant cells (Gelvin, 2017). Different strains of *Agrobacterium* can infect different types of dicotyledonous plants, but also monocotyledonous plants, yeasts, ascomycetes or basidiomycetes (Gelvin, 2003).

There is a strong research interest to improve alfalfa agronomic traits (Riday and Brummer, 2002). Several studies reported successful *A. tumefaciens*-mediated transformation of alfalfa (Du *et al.,* 1994; Samac, 1995; Shanin *et al.,* 1986; Tohidfar *et al.,* 2013). Shahin *et al.* (1986) performed first successful experiment using co-cultivation of stem sections of alfalfa variety CUF101 with disarmed *A. tumefaciens* strain LBA4404,

and obtained kanamycin-resistant calli. Later, Chabaud *et al.* (1988) successfully transformed *M. sativa* ssp. *varia* using disarmed strains of *A. tumefaciens* LBA4404 and A281. Du *et al.* (1994) tested four different strains of *A. tumefaciens* (three armed strains A281, C58, C58-R1000 and one disarmed strain GV3101) and three alfalfa genotypes. They observed low regeneration of alfalfa after co-cultivation with *A. tumefaciens.* Nevertheless, genotype C2-4 (petiole) cocultivated with *A. tumefaciens* strain (A281) could produce transgenic plants. Samac (1995) tested nine distinct sources of alfalfa germplasm introduced into North America and three disarmed *Agrobacteria* strains. Authors also tested the length of the co-cultivation period affecting transformation frequency. They created transgenic alfalfa plants expressing *Bacillus licheniformis* alpha-amylase and manganese-dependent lignin peroxidase (Mn-P) from *Phanerochaete chrysosporium* using the transformation system based on *A. tumefaciens*. Longer periods of co-cultivation increased the frequency of transformation due to the stronger selection pressure (Austin *et al.,* 1995; Samac, 1995). So far, the most rapid and efficient transformation method is leaf explant co-cultivation with *A. tumefaciens*. Several *Medicago* species, including alfalfa, are able to regenerate through somatic embryogenesis. Thus, the *A. tumefaciens*-mediated transformation can be accomplished using this highly efficient regeneration approach (Austin *et al.,* 1994, 1995; Austin and Ziegelhoffer, 2001; Austin-Philips *et al.,* 1999; Ziegelhoffer *et al.,* 1999). Optimized protocol published by Samac and Austin-Phillips (2006) can easily generate thousands of alfalfa (variety Regen-SY) plants transformed by *A. tumefaciens* strain LBA4404. Trifoliates clonally propagated in growth chamber contained much less bacterial and fungal contaminants. On average, 60 to 80 % of inoculated leaf explants produced somatic embryos and a high number of transgenic plants containing T-DNA developed from them (Samac and Austin-Phillips, 2006). Tohidfar *et al.* (2013) tried to introduce genetic resistance against alfalfa weevil (*Hypera postica*). They have used three commercial alfalfa genotypes (Km-27, Kk-14 and Syn-18) and *A. tumefaciens* strains GV3101, LBA4404 and AGL01. Transformed explants were grown on callus-induction medium and germinated somatic embryos moved to the regeneration medium. All transgenic plants were fertile and some of them showed higher resistance against insect (Tohidfar *et al.,* 2013). Jiang *et al.* (2019) simplified transformation procedure and introduced unified *A. tumefaciens*-mediated transformation protocol for both alfalfa genotype Regen SY4D and *Medicago truncatula* ecotype R108. They used trifoliates or leaflets as explants, sonication to enhance *Agrobacterium* infection and cytokinins in the culture medium to

facilitate shoot regeneration. More than 90 % transformation efficiency was achieved for alfalfa, and relatively lower efficiency of up to 60 % for *M. truncatula* (Jiang *et al.*, 2019).

NEOMYCIN PHOSPHOTRANSFERASE II (*NPTII*) gene that confers kanamycin resistance is the most widespread selectable marker in alfalfa transformation. It was found that concentration of kanamycin over 50 $mg \cdot l^{-1}$ inhibits growth of untransformed calli. Therefore, kanamycin concentrations in the range between 50 and 100 $mg \cdot l^{-1}$ were used for the selection of transformed calli, embryos and root formation (Chabaud *et al.*, 1988). Higher amount of kanamycin, however, inhibits critical stages such as callus development, somatic embryo induction and root formation from somatic embryos. Besides kanamycin, hygromycin or phosphinothricin are also used for selection of transgenic tissues (D'Halluin *et al.*, 1990; Tabe *et al.*, 1995). Transformation of alfalfa with *BAR* gene encoding *PHOSPHINOTHRICIN ACETYL TRANSFERASE* and direct selection on medium containing phosphinothricin increases the frequency of transformation by practically eliminating non-transformed embryos (D'Halluin *et al.*, 1990; Tabe *et al.*, 1995).

2.1.2.2 Transformation by biolistics method

Biolistics, also known as particle bombardment or gene gun technique, is based on the acceleration of high-density carrier particles, covered with genes of interest that pass through the cell walls and leave the DNA inside of cells (Rivera *et al.*, 2012). Many factors can affect stable transformation by this method, including the target tissue, the tissue culture system or the DNA construction (Birch and Franks, 1991; Christou, 1992; Pereira and Erickson, 1994). Pereira and Erickson (1994) have used the embryogenic clone of alfalfa cultivar C2-4 and obtained only seven transformants, which were positive for the *NPTII* gene in PCR test (Pereira and Erickson, 1994). Ramaiah and Skinner (1997) used another approach of biolistic alfalfa transformation when they applied direct delivery of DNA into pollen with plasmid carrying the *β-GLUCURONIDASE* (*GUS*) reporter gene. Male-sterile alfalfa plants were pollinated with biolistically-prepared transgenic pollen in the effort to produce fertile seeds. Incorporation of *GUS* gene by particle bombardment was confirmed by PCR and Southern blot analysis. Later analysis revealed presence of multiple inserts or truncated copies of *GUS* gene. Unfortunately, some lines lost this gene, or numbers of copies decreased after vegetative propagation. Although the reason for the loss of incorporated DNA in the next generations was not

revealed, particle bombardment-based delivery of gene constructs represents an alternative alfalfa transformation strategy for biotechnological purposes (Ramaiah and Skinner, 1997). Finally, Wei *et al.* (2011) transformed leaves and calli chloroplasts of alfalfa. The efficiency of transformation was 1.3 % in the case of callus explants and 2.7 % for leaf explants. PCR and Southern blot analyses revealed presence of *AADA* and *GFP* genes in transgenic chloroplasts.

2.1.2.3 Transformation by electroporation

Electroporation is a transformation method of high potential, because it is simple, highly efficient and fast. It is used to transport biochemical substances like proteins, lipids, RNA or DNA into the host cells (Harrison *et al.,* 1991; Rivera *et al.,* 2012). The principle of this method is enhanced formation of pores in the membrane, caused by electrical field applied to a suspension of plant cells (Bates *et al.,* 1983; Saulis *et al.,* 1991). An electrical field applied to a cellular suspension induces a dipolar moment inside the cells and potential difference across the plasmatic membrane (Barnett and Weaver, 1991; Rivera *et al.,* 2012; Weaver and Chizmadzhev, 1996). The pulse length, duration, number of pulses, electroporation solution, and concentration of plasmid have usually a strong effect on the transformation efficiency of individual cells (Joersbo and Brunstedt, 1990; Kosturkova, 1993; Kubiniec *et al.,* 1990; Weaver and Chizmadzehev, 1996). Choudhary *et al.* (1990) and Harrison *et al.* (1991) successfully electroporated alfalfa protoplasts isolated from suspension cells by chimeric gene consisting of a bean (*Phaseolus vulgaris* L.) *CHALCON SYNTHASE* (*CHS*) promoter fused with bacterial *CHLORAMPHENICOL ACETYLTRANSFERASE* (*CAT*) reporter gene. Later on, Kosturkova (1993) transformed hypocotyl protoplasts from *M. sativa* and mesophyll protoplasts from *Medicago varia* by *pDW$_2$-DNA* using electroporation. Experiments were carried out to identify the electroporation parameters allowing good protoplast viability. Mesophyll and cotyledon protoplasts remained green and healthy after selection on kanamycin (Kosturkova, 1993).

2.2 Biotechnological perspectives of genomic approaches in alfalfa

For several decades, researchers are working to develop improved major crops with better adaptability and tolerance to environmental stresses. Abiotic and biotic stresses are main factors limiting legume production, however, alfalfa (*M. sativa* L.) shows relatively high level of tolerance to drought and salt stress. The identification of

genes that affect legume crop production represents an important aim of current genomic studies (Bevan *et al.*, 2017), and this requires knowledge of their full genomic sequences. The use of modern biotechnology tools is facilitated in alfalfa and its close relative barrel medic (*M. truncatula* Gaertn.), since full genomes were released (Tang *et al.,* 2014). Technologies for sequencing DNA and RNA have undergone revolutionary improvements (Ari and Arikan, 2016). It is known that early after split between monocots and eudicots during the evolution, several whole-genome duplications and triplications occurred in legumes (Severin *et al.,* 2011; Masonbrink *et al.,* 2017). Next-generation sequencing (NGS) is less costly and has a faster turnaround time compared to classical sequencing methods. New NGS platforms, such as the Roche/454 system (Margulies *et al.,* 2005), Illumina platform (Wang *et al.,* 2012), real-time DNA sequencing by Pacific Biosciences (Eid *et al.,* 2009), Oxford Nanopore system (Lu *et al.,* 2016), and Ion Torrent system (Rothberg *et al.,* 2011), were used for sequencing crop and legume genomes. They have had a major impact on plant research, since they help to understand the genome complexity, to look into genomic variations, such as single nucleotide polymorphisms (SNPs) or insertions/deletions (INDELs; Abdelrahman *et al.,* 2018; Valliyodan *et al.,* 2017).

2.2.1 Next generation sequencing for genomics and transcriptomics

Genome sequencing and assembly have been applied to many plant species, including crops. Such genome assemblies serve as common references for alignment with re-sequenced plants (Huang *et al.,* 2012; Schreiber *et al.,* 2018). Large-scale systematic genome sequencing has been carried out in leguminous plants such as *Lotus japonicus* (Sato *et al.,* 2008), *M. truncatula* (release 3.0; jcvi.org/research/medicago-truncatula-genome-database), and *Glycine max* (Schmutz *et al.,* 2010). The genome sequence of alfalfa has been published (Alfalfa Breeder's Toolbox, alfalfatoolbox.org; Chen *et al.,* 2020; Shen *et al.,* 2020), but most of the current transcriptomic studies still rely on the genome sequence alignment of its closest relative *M. truncatula* (genome version *Mt4.*0v1; phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Mtruncatula; Young *et al.,* 2011; Tang *et al.,* 2014). Currently, the most advanced genome sequencing method is NGS. It has become the major tool for the development of new molecular markers and for gene identification (Edwards and Batley, 2010). Together with the rapid development of NGS, the number of plants with completely sequenced genomes has dramatically increased (Van *et al.,* 2013; Le Nguyen *et al.,* 2018; Kersey, 2019). Advantages of NGS

include lower costs and shorter time requirements. The development of NGS technology contributed to the identification of new genes that had evolved by whole-genome duplication and structural variations in chromosomes (Barabaschi *et al.,* 2012; Van *et al.,* 2013). Reference genome sequences of several legume and crop species are now available, and candidate genes of important SNPs can be rapidly and easily identified (Gao *et al.,* 2012; Van *et al.,* 2013; Le Nguyen *et al.,* 2018; Scheben *et al.,* 2019). Alfalfa is an outbred, tetrasomic tetraploid (2n = 4x = 32) with 8 basic chromosomes and a genome size of 800 – 1000 Mbp (Blondon *et al.,* 1994). Genetic and genomic resources have been widely employed in research on *Medicago* species. Genome of closely related barrel medic is often used as a model organism (Zhou *et al.,* 2011). Barrel medic is a diploid species (2n = 2x = 16) with smaller genome (about 550 Mbp; Piano and Pecetti, 2010). Molecular genetics and breeding research was challenging due to lack of a reference alfalfa genome. Chen *et al.* (2020) generated an allele-aware chromosome-level genome assembly for the cultivated alfalfa, which consists of 32 allelic chromosomes by using high-fidelity single-molecule sequencing and Hi-C data. They reported about *de novo* assembled high-quality and chromosome-level haploid genome sequence for a heterozygous alfalfa autotetraploid, cultivar Zhongmu 1. NGS technologies could speed up the discovery of quantitative trait loci (QTLs) and candidate SNPs, which represent common sequence variations among plants and are functionally important. Numerous molecular markers are used in high-throughput genotyping by sequencing (GBS) platforms associated with alfalfa mapping (Hawkins and Yu, 2018), population diversity studies (Herrmann *et al.,* 2018), and genomic selection (Annicchiarico *et al.,* 2016). In the past years, low density linkage maps were constructed on diploid alfalfa (Brummer *et al.,* 1993; Kiss *et al.,* 1993; Echt *et al.,* 1994; Julier *et al.,* 2003). Although several genetic linkage maps have been constructed for tetraploid alfalfa, most of them were framework maps with only few markers (Brouwer and Osborn, 1999; Julier *et al.,* 2003; Musial *et al.,* 2007; Robins *et al.,* 2007; Khu *et al.,* 2013). Li X. *et al.* (2014) have constructed a saturated genetic linkage map of autotetraploid alfalfa by using GBS. They have shown high synteny between linkage groups of alfalfa and barrel medic, and clearly identified translocations between chromosomes 4 and 8, and small inversion on chromosome 1. The high-density linkage maps contained 3591 SNP markers on 64 linkage groups across both maternal and paternal genomes of an autotetraploid alfalfa F_1 population (Li X. *et al.,* 2014).

Genome-wide associated studies (GWAS) represent a modern and powerful strategy that could be used to overcome the limitations of conventional QTL mapping. GWAS map genetic loci in a breeding population, relying on linkage disequilibrium (LD; Liu X. P. *et al.,* 2019). Recently, GWAS have been used in the identification of genetic loci in crop species such as soybean (Hwang *et al.,* 2014), maize (Olukolu *et al.,* 2016), barrel medic (Kang *et al.,* 2015) and alfalfa. Zhang T. *et al.* (2015) evaluated two important features associated with drought resistance, namely drought resistance index (DRI) and relative leaf water content (RWC) under greenhouse conditions in 198 alfalfa cultivars and landraces. These results were then correlated with genomic data obtained through genotyping by sequencing. Subsequent to the QTL mapping approach, GWAS provided identification of fifteen loci associated with DRI and RWC. Markers associated with DRI are located at all chromosomes, whereas markers associated with RWC are located at chromosomes 1, 2, 3, 4, 5, 6, and 7. Co-localization of markers for DRI and WRC were found on chromosomes 3, 5, and 7 (Zhang T. *et al.,* 2015). A GWAS approach using more than 15,000 genome-wide SNPs obtained through genotyping by sequencing was applied to examine forage yield and nutritive value-related traits. Five genes, containing known SNPs aligned to the barrel medic genome, were found as candidates in determining fall dry matter yield (*TUBBY-LIKE PROTEIN*), summer dry matter yield (*E3 SUMO-PROTEIN LIGASE SIZ1, RNA-DEPENDENT RNA POLYMERASE FAMILY PROTEIN*), fall stem weight (*UBIQUITIN-LIKE-SPECIFIC PROTEASE ESD4-LIKE PROTEIN*), and cell wall biogenesis (*NUCLEOTIDE-DIPHOSPHO-SUGAR TRANSFERASE FAMILY PROTEIN*; Sakiroglu and Brummer, 2017). Aiming to find markers for alfalfa forage quality, 154 plants originating from the second generation prepared by the outcrossing of three alfalfa cultivars were subjected to genotyping by sequencing, while their half-sib progenies were phenotyped for forage quality parameters under three different growing conditions. Subsequently, GWAS of SNPs was carried out using barrel medic as a reference genome, confirming a polygenic control of quality traits and indicating a substantially different genetic control of a given trait in stems and leaves (Biazzi *et al.,* 2017). Similar marker-trait association using a GWAS approach identified important alfalfa loci for salt tolerance during germination (Yu *et al.,* 2016). Remarkably, they used 198 different accessions with potential drought tolerance, whereas DNA libraries were sequenced in two lanes of an Illumina Hi-Seq2000 instrument. Identified SNP markers were located on all chromosomes, with the exception of chromosome 3. Several alfalfa loci showed similar genetic locations to the reported QTLs associated with

salt tolerance in barrel medic. The results suggest the similarity of mechanisms controlling salt stress responses in these two species. This study resulted in the identification of 14 genes connected to 23 markers associated with salt tolerance during seed germination. These include *PEROXYGENASE (POG), B3 DNA-BINDING PROTEIN*, and *CPR5 PROTEIN*, which are linked to cuticle wax biosynthesis and ABA signaling (Yu *et al.,* 2016).

Over the last two decades, several methods have been developed that allowed the examination of global transcriptional changes. The most used ones are the hybridization of cDNAs (DNA microarrays) and the deep sequencing of cDNA (RNA-Seq; Schena *et al.,* 1995; Wang *et al.,* 2009; Lardi and Pessi, 2018). RNA-Seq, a massive parallel sequencing method for transcriptome analysis, was developed over ten years ago (Wang *et al.,* 2009). Transcriptomic studies analyze only the transcribed portion of the genome and provides in-depth sequencing coverage and additional qualitative information such as isoform-specific expression (Abdelrahman *et al.,* 2018). In contrast to microarrays, ribosomal RNA (rRNA) does not hybridize to the chip, as homologous probes are not present. In RNA-Seq, the abundant rRNA is removed (Lardi and Pessi, 2018). Originally, transcriptomic studies were based on Sanger sequencing of expressed sequence tags (ESTs) or microarrays, which was used in alfalfa and barrel medic (Aziz *et al.,* 2005; Cheung *et al.,* 2006; Yang *et al.,* 2010). It has also been applied for other legumes such as *G. max* (Le *et al.,* 2012; Ha *et al.,* 2015; Tripathi *et al.,* 2016), *L. japonicus* (Asamizu *et al.,* 2004), and *Cicer arietinum* (Deokar *et al.,* 2011).

Several studies reported on transcriptome sequencing of alfalfa with various coverage. Most recent ones relied on the NGS technologies such as 454 technology (Han *et al.,* 2011) or RNA-Seq (Yang *et al.,* 2011; Li and Brummer, 2012; Liu *et al.,* 2013; O'Rourke *et al.,* 2015). Liu *et al.* (2013) performed *de novo* transcriptome sequencing of *M. sativa* L. subsp. *sativa* using Illumina paired-end sequencing. Plant material included 15 tissue types, and the transcriptome coverage was 5.64 Gbp of clean nucleotides. About 40,433 unigenes were obtained and 1649 potential expressed sequence tags simple sequence repeat markers (EST-SSRs) were annotated by alignment with the following databases: the National Center for Biotechnology Information (NCBI) nonredundant protein (Nr) database, the NCBI non-redundant nucleotide sequence (Nt) database, Swiss-Prot, The Kyoto Encyclopedia of Genes and Genomes (KEGG), the Clusters of Orthologous Group (COG), Translated EMBL (TrEMBL) and the InterPro (Ipr) database

(Liu *et al.,* 2013). RNA-Seq analysis of two alfalfa subspecies, namely *M. sativa* ssp. *sativa* (B47) and *M. sativa* ssp. *falcata* (F56) using roots, nitrogen-fixing root nodules, leaves, flowers, elongating stem internodes, and post-elongation stem internodes resulted in 112,626 unique transcript sequences, which were assembled into the alfalfa Gene Index 1.2 (MSGI 1.2; O'Rourke *et al.,* 2015). Chao *et al.* (2019) used PacBio SMRT technology and identified 72,606 open reading frames (ORFs) including 46,616 full-length ORFs, 1670 transcription factors and 44,040 SSRs. A total of 7568 alternative splicing events and 17,740 long non-coding RNAs supported the feasibility of deep sequencing full length RNA from alfalfa transcriptome on a single-molecule level (Chao *et al.,* 2019). Another approach developed to provide long-read sequencing of transcripts is Oxford Nanopore Technologies®. The MinION device, which was developed by Oxford Nanopore, is a portable apparatus compatible with a PC or laptop (Jain *et al.,* 2016; Lu *et al.,* 2016). Fleming *et al.* (2018) evaluated changes in mRNA in dry soybean seeds with use of MinION-based pipeline technology. Li *et al.* (2019) used MinION-based technology for high-throughput mapping of transgenic alleles in soybean. They rapidly mapped the transgene insertion positions in 51 transgenic soybean plants in a single 1D sequencing run. This method was optimized using a population of soybean lines, but it can be adapted to map the transgenes in any other crops.

2.2.2 Transcriptomic approaches and gene expression modifications

2.2.2.1 Resistance to abiotic stress

Salinity stress interferes with plant growth because it causes two main stressors on plants: hyperosmotic pressure and ion toxicity, especially due to Na^+ (Volkov *et al.,* 2004). High salinity often triggers an increase in cytosolic Ca^{2+}, reactive oxygen species (ROS), abscisic acid (ABA) and mitogen activated protein kinase (MAPK) signaling (Ovečka *et al.,* 2014; Mittler and Blumwald, 2015). These activated signal molecules affect plant transcriptomes by regulating transcription factors (Xiong *et al.,* 2002; Zhu, 2002). One of the basic strategies in plant stress responses is the accumulation of water-soluble compounds of low molecular weight, such as betaines, polyols, sugars and amino acids (Chen and Murata, 2002). These compounds accumulate to high concentrations under water or salt stress and protect plants via ROS detoxification and membrane integrity maintenance (Bohnert and Jensen, 1996). For example, glycinebetaine (GB) is a particularly effective protectant against abiotic stress (Chen and Murata, 2008), and

accumulates rapidly in plants exposed to salt, drought, and low temperature stresses (Rhodes and Hanson, 1993).

Previous studies have shown that overexpression of stress-related genes caused enhanced tolerance of alfalfa to the salinity stress (Luo *et al.,* 2019b). Li H. *et al.* (2014) successfully targeted *CHOLINE OXIDASE* (*CODA)* cDNA derived from *Agrobacterium globiformis* to alfalfa chloroplasts under the control of the strong stress inducible *SWEETPOTATO PEROXIDASE ANIONIC 2* (*SWPA2*) promoter (Kim *et al.,* 2003). Such transgenic alfalfa plants exhibited increased tolerance to oxidative, drought, and salt stress. Because salinity also causes cellular ionic imbalances, the Na^+/H^+ antiporter in the plasma membrane (SOS1 SALT OVERLAY SENSITIVE 1) and tonoplast (NHX2 – SODIUM/HYDROGEN EXCHANGER 2) can maintain higher K^+/Na^+ ratios in the cytoplasm as a protection against sodium toxicity (Fukuda *et al.,* 1999; Xia *et al.,* 2002; Zhang *et al.,* 2014). Moreover, the expression of foreign genes, such as *TaNHX2* (*Triticum aestivum NHX2*), *AhBADH* (*Artiplex hortensis hortensis BETAINE ALDEHYDE DEHYDROGENASE*), *SsNHX1 (Suaeda salsa NHX1),* and *GmDREB1* (*G. max DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 1*), can increase salt tolerance in transgenic alfalfa plants (Zhang *et al.*, 2012). As such, Zhang *et al.* (2014) transformed the exogenous gene *SeNHX1* (*Salicornia europaea NHX1*) into alfalfa using *Agrobacterium*-mediated transformation. This enhanced tolerance to salt stress, which was manifested by improved photosynthesis and membrane stability. Another attempt to improve salt tolerance in alfalfa was reported by Jin *et al.* (2010) using transformation with the soybean *DREB* ortholog, *GmDREB1*, under the control of *Arabidopsis* stress-inducible *RD29A* (*RD – RESPONSIVE TO DESICCATION*) promoter. Ion leakage, chlorophyll fluorescence, total soluble sugars, transcript level of *Δ1-PYRROLINE-5-CARBOXYLATE SYNTHASE* (*P5CS*), and free proline contents were-correlated with the higher salt tolerance of transgenic lines (Jin *et al.,* 2010). Wang *et al.* (2014) generated and characterized transgenic alfalfa plants with heterologous expression of *AtNDPK2* (*NUCLEOSIDE DIPHOSPHATE KINASE 2*) under the control of oxidative stress inducible *SWPA2* promoter. These transgenic plants showed increased tolerance to oxidative, high temperature, salt and drought stresses. Such enhanced tolerance was mediated by activation of ROS scavenging, enhanced activity of NDPK2 enzyme, improved protection of membrane integrity, and increased proline accumulation (Wang *et al.,* 2014).

First studies on drought responses of alfalfa started in the 1990s (Laberge *et al.,* 1993; Luo *et al.,* 1991, 1992). Metabolite profiling and proteomic approaches identified soluble sugars, amino acids and proteins that respond to drought in leaves and nodules of alfalfa variety Magali (Aranjuelo *et al.,* 2011). Simultaneously, Kang *et al.* (2011) have shown systematic analysis of two alfalfa varieties, Wisfal and Chilean, with different tolerance/sensitivity to the drought stress. They have identified many genes involved in adaptation to the drought stress, including genes encoding transcription and regulatory factors, or genes involved in the biosynthesis of osmolytes and antioxidants. Knowledge of such genes can help in breeding programs. A number of microRNAs have been used to improve various crop species via genetic engineering (Macovei *et al.,* 2012; Zhou and Luo, 2013; Aung *et al.,* 2015). Researchers also characterized microRNAs and their target genes that respond to hypoxia, wounding, heat or oxidative stress (Zhao *et al.,* 2007; Budak *et al.,* 2015). Recent study by Arshad *et al.* (2017) suggested that overexpression of microRNA156 (*miR156OE*) is an emerging tool to improve drought tolerance of alfalfa since it silenced *SPL13i* (*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 13*) leading to reduced water loss and enhanced stomatal conductance and photosynthetic assimilation. Another study proposed roles of *miR156OE* and *SPL13i* in heat stress tolerance since plants carrying these constructs showed increased antioxidant levels (Matthews *et al.,* 2019). As found by NGS, plants possessing *miR156OE* exhibited broad enhancement in gene expression, including genes involved in nodulation, root development and phytohormone biosynthesis (Aung *et al.,* 2017). Taking together, miR156 can improve drought or heat stress tolerance in alfalfa, at least partially by silencing *SPL13* (Matthews *et al.,* 2019; Feyissa *et al.,* 2019).

RNA-Seq analysis was utilized for alfalfa transcriptome profiling in order to study molecular mechanisms underlying frost (Song *et al.,* 2016), salinity (Postnikova *et al.,* 2013; An *et al.,* 2016; Lei *et al.,* 2018), drought (Arshad *et al.,* 2018), fall dormancy (Zhang S. *et al.,* 2015) and resistance to aluminium (Liu W. *et al.,* 2017), lead (Xu B. *et al.,* 2017) or waterlogging (Zeng *et al.,* 2019). For example, genes encoding membrane proteins and proteins involved in hormonal signal transduction or ubiquitin-mediated proteolytic pathways contribute to the freezing adaptation mechanisms in alfalfa (Song *et al.,* 2016). Using high-throughput sequencing technology, Postnikova *et al.* (2013) have demonstrated that salinity stress affects a variety of alfalfa genes. Among the most affected ones were genes of known function, such as *DIHYDROFLAVONOL*

REDUCTASE (*DFR)*, transcription factor *MYB59*, *SUGAR TRANSPORTER ERD6-like 16* (*ERD – EARLY RESPONSE TO DEHYDRATION*), and *INOSITOL-145-TRISPHOSPHATE 5-PHOSPHATASE* (*IP5P2*). This study revealed that 86 transcription factors responded to salinity stress; among them are those belonging to GRAS, ARR, JUMONJI, and MYB families that were preferentially upregulated in the tolerant alfalfa cultivar (Postnikova *et al.,* 2013). Alfalfa fall dormancy is determined by proteins involved in auxin (e.g. AUXIN-INDUCED PROTEIN 5NG4*)* and ethylene signaling (ethylene responsive TF *RAP2-11)* and carbohydrate transport (ERD6-LIKE PROTEIN; Zhang S. *et al.*, 2015). Genes encoding proteins such as BETA-AMYLASE, ETHYLENE RESPONSE FACTOR (ERF), CALCINEURIN B-LIKE (CBL) INTERACTING PROTEIN KINASES (CIPKs), GLUTATHIONE PEROXIDASE (GPX), and GLUTATHIONE S-TRANSFERASE (GST) are among those important for waterlogging stress resistance in alfalfa (Zeng *et al.,* 2019).

Plant damage caused by saline stress is usually divided into three categories: high pH damage, osmotic shock, and toxic cation stress. Variation in pH of nutrient solution significantly affected growth of alfalfa seedlings with the optimal pH values in the range between 5.0 and 6.0, as estimated by length and fresh weight of roots, hypocotyls, epicotyls, first leaf petioles, and leaf blades (Köpp *et al.*, 2011). Alfalfa is a saline-alkaline stress-tolerant species (Zhu, 2001; Wong *et al.*, 2006; Gong *et al.*, 2014; An *et al.*, 2016). An *et al.* (2016) performed transcriptomic analysis of whole alfalfa seedlings treated with saline-alkaline solutions using ion torrent sequencing technology to study changes in the gene expression pattern. This method detects hydrogen ions that are released during DNA polymerization. DEG profiles were obtained and annotated using two methods. Firstly, generated reads were mapped to barrel medic, which has a sequence that is highly homologous to alfalfa. Secondly, functional annotations of assembled unigenes were performed using BLASTX search against the Swiss-Prot databases of barrel medic, thale cress, and soybean. Gene ontology analysis revealed 14 highly enriched pathways. Specific responses of peroxidases, the expression level of *RUBISCO*, and flavonoids indicated antioxidant capacity as one of the main mechanisms behind the saline-alkaline stress tolerance in alfalfa (An *et al.*, 2016). Another study provided a comprehensive transcriptome analysis of alfalfa roots under prolonged ABA treatment (Luo *et al.*, 2019a). Sequences were assembled for many differentially expressed isoforms (DEIs) and were analyzed for their potential role. DEIs regulated by ABA were mainly involved

in transcriptional regulation, plant immunity, plant hormone signal transduction, and anti-oxidative defense.

Nevertheless, these studies were mainly focused on genotype-specific stress mechanisms. Functional and structural genomics studies are fundamental for the understanding of plant biology. Access to high-quality genome and transcriptome sequences is important to perform studies of this kind. Recently, the third-generation sequencing technology PacBio RSII has emerged as a unique method for constructing full-length transcripts (Dong *et al.,* 2015; Nakano *et al.,* 2017). PacBio RSII is an ideal tool for whole genome sequencing, targeted sequencing, RNA-Seq, and epigenetic characterization. This technique allows the sequencing of single DNA molecules in real-time (SMRT) without amplification by PCR (Dong *et al.,* 2015). Using PacBio RSII, Luo *et al.* (2019b) studied salt stress as a major environmental factor that affects alfalfa development and production (Zhang S. *et al.,* 2015). They have constructed the first full-length transcriptome database of alfalfa root tips treated with mannitol (a non-ionic osmotic stress) and NaCl (an ionic osmotic stress), which provided evidence that the response to salinity stress includes both osmotic and ionic components. They have found 8,016 mannitol-regulated DEGs and 8,861 NaCl-regulated DEGs. These DEGs are involved in signal transduction, transcriptional regulation, anti-oxidative defense, and signal perceptions (Luo *et al.,* 2019b).

2.2.2.2 Resistance to biotic stress

Plants are subjected to a wide range of environmental stresses. Attacks by various pathogens, such as bacteria, fungi, oomycetes, nematodes, or herbivores are classified as biotic stresses. Plants have no choice to escape, so they developed various mechanisms protecting them against environmental cues (Gull *et al.,* 2019). Many pathogens can be problematic for alfalfa production.

Weeds affect the quality of alfalfa hay. An annual parasitic weed infesting alfalfa is dodder belongs to the *Cuscuta* species. Another poisonous weed for alfalfa is oleander from the *Nerium oleander* species. Therefore, producers choose to grow weed-free alfalfa. Insects and pests cause a significant reduction in the yield and quality of forage. Most dangerous are spotted alfalfa aphid (*Therioaphis maculate* B.), alfalfa weevil (*Hypera postica* G.), pea aphid (*Acrythosiphon pisum* H.), potato leaf hopper (*Empoasca fabae* H.), and blue alfalfa aphid (*Acyrthosiphon kondoi* S.) (Liu *et al.,* 2008). Larvae

stadium of the weevil is considered most damaging due to skeletonization of leaves, and consequently reducing forage yield and quality (Chandra, 2009). Modern biotechnological approaches, e.g. somatic hybridization, embryo rescue, or in-planta gene transfer, can be used for interspecific hybrids and shifting of the traits. One of the hybrid was prepared by the polyethylene glycol (PEG) mediated protoplast fusion of *M. sativa* and *M. falcata* (Mendis *et al.,* 1991; Weeks and Rommens, 2008). McCaslin *et al.* (2002) genetically transformed alfalfa with *Bt* (*Bacillus thruringiensis*) gene to prove this effective strategy. Strizhov *et al.* (1996) showed high resistance of alfalfa against weevil and beet armyworm when expressing *Cry1C* encoding a *B. thruringiensis δ-ENDOTOXIN* as compared to the wild type. Another study revealed that alfalfa could be resistant to weevil by expressing the synthetic *Cry3a* gene (Tohidfar *et al.,* 2013).

Alfalfa production has been dramatically impacted by pathogens. Since most of the alfalfa pathogens are the same as pathogens of barrel medic, it is expected that barrel medic can serve as a tool for searching resistance genes for many diseases of alfalfa (Wu *et al.,* 2016; Yang *et al.,* 2008). Fungus *Colletotrichum trifolii* cause anthracnose, one of the destructive diseases in alfalfa. It starts with stem and leaf lesions, continue with root and crown rot, and results in plant death (Yang *et al.,* 2007). Defense responses were observed against *C. trifolii* in *M. truncatula* and other *Medicago* species (Torregrosa *et al.,* 2004). *C. trifolli* is an agent of a highly destructive and prevalent foliar disease, (Annicchiarico *et al.,* 2015), which can cause up to 30 % decrease in alfalfa yield (Yang *et al.,* 2008). Recognition of this pathogen and induction of response in alfalfa are understudied and need further characterization by gene cloning techniques. *C. trifolii* races 1 and 2 were identified in North America (1979), race 2 in 1982, and race 4 in Australia and the USA in 2003 and 2006. Two dominant genes, *An1* and *An2* for the races 1, and 2, control resistance to *C. trifolii* in alfalfa (Mackie *et al.,* 2007, Tesfaye *et al.,* 2007). Yang *et al.* (2008) showed that map-based cloning of *RCT1* (*RESISTANCE TO C. trifolli RACE 1*) gene for R protein encoding wide spectrum anthracnose resulted in host resistance against *Colletotrichum*, and also helped to understand translational research progress from *M. truncatula* to alfalfa plants. Another study tested alfalfa for fungal *ENDOCHITINASE GENE* (*ECH4)* including resistance and antifungal activity. The chitinase activity was several times higher in transgenic alfalfa (Tesfaye *et al.,* 2005).

Plants show sophisticated defense responses to pathogens. Disease resistance mechanisms in plants after encountering a pathogen have been well-described (Roumen,

1994; Zipfel, 2014; Rubiales *et al.*, 2015). Plant infection is facilitated by effector molecules produced by pathogens, which can overcome the first line of plant defense, such as pathogen-associated molecular pattern (PAMP) triggered immunity, whereby a plant perceives conserved molecules through the use of a pattern recognition receptors, and triggers a downstream responses (Zipfel, 2014). On the other hand, specific plant resistance (R) proteins have been evolutionarily co-evolved and can provide protection against specific pathogen effectors (Jones and Dangl, 2006; Singer *et al.*, 2018). Nowadays, genes encoding R proteins are widely manipulated for introducing and enhancing plant resistance to a specific pathogen (Rubiales *et al.*, 2015).

Plant defense-related peptides are composed of five main groups: proteases, α-amylase inhibitors, lectins, chitinases and polyphenol oxidases (Fürstenberg-Hägg *et al.*, 2013). Singer *et al.* (2018) report about transgenic approaches involving the heterologous gene expression with anti-pathogenic or anti-bacterial properties, such as *AGLUL* encoding β-1,3-GLUCANASE (Masoud *et al.*, 1996), *IOMT* – ISOFLAVONE-O-METHYLTRANSFERASE (He and Dixon, 2000), *LF* – encoding LACTOFERRIN (Stefanova *et al.*, 2013) and *RS* – encoding RESVERATROL SYNTHASE (Hipskind and Paiva, 2000).

Transcriptomic studies contributed to the knowledge of alfalfa resistance to nematodes, aphids and strips. Alfalfa is the host of several important nematode species, especially stem nematode (*Ditylenchus dipsaci*), cyst nematode (*Heterodera* spp.), root lesion nematode (*Pratylenchus* spp.) and root-knot nematode (*Meloidogyne* spp.). These nematodes are an economic thread to alfalfa production (Griffin, 1990; Hafez and Sundararaj, 2009). Postnikova *et al.* (2015) have used RNA-sequencing to study alfalfa-root-knot nematode interactions. They have performed root transcriptomic analysis of resistant (cv. Moapa 69) and susceptible (cv- Lahontan) alfalfa cultivars infected by root-knot nematode *Meloidogyne incognita.* LRR and NB-ARC DOMAIN DISEASE RESISTANCE PROTEIN (Medtr3g023030.1), RECEPTOR-LIKE PROTEIN (Medtr5g087320.1) and DISEASE RESISTANCE PROTEIN (TIR-NBS-LRR class, Medtr0277s0020.3) were up-regulated in the resistant cultivar, while susceptible one showed their down-regulation (Postnikova *et al.*, 2015). Aphids are major insect pests of alfalfa, consuming sap from phloem tissue, leading to plant death. Aphids are highly mobile, have high reproductive rates and are able to damage plants. Tu *et al.* (2018) performed a transcriptomic analysis of two alfalfa cultivars (resistant Zhongmu 1 and

susceptible Soca) differing in aphid resistance. Genes involved in salicylic acid biosynthesis represented an important defense mechanism in both cultivars. The alfalfa resistance against aphids was mainly determined by induction of genes involved in linoleic acid synthesis important for jasmonic acid and flavonoid biosynthesis (Tu *et al.*, 2018).

Another introducer influencing alfalfa growth and development is leaf rust caused by *Uromyces striatus. U. striatus* belongs to the *Pucciniaceae* family (Webb and Nutter, 1997). Environmental factors, such as leaf wetness, humidity, temperature, or light are crucial for rust infection and outbreak in alfalfa (Webb and Nutter, 1997). With the increase of NGS and another genomic tools, marker-assisted selection (MAS) has become a popular approach for developing plant cultivars with desirable characteristics. Alfalfa is an outcrossing species, QTL analysis in F1 population uses single dose allele (SDA) markers (Yang *et al.,* 2018). Adhikari and Missaoui (2019) identified eight QTLs associated with leaf rust in alfalfa.

Generally, the most frequently appearing pathogens are bacteria and fungi belonging to Ascomycetes and Basidiomycetes. Improved fungal disease resistance is the critical trait in alfalfa persistence (Volenec *et al.,* 2002). Considerable declines in alfalfa production have been observed mostly due to root infections leading to root diseases caused by the bacterial wilt (*Clavibacter michiganensis* subsp. *insidiosus*), Verticillium wilt (*Verticillium alfalfae*), Phytophthora root rot (*Phytophthora medicaginis*), Fusarium wilt (*Fusarium oxysporum*), and the foliar disease anthracnose caused by *Colletotrichum trifolii* (Annicchiarico *et al.,* 2015; Singer *et al.,* 2018). There are also other diseases caused by *Phoma sclerotioides, Phoma medicaginis varieties,* and *Sclerotinia trifoliorum* (Rubiales *et al.,* 2015). Alfalfa varieties resistant to these diseases have been obtained by common breeding methods over decades (Toth and Bakheit, 1983; Elgin *et al.,* 1988; Pratt and Rowe, 2002). García *et al.* (2014) proposed that the use of SNAKIN-1 PEPTIDE (SN1) could improve alfalfa tolerance to virulent fungal pathogens. SN1 was isolated from a crude cell wall preparation of potato (*Solanum tuberosum*) tubers (*St*N1) (Lopez-Solanilla *et al.,* 1998). It is cysteine-rich peptide, which could be a component of constitutive defense barriers (Segura *et al.,* 1999). A second snakin peptide (*St*N2) with 30 % acid identity was isolated from potato tubers (Berrocal-Lobo *et al.,* 2002). Three independent transgenic lines carrying the *CaMV35S:MsSN1* construct showed

significantly lower amounts of infected leaves than wild type plants when treated by *C. trifolii* and with the oomycete *P. medicaginis* (García *et al.*, 2014).

2.3 Legume-rhizobial symbiosis

Many plant species are able to manage beneficial interactions with a wide range of microorganisms. The most and best-studied symbiotic interactions are those between legumes and nitrogen-fixing rhizobial bacteria, and between plants and arbuscular mycorrhizal fungi (Oldroyd, 2013). Nitrogen is a major essential element for all living organisms. It is a main constituent of cellular macromolecules, such as nucleic acids and proteins, and part of low-molecular mass compounds like amines, vitamins and chlorophylls (Stambulska and Bayliak, 2020).

Fabaceae is the third largest family of flowering plants. Diverse plant species of this family developed during their evolution symbiotic interactions with nitrogen-fixing soil bacteria collectively called rhizobia (e.g. *Bradyrhizobium* or *Sinorhizobium*) which can reduce atmospheric dinitrogen (N_2) into ammonium (NH_3) in specialized organs, the so called root nodules (Wang *et al.,* 2018). Host legumes and rhizobia show a strong specificity, presumably because of their coevolution (Stambulska and Bayliak, 2020). This relationship provides nutrient benefits for both partners, plant provides carbon sources for bacteria, and bacteria supply NH_3 for plant growth (Clúa *et al.,* 2018; Dupont *et al.,* 2012; Halbleib and Ludden, 2000). Two symbiotic partners have to be compatible with each other to establish effective symbiosis (Brencic and Winans, 2005; Clúa *et al.,* 2018, Wang *et al.,* 2018). The successful establishment of legume-rhizobial symbiosis can increase plant biomass and crop yield and help to enrich nitrogen in the soil (Clúa *et al.,* 2018; Dupont *et al.,* 2012). There are several agronomically important legumes performing symbiosis with rhizobia, such as common bean (*P. vulgaris*), pea (*Pisum sativum*), alfalfa (*M. sativa*), soybean (*G. max*) or lentil (*Lend culinaris*) (Clúa *et al.,* 2018; Dupont *et al.,* 2012).

Compatibility of both symbiotic partners depends on mutual recognition through chemical signals released from the host and from rhizobia (Brencic and Winans, 2005; Clúa *et al.,* 2018, Wang *et al.,* 2018). Specialized secondary metabolites (e.g. flavonoids) produced by legume roots are attractive to rhizobia. Rhizobia adhere to the cell wall of the root hair (Bais *et al.,* 2006; Brencic and Winans, 2005; Li *et al.,* 2016; Liu *et al.,* 2017; Liu and Murray, 2016). Flavonoids secreted from roots are important in the initiation of

symbiosis with rhizobia (Cooper, 2004, 2007; Janczarek *et al.,* 2014; Mus *et al.,* 2016). They activate bacterial transcription factors and nodulation (Nod) factors which trigger the expression of nodulation genes (*nod* genes) (Gibson *et al.,* 2008; Janczarek *et al.,* 2014; Liu and Murray, 2016; Long, 2001). Nod factors and exopolysaccharides trigger curling of root hairs and formation of infection threads filled with rhizobia. These infection threads penetrate towards dividing cells of inner cortical tissue, which form nodule and invading bacteria are internalized to the nodule cells and form symbiosomes (Jones *et al.,* 2007).

2.3.1 Classification of rhizobial strains

Rhizobia are medium-sized, rod-shaped, gram-negative bacteria. They represent a genetically diverse and physiologically heterogenous group of bacteria (Somasegaran and Hoben, 2012). In the past, the classification of rhizobial strains in different species was based on the legume they have nodulated. Following this criterion, Dangeard (1926) described three fast-growing species *Rhizobium phaseoli*, *Rhizobium trifolii* and *Rhizobium meliloti* nodulating *Phaseolus, Trifolium* and *Melilotus*, and Buchanan (1926) described the slow-growing species *Rhizobium japonicum*, nodulating *G. max.* In 1980s, the genus *Rhizobium* contained five species and was included in the family *Rhizobiaceae* (Conn, 1942). *Rhizobiaceae* contained several old genera, such as *Alcaligenes*, which was by Conn (1942) reclassified as *Agrobacterium*. Later, numerical taxonomy was introduced into the taxonomy of rhizobia (Graham, 1963). This technique used computers for comparison of a substantial number of phenotypic characters, spectra of C and N resources, metabolic features, or growth conditions, such as pH, temperature or salinity ranges (Graham, 1963; Moffet and Colwell, 1968; Mannetje, 1967). In the 1980s-1990s, numerical taxonomy was widely applied for investigating phenotypic similarities among rhizobial strains. Jordan (1984) published new list of *Rhizobiaceae*, which contained genera *Rhizobium, Agrobacterium, Bradyrhizobium* and *Phyllobacterium*. Chen *et al.* (1988) described fast-growing soybean rhizobium, called *Sinorhizobium*. Over last decades, several approaches have been used to address the taxonomy of *Rhizobia* (Suneja *et al.,* 2017). The best taxonomic consensus showed to be polyphasic taxonomy based on combination of genotypic, phenotypic and phylogenetic data. Strains sharing a high level of similarity can be divided using this approach (Vandamme *et al.,* 1996). Genotypic data involve information obtained from DNA, RNA, restriction-fragment-length polymorphism, DNA hybridization or PCR DNA finger printing. The phenotypic data

involve biochemical and physiological traits (Stackebrandt *et al.,* 2002). Conserved housekeeping genes were used for deeper analysis. These genes include *nod* genes and nitrogen fixation genes (*nif* and *fix* genes, or *x* genes) (Suominen *et al.,* 2001). Another approach used in rhizobia classification is multilocus sequence analysis (MLSA) methodology. The major focus of this analysis is identification of prokaryotic organisms and the delineation of species for the taxonomy of microorganisms (Azevedo *et al.,* 2015). Another approach is mass spectrometry (Jia *et al.,* 2015). The most recent innovation in rhizobial taxonomy has been the whole-genome sequencing (Rashid *et al.,* 2015).

About 770 genera covering more than 19,500 plant species have been recognized in the family *Fabaceae*, also known as *Leguminosae* (LPWG, 2017). Rhizobia are generally classified according to a host-based system and their ability to nodulate members of the *Leguminosae* (Suneja *et al.,* 2017). The *Leguminosae* is divided into three subfamilies: *Caesalpinoideae, Mimosoideae* and *Papilionoideae*. The *Caesalpinoideae* includes mostly woody plants and nodulation is limited to very small number of species. The *Mimosoideae* consists mostly of woody species and nodulation occurs at a higher frequency. Nodulation is typical for *Papilionoideae* because most genera in this subfamily are nodulated. However, molecular studies, especially the extensive phylogenetic analysis of plastid *matK* genes, showed that monophyletic *Mimosoideae* and *Papilionoideae* are nested within a paraphyletic *Caesalpinioideae* (LPWG, 2017). Therefore, a new system combined morphological data and suggested division into six subfamilies in *Leguminosae, namely Caesalpinioideae, Cercidoideae, Detarioideae, Dialioideae, Duparquetioideae* and *Papilionoideae* (LPGW, 2017). Nodulation is typical for subfamilies *Papilionoideae* and *Caesalpinioideae* (Doyle, 2016).

Based on biogeographic and genetic studies, rhizobial diversity depends of four factors such as environmental selection for survival, long evolutionary history, lateral transfer of symbiotic genes and host selection for nodulation (Wang, 2019). Currently, all the symbiotic nitrogen-fixing bacteria belong to the phylum *Proteobacteria*, within the classes *Alphaproteobacteria* (α-rhizobia), *Betaproteobacteria* (β-rhizobia) and perhaps also *Gammaproteobacteria* (γ-rhizobia). The most common group are α-rhizobia (Suneja *et al.*, 2017; Wang, 2019), which are distributed in 16 genera of seven families: *Agrobacterium, Allorhizobium, Ensifer* (formerly *Sinorhizobium*), *Neorhizobium, Pararhizobium, Rhizobium* and *Shinella* in the family *Rhizobiaceae*; *Aminobacter, Phyllobacterium* and *Mesorhizobium* in *Phyllobacteriaceae; Bradyrhizobium* from

Bradyrhizobiaceae; *Microvirga* and *Methylobacterium* in *Methylobacteriaceae; Ochrobactrum* in *Brucellaceae; Devosia* in *Hyphomicrobiaceae;* and *Azorhizobium* in *Xanthobacteraceae* (Suneja *et al.,* 2017; Wang, 2019). Nevertheless, the complete list of rhizobial species is constantly updated and recorded in the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.cict.fr).

2.3.2 Nod factors

Nod factors are specific rhizobial lipochitooligosaccharides and *nod* genes control their production. They are signaling molecules essential for bacterial invasion and initiation of the nodule formation in the root cortex (Dupont *et al.,* 2012; Gourion *et al.,* 2015; Long, 2001; Ribeiro *et al.,* 2015). Without them rhizobia cannot enter legume roots (Cooper, 2007). They form oligomers consisting of usually four or five β-(1,4)-linked N-acetyl-glucosamine residues with attached fatty acyl chain of varying length and varying degrees of unsaturation at the nonreducing terminus (Debellé *et al.,* 2001; Janczarek *et al.,* 2014). Different rhizobial species produce various Nod factors. For example, each species of *Rhizobium* has a specific set of *nod* genes that make the Nod factors specific to the host plant due to determination of length of the lipochitooligosacccharide skeleton (Cooper, 2007). Rhizobia possess common and specific *nod* genes. The group of *nod* genes (*nod*ABC) encodes the core Nod structure typical for all rhizobial species (Bonaldi *et al.,* 2010; Debellé *et al.,* 2001). The *nod*ABC genes are usually assembled in an operon (Long, 1996). *NodA* gene encodes an acetyltransferase, *nodB* encodes a deacetylase, and *nodC* encodes an *N*-acetyl-glucosaminyltransferase (Bonaldi *et al.,* 2010). Another group of *nod* genes (*nod*PQ, *nod*EF, *nod*X, *nod*H) shows a strong species specifity (Franche *et al.,* 2009). Genes encoding enzymes involved in the Nod factor synthesis and genes of symbiotic nitrogen fixation are called *nif* and *fix* genes. They are located on a symbiotic plasmid (pSym) (e.g. *S. meliloti*) or grouped in a large chromosomal symbiotic island (e.g. *B. japonicum*) (Franche *et al.,* 2009; Janczarek *et al.,* 2014). The substituents of Nod factors can also strongly affect their stability in the rhizosphere and protect against degradation by plant chitinases or other glycosyl hydrolases (Schultze and Kondorosi, 1998; Staehelin *et al.,* 2000).

The flavonoid-activated NodD proteins are LysR-type transcription factors and mediate induction of *nod* gene expression (Long, 1996). Such *Nod* gene expression is mediated through binding of NodDs in a complex with a flavonoid to the conserved DNA motifs (*nod* boxes) upstream of the *nod* operons (Fisher *et al.,* 1988; Rostas *et al.,* 1986).

Direct binding of rhizobial Nod factors to the LysM domains of the plant receptor complex leads to subsequent events (Broghammer *et al.,* 2012) such as Ca^{2+} oscillation and activation of downstream singaling pathway (Janczarek *et al.,* 2014; Limpens *et al.,* 2003). The synthesis of EARLY NODULINS (ENODs) is induced and Ca^{2+} CALMODULIN-DEPENDENT PROTEIN KINASE (CCaMK) affects nodule primordia formation and establishment of symbiosis. Downstream, two GRAS-type transcription factors, NODULATION SIGNALING PATHWAY 1 (NSP1) and NSP2 are required (Madsen *et al.,* 2010; Oldroyd *et al.,* 2011; Oldroyd and Downie, 2008; Suzaki *et al.,* 2015). A negative regulation of *NOD* genes has been reported. The No1R protein binds to *NOD* promotors and negatively controls their expression in the presence of luteolin from *S. meliloti* (Cren *et al.,* 1995).

2.3.3 Flavonoids as infection signals

Flavonoids are low-molecular weight secondary metabolites that are produced by plants. They possess fifteen-carbon skeleton consisting of two benzene rings biosynthesized by phenylpropanol pathway (Liu and Murray, 2016). Secondary modifications like methylation, hydroxylation and glycosylation can strongly affect flavonoid function, mobility and solubility in the plant and the soil (Dixon and Steele, 1999; Winkel-Shirley, 2001). Isoflavonoids are typical for legumes. Despite massive production of diverse flavonoids by legumes, only specific subsets have function in nodulation. Roots secrete nodulation-specific flavonoids into the rhizosphere, at the root surface and inside infection threads, where they induce expression of *nod* genes (Chovanec and Novák, 2005; Kape *et al.,* 1992; Peters *et al.,* 1986; Redmond *et al.,* 1986; Subramanian *et al.,* 2007; Zuanazzi *et al.,* 1998). Flavonoids play important roles in legume-rhizobium symbiosis. They serve as chemoattractants for compatible species of rhizobia and primary plant signals that regulate expression of several rhizobial genes (Liu and Murray, 2016; Oldroyd *et al.,* 2011). Compatible rhizobia can respond to plant flavonoids by elicitation of quantitative and qualitative composition of these compounds in root exudates (Cooper, 2004; Lawson *et al.,* 1996). Some studies showed increased quantities of naringenin, daidzein, isoliquiritigenin and liquiritigenin in root exudates of *P. vulgaris* after treatment with compatible rhizobia (Bolaños-Vásquez and Werner, 1997). Different flavonoids can have distinct roles in the nodulation process of *M. truncatula* inoculated by *S. meliloti* (Zhang *et al.,* 2009). Rhizobia can degrade plant flavonoids to derivatives and phenolic metabolites (Brencic and Winans, 2005).

Plant flavonoids activate bacterial NodD proteins, which respond to different sets of flavonoids, e.g. the daidzein and genistein from soybean, and induce *NOD* gene expression in *B. japonicum*. At the same time, daidzein prevents production of Nod factors in the *S. meliloti*, which responds positively to the flavone luteolin (Gibson *et al.*, 2008; Peck *et al.*, 2006). The isoflavonoids cumesterol and medicarpin have been reported to negatively control synthesis of Nod factors by *S. meliloti* (Zuanazzi *et al.*, 1998). Flavonoids can protect the nitrogen-fixing rhizobia from oxidative stresses during rhizobial infection and plant colonization (Cooper, 2004). There is also evidence that some flavonoids might be involved in the accumulation of auxin in cortical cells, which is important for nodule development and organogenesis (Skorupska *et al.*, 2017; Subramanian *et al.*, 2007; Wasson *et al.*, 2006).

2.3.4 Other rhizobial compounds involved in legume-rhizobia interactions

There are several other compounds secreted by rhizobia, which can be involved in successful progression of root colonization and development of nodules (Cooper, 2007; Downie, 2008). These compounds include hopanoids, indole-3-acetic acid (IAA), hydrogen peroxide (H_2O_2), nitric oxide (NO), quorum sensors, bradyoxetin, and lumichrome (Janczarek *et al.*, 2015).

Hopanoids are pentacyclic triterpenoid lipids widely distributed in both gram-negative and gram-positive bacteria with function in membrane reinforcement conferring resistance to several environmental stresses (Cooper, 2007; Janczarek *et al.*, 2015; Kannenberg *et al.*, 1995). For bradyrhizobia is typical that hopanoids and their derivatives are present in amounts detectable by chromatographic and spectrometric analyses. Other fast growing rhizobia does not contain detectable amounts of hopanoids (Kannenberg *et al.*, 1995).

Some rhizobial strains are also able to synthetize IAA (Prinsen *et al.*, 1991, Theunis *et al.*, 2004). The production of IAA in *Sinorhizobium* sp. NGR234 is controlled by flavonoids and NodD proteins (NodD1, NodD2, SyrM2). It was expected that an IAA-deficient mutant will produce lower IAA amounts, but Theunis *et al.* (2004) suggested that synthesis of this compound by the microsymbiont did not play a significant role in symbiosis.

H_2O_2 and NO play important signaling roles in early stages of symbiotic interactions and nodule formation (Boscari *et al.*, 2013, Puppo *et al.*, 2013). H_2O_2 controls

infection process and differentiation of rhizobial bacteria into bacteroids. NO is an inhibitor of nitrogenase activity and N_2 fixation in nodules (Boscari *et al.,* 2013; Meilhoc *et al.,* 2011). The balance between ROS and NO levels seems to be important in the redox regulation during nodulation (Puppo *et al.,* 2013).

Regulation of gene expression pattern in response to the change in the bacterial population density is affected by small and diffusible autoinducers called quorum sensors (Rinaudi-Marron and González, 2013). The most common signals of this type are *N*-ACETYL HOMOSERINE LACTONES (AHLs) (Downie and González, 2008). Bradyoxetin is a non-AHL quorum sensor of a two component regulatory system in *B. japonicum*. It has ability to repress *NOD* genes when the bacterial population grows up (Loh *et al.,* 2002). Moreover, *S. meliloti* is able to produce lumichrome, a riboflavin degradation product. Lumichrome enhances root respiration and improves alfalfa growth before initiation of the nitrogen fixation (Matiru and Dakora, 2005a,b; Phillips *et al.,* 1999).

2.3.5 Legume inoculation

Symbiosis between rhizobium and legume plants requires a mutual recognition of both partners. Recognition starts when flavonoids are recognized as specific inducers of *NOD* genes in rhizobia (Peters *et al.* 1986; Subramanian *et al.* 2007). The early steps in the invasion of barrel medic (*M. truncatula*) and alfalfa roots by *S. meliloti* are characterized by the mutual exchange of signals that allow the bacteria enter the plant root hair cells (Jones *et al.,* 2007).

Alfalfa derived flavonoid luteolin stimulates binding of an active form of NodD1 to an *S. meliloti NOD*-box promoter, and thereby activation of downstream *NOD* genes transcription (Peck *et al.,* 2006). Genome of *S. meliloti* contains two other genes for NodD proteins – NodD2, which is activated by as-yet-unpurified plant compounds, and NodD3, which does not require plant-derived compounds for activation of genes from *NOD* box promoters (Perret *et al.,* 2000). Any of these NodD proteins can start nodulation of alfalfa with *S. meliloti* and flavonoids from non-host plants can inhibit the transcription of *NOD* genes (Peck *et al.,* 2006). Several *NOD* genes encode enzymes responsible for production of Nod factors (Oldroyd and Downie, 2004). One of the earliest plant responses to the matching Nod factor structure is an increase of intracellular Ca^{2+} in root hairs, followed by strong calcium oscillations (Oldroyd and Downie, 2004), and modifications of root

hair cytoskeleton (Cárdenas *et al.,* 2003; de Ruijter *et al.,* 1999; Sieberer *et al.,* 2005; Timmers *et al.,* 1999). Calcium spiking and transcript induction are dependent on phospholipid signaling pathway (Charron *et al.,* 2004). Microtubules and actin filaments work together in coordinated manner. Actin cytoskeleton is important for root hair growth (Ringli *et al.,* 2002) and is reorganized by the polymerization of G-actin monomers to the new filaments through the ACTIN RELATED PROTEIN 2/3 (ARP2/3) complex (Smith and Oppenheimer, 2005). The ARP2/3 complex depends also on SCAR/WAVE complex (Miyahara *et al.,* 2010; Yokota *et al.,* 2009). Morphologically, polarized root hair growth shows deformation, such as swelling of the root hair tip caused by Nod factors (**Figure 1**; de Ruijter *et al.,* 1999; Heidstra *et al.,* 1994). Curled root hairs entrap rhizobia (Esseling *et al.,* 2003; Gage, 2004) which enter root hair via specific thin tubular structure called infection thread (IT). Nuclei of root hairs are relocalized, which is accompanied by changes of cytoskeletal organization (Genre *et al.,* 2005). The ITs elongate into the root cortex of plant host (**Figure 1**). Microscopic analysis of fluorescently tagged bacteria in ITs showed that these bacteria are actively dividing (Gage, 2004). *S. meliloti* produces the exopolysaccharides succinoglycan (also known as exopolysaccharide I, EPSI) and galactoglucan (EPSII), which promote IT formation. Succinoglycan is more efficient than galactoglucan concerning IT development in alfalfa (Glazebrook and Walker, 1989; Pellock *et al.,* 2000).

Cytokinin- and the Nod-factor-dependent reinitiation of the cell cycle are involved in directing ITs to the plant cortex. Cortical cells initiate cell divisions and form nodule primordia. ITs grow toward the nodule primordia and release rhizobia into the dividing cells in the nodule. Cells in the primordium give rise to a persistent nodule meristem and nodules gradually expand in their size (Foucher and Kondorosi, 2000; Timmers *et al.,* 1999). Internalized rhizobia are surrounded by a plant membrane and perform nitrogen fixation inside nodules (Oldroyd and Downie, 2008). In legume plants, indeterminate or determinate type of nodules can be formed. Indeterminate nodules initiate from inner cortex and pericycle and they have a sustained nodule meristem originating from the third cortical layer. Barrel medic and alfalfa are examples of indeterminate legumes, which form nodules with such persistent meristem (Xiao *et al.,* 2014). Mature indeterminate nodules have different nodule zones: I – the apical meristem, II – the invasion zone, III – the nitrogen-fixing zone and IV – the senescence zone (**Figure 1**; Timmers *et al.,* 1999). Determinate nodules initiate from the outermost one or two layers of cortical cells and do

not maintain a nodule meristem. Such nodules usually proliferate, differentiate and senesce synchronously (Mergaert *et al.*, 2006). Example of determinate legume is *L. japonicus* (Calvert *et al.*, 1984). Development of both types of nodules is controlled by plant hormones (Oldroyd, 2013).

The nodule provides good conditions for effective nitrogen fixation (Lin *et al.*, 2020), which is a highly energy-dependent process. It requires at least 16 molecules of ATP for every two molecules of NH_3 synthetized. Nif proteins involved in nitrogenase synthesis also function as regulatory molecules. NifH is a dinitrogenase reductase, or Fe-protein. NifD and NifK are α- and β-subunits of dinitrogenase and form functional complexes with the FeMo cofactor. Prosthetic groups contain 4Fe-4S clusters covalently bound to the FeMo protein bridging the α- and β-subunits. The 4Fe-4S group is linked to the Fe-protein (Dixon and Kahn, 2004; Khan *et al.*, 2010; Newton, 2007). NifA regulates expression of *nif* genes, and is regulated by oxygen.

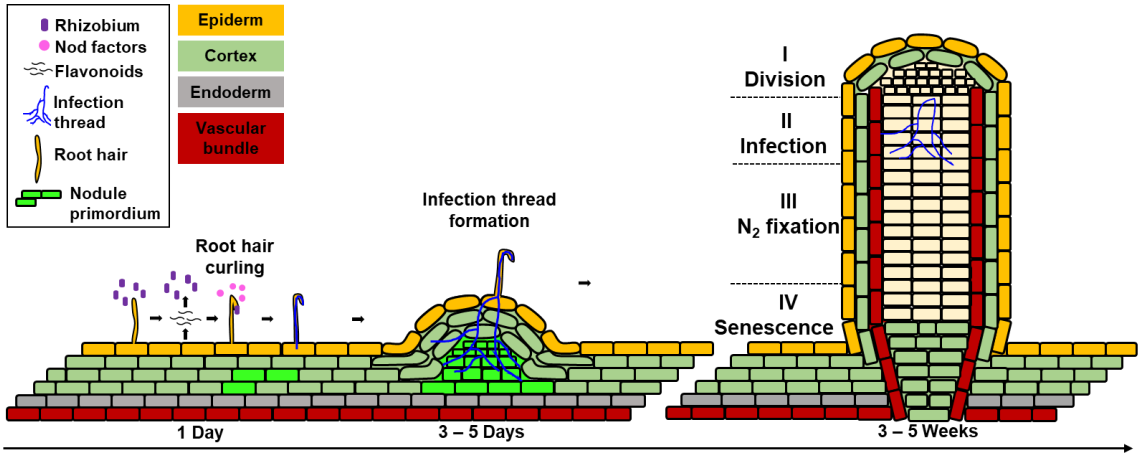

Figure 1 Symbiotic signaling in the legume-rhizobial interaction. The process of bacterial infection and nodule development. A mature indeterminate nodule contains: I – a division zone, II – an infection zone, III – a nitrogen fixing zone and IV – a senescent zone. Adapted from Berger *et al.* (2019) and Wang *et al.* (2018).

Nodule development is locally regulated by feedback between plant and rhizobia. The total number of nodules on roots is controlled by a systemic mechanism called autoregulation of nodulation (Delves *et al.*, 1986; Lin *et al.*, 2020; Reid *et al.*, 2011) or by long-distance communication from shoots. Both the local and the systemic mechanisms are regulated by phytohormones. The host plant is affected by two mechanisms including direct synthesis of phytohormones by rhizobia or indirect manipulation of the phytohormonal balance in the plant by bacterial Nod factors

(Ferguson and Mathesius, 2014). In this respect, cytokinin and auxin are the two major phytohormones that help to coordinate nodulation process. Overall, auxin and cytokinin are important for cortical cell proliferation and differentiation followed by nodule development. In many developmental processes, cytokinin and auxin interact with each other and regulate each other's metabolism, signaling and transport (El-Showk *et al.*, 2013). Cytokinin signaling is essential for the induction of nodule morphogenesis (Oldroyd *et al.*, 2011) and local accumulation of auxin can promote nodule development (Liu *et al.*, 2018). Cytokinin signaling pathway is also important for nodulation in *M. truncatula* and *L. japonicus* (Liu *et al.*, 2018). Gain-of-function mutant in the cytokinin receptor gene *LOTUS HISTIDINE KINASE 1* (*LHK1)* of *L. japonicus* activated spontaneous nodule formation (Tirichine *et al.*, 2007). Loss-of-function mutant of *LHK1* and its ortholog *CYTOKININ RESPONSE 1* (*CRE1)* in *M. truncatula* blocked nodule formation, but bacterial infection still worked (Murray *et al.*, 2007; Plet *et al.*, 2011). *CRE1*-dependent change in polar auxin transport is a direct result of cytokinin signaling, which is suppressed by Nod factors and rhizobia (Oldroyd *et al.*, 2011; Plet *et al.*, 2011; van Noorden *et al.*, 2006). Auxin regulates many important developmental processes in plants (Teale *et al.*, 2006). Inoculation of white clover with rhizobia induced a rapid and local downregulation of *GH3::β-GLUCURONIDASE* (GUS), which is an auxin responsive reporter construct (Mathesius *et al.*, 1998). These induced changes are highly significant because auxin transport inhibitors can trigger nodule organogenesis (Hirsch *et al.*, 1989). Transcript abundance of the auxin-responsive genes *MtIaa9* (*PUTATIVE AUX/IAA PROTEIN* 9) and *MtArf16a* (*AUXIN RESPONSE FACTOR 16a*) is increased in *M. trunctatula* infected by *S. meliloti*. Mutant *arf16* showed reduction of infection events (Breakspear *et al.*, 2014). A gaseous hormone ethylene negatively regulates nodulation in most legumes (Lin *et al.*, 2020). Moreover, ethylene and cytokinin balance is important for feedback loop regulating nodule formation (van Zeijl *et al.*, 2015). Also levels of active gibberellins must be precisely coordinated to assure correct signaling in nodulation (Maekawa *et al.*, 2009). Finally, jasmonic acid, brassinosteroids and abscisic acid suppress nodule formation and development (Liu *et al.*, 2018).

Several small signaling molecules such as CLE (CLAVATA (CLV)/EMBRYO SURROUNDING REGION (ESR)-RELATED PROTEIN) peptides mediate systemic autoregulation of nodulation (Delves *et al.*, 1986; Lin *et al.*, 2020; Reid *et al.*, 2011). They also play roles in the regulation of cell division and differentiation (Concha and Doerner,

2020; Hirakawa and Sawa, 2019; Ito *et al.*, 2006). CLE peptides move as long-distance signals from roots to shoots where specifically interact with shoot receptors. One of them is leucine-rich-repeat receptor SUPER NUMERIC NODULES (SUNN) in *M. truncatula* (Schnabel *et al.*, 2005), which negatively autoregulate the nodule number. Otherwise, C-terminally ENCODED PEPTIDES (CEPs) regulate an independent systemic pathway in *M. truncatula*, that positively promote rhizobial infections and nodule number (Huault *et al.*, 2014; Imin *et al.*, 2013; Laffont *et al.*, 2020; Mohd-Radzman *et al.*, 2016). Root competence for nodulation is controlled also by the microRNAs like miR2111 that is produced upon activity of the receptor COMPACT ROOT ARCHITECTURE 2 (CRA2) in shoots. CRA2 positively affects root nodulation as systemic regulation signal (Gautrat *et al.*, 2020).

2.4 Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are one of the largest group of phospho-transferases, catalyzing phosphorylation of appropriate protein substrates on serine and threonine residues. MAPK pathways represent universal signal transduction modules in all eukaryotes, including mammals, plants and yeasts (Ichimura *et al.*, 2002; Zanke *et al.*, 1996; Xu C. *et al.*, 2017). These protein phosphorylation cascades mediate the intracellular transmission and amplification of extracellular signals for induction of cellular responses. A typical MAPK cascade consists of at least three sequentially acting serine/threonine kinases, namely MAPK kinase kinase (MAPKKK, MAP3K), MAPK kinase (MAPKK, MAP2K) and MAPK, each subsequently phosphorylating, and hence activating, downstream kinase (**Figure 2,** Lewis *et al.*, 1998; Madhani and Fink, 1998; Schaeffer and Weber, 1999; Colcombet and Hirt, 2008; Raja *et al.*, 2017; Šamajová *et al.*, 2013b). MAPKs are mostly present in the cytoplasm and nucleus (Danquah *et al.*, 2015; Gupta and Chakrabarty, 2013; Pitzschke *et al.*, 2009; Seguí-Simarro *et al.*, 2005; Sheikh *et al.*, 2013; Wang Z. *et al.*, 2014).

2.4.1 MAPK cascades in plants

MAPK pathway is usually triggered by the activation of a MAPKKK after ligand-based stimulation of plasma membrane receptor. The MAPKKK then activates a downstream MAPKK by phosphorylation of serine or threonine residues in the S/T-X_5-S/T (X as any amino acid) motif in the activation loop. After activation, MAPKK acts as a dual-specifity kinase, which phosphorylates T-X-Y motif of MAPK in the activation loop between subdomains VII and VIII of its catalytic domain (Jagodzik *et al.*, 2018;

Hettenhausen *et al.,* 2014; Rodriguez *et al.,* 2010). Last member of cascade is MAPK, a serine/threonine kinase that can activate a broad spectrum of proteins in the nucleus or cytoplasm, as well as other kinases, enzymes or transcription factors (Rodriguez *et al.,* 2010). In general, the specifity of MAPK substrates is affected by the presence of MAPK docking motif. The interaction between MAPKs and MAPKKs require a "D-domain" [or D-site, D-box, D-motif] located <100 amino acids upstream (in the N-terminal part) from the phosphorylation site (Bigeard and Hirt, 2018; Biondi and Nebreda, 2003; Ubersax and Ferrell, 2007) and a complementary CD-domain in its cognate MAPK (Dóczi *et al.,* 2012; Šamajová *et al.,* 2012). Besides docking motifs, MAPK specifity can be altered also by adaptors or scaffold proteins, MAPK substrates, protein tyrosine phosphatases or type 2C phosphatases (Bhattacharyya *et al.,* 2006; Good *et al.,* 2011; Šamajová *et al.,* 2012; Zeke *et al.,* 2009). So far, little is known about scaffolding in the context of plant MAPK signaling. Scaffold role has been proposed for two MAPKKKs, namely oxidative stress activated MAPKKK in alfalfa (MsOMTK1) and its *Arabidopsis* ortholog MEKK1. MEKK1 directly interacts with AtMPK4, AtMPK5 and AtMPK13 (Ichimura *et al.,* 1998). MsOMTK1 directly interacts with MsMMK3 (Nakagami *et al.,* 2004). Both OMTK1 and MEKK1 have likely scaffolding roles in the MAPK pathways activated by oxidative stress (Nakagami *et al.,* 2004, 2006).

Inactivation of MAPKs is equally important in establishing physiological equilibrium in living cells. This inactivation is carried out via dephosphorylation of the MAPK activation motif by specific phosphatases (Brock *et al.,* 2010; Camps *et al.,* 2000; Carrasco *et al.,* 2014; Mishra *et al.,* 2006). Protein tyrosine phosphatases (PTPs), dual-specifity (Ser/Thr and Tyr) phosphatases (DSPs), protein phosphatase of type 2C (PP2C) and serine/threonine phosphatases (PSTPs) (Andreasson and Ellis, 2010; Bartels *et al.,* 2010) belong to them. Phosphatase function is essential in order to control intensity and duration of MAPK activation (Tsuda *et al.,* 2013). Moreover, the abundance and activity of such phosphatases are often regulated by respective MAPKs they dephosphorylate, in the form of feedback mechanism (Bartels *et al.,* 2010; González Besteiro and Ulm, 2013). The MP2C, a wound-induced alfalfa PP2C is a negative regulator of MAPK pathway. Stress-induced MAPK (SIMK) and stress-activated MAPK (SAMK) in alfalfa are activated by wounding and MP2C directly inactivates SIMK, but not SAMK (Meskiene *et al.,* 2003).

Although the mechanism of plant MAPK activation is consistent, the kinetics of activation is variable. The kinetics of MAPK activation depends on the upstream signaling events leading to MAPK activation, mechanism of deactivation (Bigeard and Hirt, 2018; Gómez-Gómez and Boller, 2000) and MAPK protein turnover (Zhao *et al.*, 2014).

Signaling by MAPK cascades can regulate various cellular and developmental processes in plants (Komis *et al.*, 2018; Šamajová *et al.*, 2013a). MAPKs phosphorylate and regulate (activate or deactivate) broad range of substrates such as other protein kinases, nuclear transcription factors, cytoskeletal components, and proteins involved in metabolism and vesicular trafficking (Šamajová *et al.*, 2013b; Smékalová *et al.*, 2014). Plant MAPKs can be activated by several abiotic stimuli such as cold, drought or salinity (Sinha *et al.*, 2011; Šamajová *et al.*, 2013a; Ovečka *et al.*, 2014) and biotic stimuli such as pathogens, pathogen-derived toxins, or generally by microbe-associated molecular patterns (MAMPs; Pitzschke *et al.*, 2009; Rasmussen *et al.*, 2012).

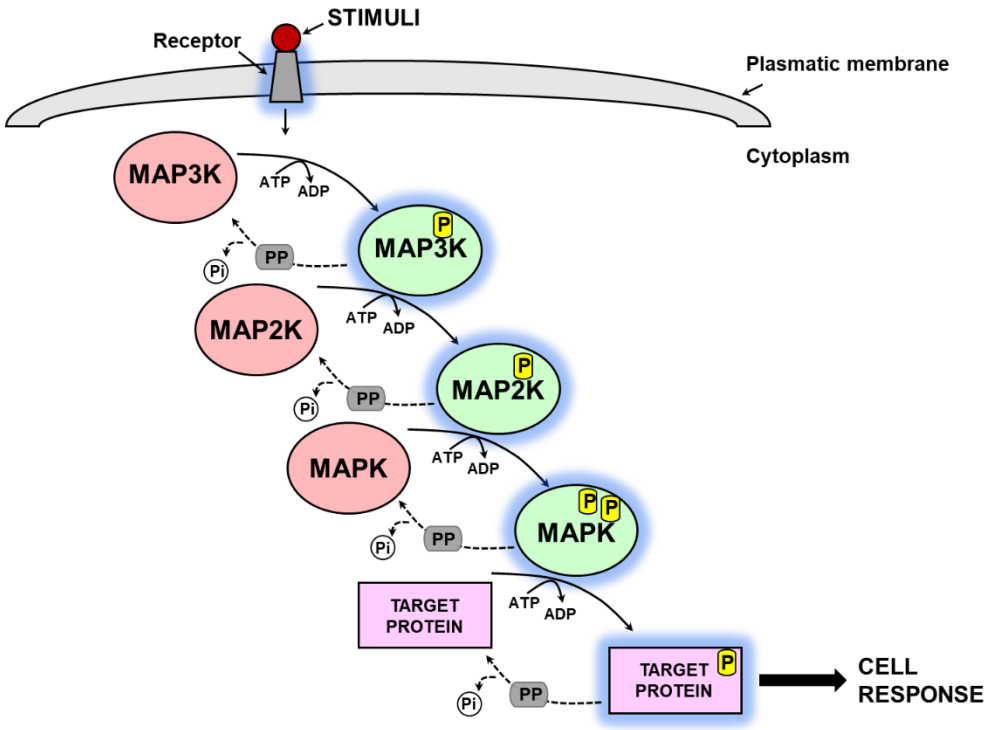

Figure 2 Schematic presentation of a MAPK cascade. Signals including chemical or physical environmental stimuli are recognized by receptors. This leads to the activation of a MAPK kinase kinase (MAP3K) that mediates the phosphorylation of a MAPK kinase (MAP2K) and finally phosphorylation of a MAPK within the MAPK signaling module. Regulation of target proteins by activated MAPK leads to cellular response.

2.4.1.1 Nuclear localization of MAPKs

Comparing to animals, only a few strictly nuclear protein kinases are identified in plants (Dahan *et al.*, 2010). Larger part of known nuclear plant protein kinases are also localized in the cytoplasm. Several studies have shown GFP-tagged MAPKs localized in nuclei and cytoplasm (Bigeard and Hirt, 2018; Hrbáčková *et al.*, 2021; Kumar and Kirti, 2010; Šamaj *et al.*, 2002; Ovečka *et al.*, 2014; Umbrasaite *et al.*, 2010; Yoo *et al.*, 2008; Zaïdi *et al.*, 2010). Generally, the nuclear localization of MAPKs can be driven by the presence of nuclear sorting signals such as nuclear localization signal (NLS) (Chuderland, 2008; Šamajová *et al.*, 2012). Subcellular localization of plant MAPK was shown in pepper and onion cells with the orthologs of the tobacco MAPK called NTF6, which localized in both cytosol and nucleus (Préstamo *et al.*, 1999). Since that, several members of *Arabidopsis* and other plant MAPK modules were homologously or heterologously expressed as GFP fusions and used for nuclear or cytosolic subcellular localization studies. AtMPK3, AtMPK6 and AtMKK9 are located in the cytosol, nucleus, and also associated with membranes (Ahlfors *et al.*, 2004; Brock *et al.*, 2010; Carrasco *et al.*, 2014; Maldonado-Bonilla *et al.*, 2013; Müller *et al.*, 2010; Persak and Pitzschke, 2013; Pitzschke *et al.*, 2014; Umbrasaite *et al.*, 2010). AtMPK4 localized to the cytosol, the nucleus, the plasma membrane, the cell plate and to microtubules (Beck *et al.*, 2011; Brock *et al.*, 2010; Gao *et al.*, 2008; Kosetsu *et al.*, 2010; Schweighofer *et al.*, 2007; Umbrasaite *et al.*, 2010). AtMPK12 is localized in the nucleus and in the cytosol of guard cells (Jammes *et al.*, 2009). Similarly, nuclear localization was reported for orthologs of *Arabidospis* MAPKs in other plant species. In *N. benthamiana*, the NbWIPK (AtMPK3 ortholog), NbSIPK (AtMPK6 ortholog), NaMPK4 (AtMPK4/AtMPK11 ortholog), NTF4, and NTF6 were all localized in the cytosol and in the nucleus (Hettenhausen *et al.*, 2012; Ishihama *et al.*, 2011; Menke *et al.*, 2005; Yap *et al.*, 2005). In maize, ZmMPK7 (AtMPK7/AtMPK14 ortholog) and ZmMPK17 (AtMPK15 ortholog) were located in the nucleus (Pan *et al.*, 2012; Zong *et al.*, 2009). In alfalfa, SIMK (AtMPK6 ortholog) was located predominantly in the nucleus but also in the cytoplasm (Munnik *et al.*, 1999; Hrbáčková *et al.*, 2021; Šamaj *et al.*, 2002; Ovečka *et al.*, 2014), and in *M. truncatula* MtMPK3 (AtMPK3 ortholog) and MtMPK6 (AtMPK6 ortholog) were localized in the cytosol, in the nucleus and on the membranes (Chen *et al.*, 2017). It should be also mentioned that the nuclear localization was also reported for some MAPKKs and MAPKKKs (Yoo *et al.*, 2008).

2.4.1.2 Classification of MAPKs

There are around 110 genes encoding MAPK cascade kinases in the genome of *Arabidopsis thaliana*. Among them 80 genes encode MAPKKKs, 10 genes encode MAPKKs and 20 genes encode MAPKs (Colcombet and Hirt, 2008; de Zélicourt *et al.*, 2016; Raja *et al.*, 2017). Comparable numbers of MAPK cascade members have been identified in genomes of forage crop species, such as rice (*Oryza sativa*) containing 75 *MAPKKK*, 8 *MAPKK* and 17 *MAPK* genes (Singh *et al.*, 2012; Wankhede *et al.*, 2013; Xiong *et al.*, 2001), or maize (*Zea mays*) possessing 74 *MAPKKK*, 9 *MAPKK* and 19 *MAPK* genes (Kong *et al.*, 2013). The genome of *G. max* contains the highest number of MAPKs published so far, namely 150 *MAPKKK*, 11 *MAPKK* and 38 *MAPK* genes (Neupane *et al.*, 2013).

From phylogenetic point of view, plant MAPKs are divided into four groups (A – D; MAPK-Group, 2002). These groups are characterized by the conserved TxY consensus motif in their activation loop (T-loop) and sequence comparison of this motif allowed classifying MAPKs into two subtypes (TEY and TDY). The TEY subtype includes groups A, B, and C; the TDY subtype forms group D (Anderson *et al.*, 1990; Bigeard and Hirt, 2018; MAPK-Group, 2002; Payne *et al.*, 1991). Group A of the subtype TEY MAPKs includes AtMPK3 in Arabidopsis and its orthologs MsSAMK (*M. sativa* SAMK), NbWIPK (*N. benthamiana* WIPK), OsMAPK2 (*O. sativa* MAPK2), ZmMPK4 (*Z. mays* MPK4); AtMPK6 (*A. thaliana* MPK6) and its orthologs MsSIMK (*M. sativa* SIMK), NbSIPK (*N. benthamiana* SIPK), ZmMPK5 (*Z. mays* MPK5); or AtMPK10 (*A. thaliana* MPK10) (MAPK-Group, 2002). Group B of the subtype TEY MAPKs includes AtMPK4 or AtMPK5 (*A. thaliana* MPK4, 5) and its ortholog MsMMK2 (*M. sativa* MMK2); AtMPK11, AtMPK13 (*A. thaliana* MPK11, 13), and its orthologs MsMMK3 (*M. sativa* MMK3), or NbNTF6 (*N. benthamiana* NTF6) (MAPK-group, 2002). Group C of the subtype TEY MAPKs includes AtMPK1, AtMPK2 (*A. thaliana* MPK1, 2) and its ortholog NbNTF3 (*N. benthamiana* NTF3); AtMPK7, AtMPK14 (*A. thaliana* MPK7, 14) and its ortholog OsMAPK3, OsMAPK4 (*O. sativa* MAPK3, MAPK4). Group D is formed by MAPKs of the TDY subtype, such as AtMPK8, AtMPK15 (*A. thaliana* MPK8, 15) and its ortholog OsMPKG2 (*O. sativa* MPKG2); AtMPK18, AtMPK19 (*A. thaliana* MPK18, 19) and its ortholog MsTDY1 (*M. sativa* TDY1) (MAPK-Group, 2002).

2.4.2 MAPKs in alfalfa

In alfalfa, stress-induced MAPK (SIMK) was identified as a 46 kDa salt stress- and elicitor-induced MAPK (Munnik *et al.,* 1999; Cardinale *et al.,* 2000, 2002). Yeast two-hybrid screen and activation studies identified SIMK kinase (SIMKK) as an upstream activator of SIMK (Kiegerl *et al.,* 2000; Cardinale *et al.,* 2002). Interaction between SIMKK and SIMK upon salt stress is quite specific, because no interaction was observed with three other MAPKs, such as MMK2 (Jonak *et al.,* 1995), MMK3 (Bögre *et al.,* 1999) and SAMK (Jonak *et al.,* 1996). Another MAPKK identified in alfalfa is PRKK. PRKK together with SIMKK showed strongest interaction with SIMK, but PRKK required activation by an upstream-activated MAPKK kinase. SIMKK has function in pathogen and elicitor signaling. PRKK is not able to activate SIMK, MMK3 or SAMK upon salt stress, but is able activate them upon elicitor treatment of cells (Cardinale *et al.,* 2002). In addition, MMK2 and MMK3 are involved in cell growth and division (Bögre *et al.,* 1999). MMK2 is one of the first alfalfa MAPKs identified as being involved in cytoskeletal regulation. MMK2 targeted a 39 kDa substrate protein residing in detergent-resistant cytoskeletal preparations from carrot suspension cells (Jonak *et al.,* 1995). Calderini and co-workers (1998) and subsequently Bögre and co-workers (1999) identified MMK3 in alfalfa and $p43^{NTF6}$ in tobacco, two MAPKs that become activated in a microtubule-dependent manner during late anaphase/early telophase and localize at the phragmoplast midzone, where sets of antiparallel MTs interdigitate through their plus ends (Otegui and Staehelin, 2000). SAMK is stress-activated MAPK identified as kinase inducible by osmotic stress (Munnik *et al.,* 1999), wounding (Bögre *et al.,* 1997) and by various fungal elicitors (Cardinale *et al.,* 2000). OMTK1 was identified as a first alfalfa MAPKKK (Nakagami *et al.,* 2004). It was activated by oxidative stress (H_2O_2) and specifically activated MMK3, which resulted in increased cell death rate (Nakagami *et al.,* 2004). SIMKK is a functional dual-specificity protein kinase that phosphorylates SIMK on both threonine and tyrosine residues of the activation loop (Kiegerl *et al.,* 2000; Cardinale *et al.,* 2002). SIMKK shares 88% amino acid similarity with SIP2 (MAPKK in *L. japonicus;* Chen *et al.,* 2012) and is highly similar to MKK4 (MAPKK4 in *M. truncatula*; Chen *et al.,* 2017). Previously, it was shown that SIMK predominantly localizes in nuclei of root trichoblast cells while it is activated and redistributed from nucleus into growing root hair tips (Šamaj *et al.,* 2002). In latrunculin B-treated root hairs, SIMK relocated back to the nucleus while after jasplakinolide treatment, SIMK co-localized with thick F-actin cables in the cytoplasm. Thus, these drugs affecting actin

cytoskeleton (Baluška *et al.*, 2000b), have a direct impact on the intracellular localization of SIMK (Šamaj *et al.*, 2002). In order to enhance SIMK expression, we cloned N-terminal fusion construct of enhanced *GFP* (*eGFP*) with *SIMK* driven under constitutive *35S* promoter (*35S::GFP:SIMK*) and stably transformed this construct into alfalfa plants (Hrbáčková *et al.*, 2021). Live cell microscopy imaging revealed GFP-SIMK localization predominantly to the nucleus and cytoplasm in cells of diverse organs such as roots and leaves. This was consistent with previously published data on SIMK immunolocalization in alfalfa roots (Baluška *et al.*, 2000a; Ovečka *et al.*, 2014). The GFP-SIMK was also localized in tips of growing root hairs in agreement with previously published SIMK localization pattern using immunofluorescence microscopy (Šamaj *et al.*, 2002).

Previously, Bekešová *et al.* (2015) showed decreased accumulation of phosphorylated SIMK in SIMKKi lines. In alfalfa, representing an important forage crop, total leaf surface area and shoot biomass are agronomical parameters of interest. In this respect, genetic manipulation of SIMK brought interesting and potentially promising results. Downregulation of *SIMKK* and *SIMK* genes in SIMKKi lines influenced negatively the development of above ground plant parts, leading to formation of smaller leaves and production of less shoot biomass per plant. Importantly, SIMK overexpression in GFP-SIMK lines, on the other hand, resulted in higher shoot biomass per plant, production of bigger size of analyzed leaves and their better distribution in shoots due to the longer petioles. This result may support a general effort on alfalfa biotechnological improvement as a forage crop (Hrbáčková *et al.*, 2021).

3 Part II - Preparation and transformation of fluorescently-tagged MAPKs of *Medicago sativa*

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Protein phosphorylation is one of the main mechanisms controlling cellular functions in response to external signals. In all eukaryotes, including plants, animals, and fungi, a specific class of Ser/Thr protein kinases (MAPKs), is involved in signal transduction. A typical feature of MAPK pathways is their composition of three functionally linked protein kinases (Cardinale *et al.,* 2002; Karin, 1998; Whitmarsh and Davis, 1998). Signaling through MAPK cascades leads to cellular responses such as differentiation, stress adaptation or cell division (Robinson and Cobb, 1997). Duration, specificity and amplitude of MAPK signaling is regulated by composition of a given MAPK module (Colcombet and Hirt, 2008), deactivation of MAPK cascade by phosphatases (Bigeard and Hirt, 2018; Gómez-Gómez and Boller, 2000), and by specific subcellular organization and localization of particular MAPK modules (Komis *et al.,* 2018; Šamajová *et al.,* 2013b). *M. sativa* is one of the several plant species with characterized MAPK-mediated signaling involved in abiotic or biotic stress. Activation of SIMK by different osmotic stresses is well known, but downstream targets are not explained. SIMK kinase (SIMKK) is well-characterized upstream activator of SIMK (Munnik *et al.*, 1999; Cardinale *et al.*, 2000, 2002).

A further subject of the study was the preparation of two types of fluorescently tagged MAPKs in alfalfa used for subcellular localization. *In vivo* imaging of GFP-SIMK and tagRFP-SIMKK fusion proteins allows obtaining information about subcellular distribution of SIMK and SIMKK. Preparation of stably transformed alfalfa lines carrying these fluorescent markers required tissue culture technique (somatic embryogenesis) and effective genetic transformation. Fluorescence signal in transiently transformed epidermal cells of *N. benthamiana* was observed by microscopic methods. Subsequently, immunoblot analysis was performed to confirm presence of GFP-SIMK fusion protein in epidermal leaf cells of *N. benthamiana* by polyclonal anti-SIMK antibody. In addition, PCR genotyping was used to check presence of vector carrying tagRFP-SIMKK fusion

in these cells. Stable transformed alfalfa lines were also tested by microscopic methods, based on the presence of specific fluorescence signal. Again, immunoblot analysis was performed to confirm presence of GFP-SIMK fusion protein using polyclonal anti-SIMK antibody. Activation status of SIMK in cells was studied by colocalization experiments using both anti-SIMK and anti-phospho-p44/42 specific antibodies.

3.1 Material and methods

3.1.1 Plant material

Somatic embryos of *M. sativa* cv. Regen-SY (RSY; Bingham, 1991) of two wild-type lines (RSY L1, L2), two independent lines of *M. sativa* cv. Regen-SY carrying *35S::GFP:SIMK* construct (GFP-SIMK L5, L6) or two independent *SIMKK RNAi* lines carrying pHellsgate12 plasmid (obtained from CSIRO Plant Industry, Australia) driven under *35S* promoter (SIMKKi L3, L4) with well-developed root poles were separated, individually transferred on root and plant development medium (MMS) or MS medium for long-term cultivation of complete plants in controlled environmental chambers. Plants were grown in the environmental chamber at 21°C and 70% humidity in a 16-h light/8-h dark cycle. Illumination intensity was 150 μmol $m^{-2}s^{-1}$.

3.1.2 Cloning of fluorescently-tagged MAPKs

Cloning of constructs for expression of N-terminal GFP-tagged SIMK or tagRFP-tagged SIMKK driven under *p35S* promoter (*35S::GFP:SIMK*, *35S::tagRFP:SIMKK*) was performed in pB7m34GW,0 by MultiSite Gateway® Three-fragment vector construction kit. For construction of *35S::GFP:SIMK* vector we have used pEN-L4-2-R1™ plasmid carrying *p35S* sequence (Karimi *et al.,* 2007), pEN-L1-F-L2™ carrying *eGFP* sequence (Karimi *et al.,* 2007) and pDONR™P2R-P3 carrying *SIMK* cDNA sequence (https://www.thermofisher.com). For construction of *35S::tagRFP:SIMKK* vector were employed pEN-L4-2-R1™ plasmid carrying *p35S* sequence, pGEM®-T Easy plasmid containing *tagRFP* sequence (obtained from Department of Molecular Biology, CRH, Olomouc) and pDONR™P2R-P3 carrying *SIMKK* cDNA sequence (https://www.thermofisher.com). In the first step, 1190 bp *SIMK* and 1133 bp *SIMKK* PCR fragments were amplified using iProof™ High-Fidelity DNA Polymerase (Bio-Rad, USA) with specific primers listed in **Table 1** and total cDNA of alfalfa as a template. Donor and destination vectors were transformed in *Escherichia coli* strain TOP10. Colonies were tested for presence of required construct by cleavage with PstI (for *35S::GFP:SIMK*) and SacI (for *35S::tagRFP:SIMKK*) restriction enzymes (Thermo Fisher Scientific, USA) at 37°C for 2 hours. Plasmid DNA was isolated by QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany) according recommended protocol. Positive colonies were tested by sequencing (SEQme, Czech Republic). Final destination vector was used for *A. tumefaciens* strain GV3101 cold-shock transformation.

Table 1 List of forward and reverse primers used for PCR amplification of *SIMK* and *SIMKK* sequence with ATT recombination sites.

Primer description	**Primer sequence 5'- 3'**
SIMKFor	GGGGACAGCTTTCTTGTACAAAGTGGGGATGG AAGGAGGAGGAGCTC
SIMKRev	GGGGACAACTTTGTATAATAAAGTTGCCTACT GCTGGTACTC AGGGTTAAAT
SIMKKFor	GGGGACAGCTTTCTTGTACAAAGTGGGGATGA GGCCGATTCAGCTTC
SIMKKRev	GGGGACAACTTTGTATAATAAAGTTGCCTAAG AAGAAAGTGATCTTGGTGGT

3.1.3 Transient transformation of *Nicotiana benthamiana* leaves

LB medium (5 ml) containing appropriate selection antibiotics was inoculated with *A. tumefaciens* GV3101 strain transformed with vectors encoding GFP-SIMK and tagRFP-SIMKK fusion proteins. Bacterial cultures grown at 28°C, 200 rpm to OD_{600} 0.7 were pelleted at 3500 g, 4°C for 15 min. Pellets were suspended in 2 ml buffer containing 10 mM $MgCl_2$, 10 mM MES (pH 5.6) and 150 μM acetosyringone, and subsequently incubated at room temperature in the dark for 2 h. Bacterial cultures containing *35S::GFP:SIMK* and/or *35S::tagRFP:SIMKK* constructs were infiltrated into six-week-old *N. bethamiana* leaves using syringe. Leaves were infiltrated by single construct or simultaneously by two constructs, and eventually treated by 500 mM NaCl for 10 min. Infiltration buffer without any construct was used as a negative control. After infiltration, plants were covered with transparent plastic bags and maintained in growth chamber for 24 h, than were uncovered. After 48 h, transformed epidermal cells were observed with CLSM (LSM710, Axio Imager2, Carl Zeiss, Germany) equipped with Plan-Apochromat 20x/0.8 objective (Carl Zeiss, Germany). Samples were imaged with 488 nm excitation laser line and appropriate detection range for GFP emission. Image post- processing was done using ZEN 2010 software.

3.1.4 Immunoblotting analysis of transiently transformed *N. benthamiana* leaves

Immunoblotting analysis was performed on *N. benthamiana* leaves transiently transformed with *35S::GFP:SIMK* construct. Plants were homogenized using liquid nitrogen to fine powder and the proteins were extracted in E-buffer [50 mM HEPES (pH

7.5), 75 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM NaF, 10 % (v/v) glycerol, Complete™ EDTA-free protease inhibitor and PhosSTOP™ phosphatase inhibitor cocktails (both from Roche, Basel, Switzerland)]. After centrifugation, supernatants were mixed with Laemmli buffer [final concentration 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 300 mM 2-mercaptoethanol]. After protein concentration measurement using Bradford assay equal protein amounts (10 ng) were separated on 12% TGX Stain-Free™ gels (Biorad, Hercules, CA). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes in a wet tank unit (Biorad) overnight at 24 V and 4°C using the Tris-glycin-methanol transfer buffer. Membranes were blocked in 4% (w/v) bovine serum albumin (BSA) in Tris-buffered-saline (TBS, 100 mM Tris-HCl, 150 mM NaCl, pH 7.4) at 4°C overnight. Following washing step with TBS-T (TBS, 0.1% Tween 20) and incubation with polyclonal anti-AtMPK6 antibody (Sigma, Life Science, USA), highly specific for SIMK detection (Bekešová *et al.*, 2015), diluted 1:15000 in TBST-T containing 1% (w/v) BSA. Following five washing steps in TBS-T and incubation with a horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody (diluted 1:5000). Signals were developed using Clarity Western ECL substrate (Biorad) and detected on Chemidoc MP Documentation system (Biorad).

3.1.5 PCR-based genotyping

Transiently transformed *N. benthamiana* leaves were used to confirm presence of *35S::tagRFP:SIMKK* construct by genotyping. Genotyping was performed by PCR with Phire® Plant Direct PCR kit according to recommended manufacturer's protocol. Destination vector pB7m34GW,0 containing *35S::tagRFP:SIMKK* was used as a positive control. Primers used for PCR-based genotyping are listed in **Table 2**.

Table 2 Forward and reverse primers used for genotyping.

Primer description	**Primer sequence 5'- 3'**
tagRFP_For	ATGGACAACACCGAGGACGT
SIMKK_Rev	CGACGAACTGATTCCTCGTG

3.1.6 Stable transformation of *M. sativa*

To obtain stable transformed line of *M. sativa* with N-terminal fusion construct of enhanced GFP (*eGFP*) with *SIMK* driven under *35S* promoter (*35S::GFP:SIMK*), leaves

of mature plants were transformed with *A. tumefaciens* GV3101 carrying *35S::GFP:SIMK* in pB7m34GW,0 expression plasmid. The transformation procedure was performed according to protocol for efficient transformation of alfalfa described by Samac and Austin-Phillips (2006). Leaves explanted from well-developed plant nodes were surface sterilized, cut in half and wounded on the surface with sterile scalpel blade, incubated with overnight *Agrobacterium* culture showing cell density between 0.6 and 0.8 at A_{600} nm for 30 minutes. Leaves were dried of the bacterial solution and transferred on appropriate cultivation media (called B5H medium). Induction of callogenesis from leaf explants, production of somatic embryos from calli (B50 medium), development of shoot poles and somatic embryo rooting were performed on the appropriate media (MMS medium) in the culture chamber at 22°C, 70% humidity, light intensity 100 $\mu mol.m^{-2}s^{-1}$ and 16/8 h light/dark photoperiod. Regenerated plants were maintained and selected on media (MS medium) with phosphinothricin (50 μg/ml) and tested for the presence of GFP-SIMK fusion protein using molecular genotyping or epifluorescence microscope. Transgenic alfalfa plants annotated as GFP-SIMK L5, L6 were propagated in sterile culture via somatic embryogenesis. Somatic embryos stably expressing *35S::GFP:SIMK* construct were used in further experiments.

Obtaining stable transformed line of alfalfa with N-terminal fusion construct of tagRFP-tagged *SIMKK* driven under *35S* promoter (*35S::tagRFP:SIMKK*) was not successful. Plants contained *35S::tagRFP:SIMKK* construct verified by PCR-based genotyping, but without fluorescent signal detectable in the microscope.

3.1.7 Live-cell subcellular localization of fluorescently-tagged SIMK and SIMKK

Agrobacteria carrying *35S::GFP:SIMK* and *35S::tagRFP:SIMKK* constructs were used for transient transformation of *N. benthamiana* leaves and *Agrobacterium* carrying *35S::GFP:SIMK* for stable transformation of alfalfa RSY L1 plants. Transgenic alfalfa lines (GFP-SIMK L5, L6) were regenerated through somatic embryogenesis and cultivated in the culture chamber at above-described conditions. Fluorescence signals were observed in transformed *N. benthamiana* epidermal leaf cells and in alfalfa lines using confocal laser scanning microscopes LSM 710 (Carl Zeiss, Germany) equipped with Plan-Apochromat 20×/0.8 (Carl Zeiss, Germany), and LSM880 with Airyscan (Carl Zeiss, Germany) equipped with Plan-Apochromat 20×/0.8 (Carl Zeiss, Germany). Samples were imaged with 488 nm excitation laser line with emission filter 493 – 598 nm

for GFP and 561 nm excitation laser line with emission filter 565 – 583 nm for tagRFP. Image post-processing was done using ZEN 2010 software.

3.1.8 Fixation and immunolabeling of SIMK and phosphorylated MAPKs in alfalfa roots

Immunolocalization of GFP-tagged SIMK and pERK in root whole-mounts of transgenic plants carrying *35S::GFP:SIMK* construct (GFP-SIMK L5) was done as described previously (Tichá *et al.,* 2020a). A double-immunolabeling with mouse anti-GFP (Abcam, UK) and rabbit anti-phospho-p44/42 (Cell Signaling, Netherlands) primary antibodies diluted 1:100 in 2.5% (w/v) BSA dissolved in PBS was performed on root tip samples. Vacuum pump was used (3×5 min) to improve antibody penetration, followed by incubation at 4°C overnight. Samples were sequentially incubated with secondary antibody solutions. First, Alexa-Fluor 488 goat anti-mouse secondary antibody (Invitrogen, USA) diluted 1:500 in 2.5% (w/v) BSA in PBS was used for incubation at 37°C for 2 h. After extensive washing with PBS and subsequent blocking [5 % (w/v) BSA in PBS for 20 min] samples were incubated with Alexa-Fluor 555 goat anti-rabbit secondary antibody (Abcam) diluted 1:500 in 2.5% (w/v) BSA in PBS at 37°C for 2 h. Nuclei were counterstained with DAPI. Immunolabeled root samples were mounted in anti-fade mounting medium [0.1 % (w/v) paraphenylenediamine in 90% (v/v) glycerol buffered with 10% (v/v) PBS at pH 8.2 - 8.6] and used for microscopy. Microscopic analysis was performed with a LSM710 CLSM platform (Carl Zeiss) or Zeiss LSM880 Airyscan equipped with a 32 GaAsP detector, using excitation laser lines at 405 nm for DAPI, 488 nm for Alexa-Fluor 488 and 561 nm for Alexa-Fluor 555. The image post-processing was done using ZEN 2014 software and final figure plates were prepared using Photoshop 6.0/CS and Microsoft PowerPoint software. Kateřina Hlaváčková and Olga Šamajová performed fixation and immunolabeling of SIMK.

3.2 Results

3.2.1 Cloning of *GFP*-tagged *SIMK* and *tagRFP*-tagged *SIMKK* under *35S* promoter

Two constructs *35S::GFP:SIMK* and *35S::tagRFP:SIMKK* were prepared using MultiSite Gateway® cloning system (**Figure 3, Figure 7A,B**). High-Fidelity Polymerase (iProof™) was used for PCR amplification from cDNA sequences (**Figure 4**). Due to the complexity of Gateway primers (50 bp fused with ATT sequences for recombination with 25 bp incorporated in primers), the PCR additives DMSO and/or betaine were used to

suppress primer secondary structure formation like hairpins or dimers. The restriction enzymes NdeI, DpnI, PvuI and XbaI were employed for restriction analyses of entry clones (**Figure 5**), and PstI and SacI for final destination vectors (**Figure 6**). Entry clones were verified by sequencing.

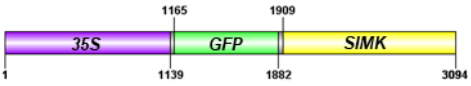
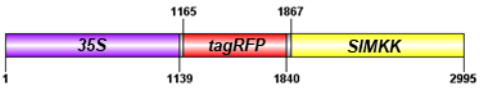
Protein fusion	Construct map	Description
GFP-SIMK		N-terminal fusion of *SIMK* with *GFP* cloned from cDNA driven by *35S* promoter
tagRFP-SIMKK		N-terminal fusion of *SIMKK* with *tagRFP* cloned from cDNA driven by *35S* promoter

Figure 3 Description of expression cassettes of *GFP*-tagged *SIMK* and *tagRFP*-tagged *SIMKK* genes under *35S* promoter prepared by Multisite Gateway® Cloning.

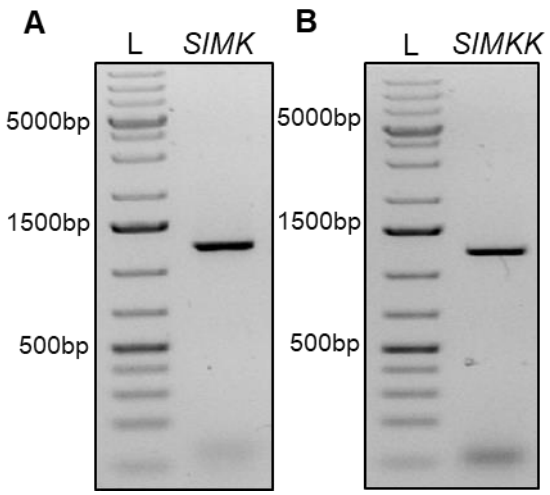


Figure 4 Representative electrophoretic images of PCR products for preparation of *SIMK* and *SIMKK* entry clones for their N-terminal fusions. **(A-B)** PCR products for BP recombination reaction by Multisite Gateway® cloning system. **(A)** PCR amplicon of *SIMK* cDNA sequence for N-terminal fusion (*SIMK*; 1190bp). **(B)** PCR amplicon of *SIMKK* cDNA sequence for N-terminal fusion (*SIMKK*; 1133bp). L – DNA ladder.

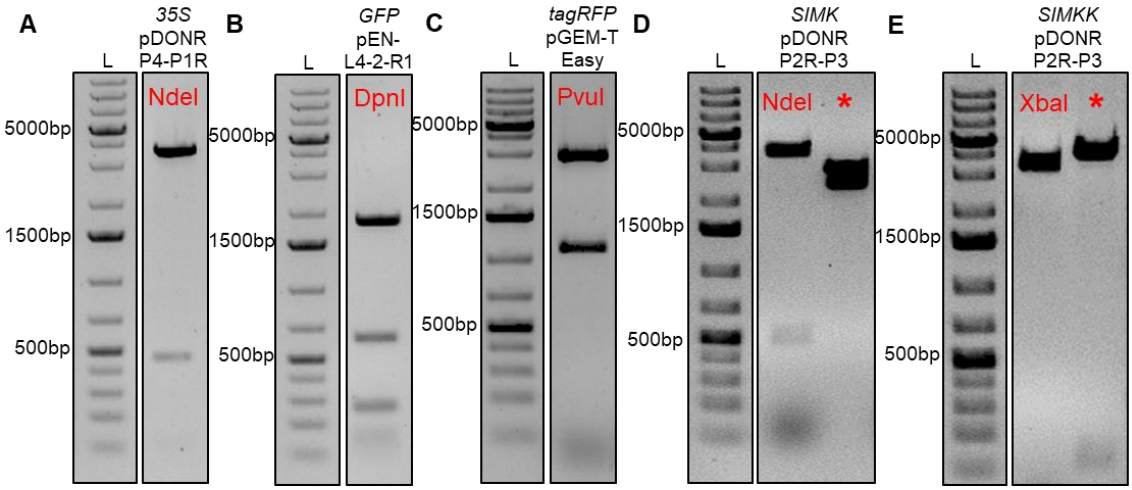


Figure 5 Representative electrophoretic figures of entry clone preparation for *GFP*-fused *SIMK* and *tagRFP*-fused *SIMKK* genes under *35S* promoter. **(A-E)** Enzymatic digestions of entry clones prepared for LR recombination reaction of Multisite Gateway® cloning system. **(A)** Enzymatic digestion of entry clone *35S* promoter sequence in pDONR P4-P1R with NdeI (3288 bp + 436 bp + 82 bp). **(B)** Enzymatic digestion of entry clone *GFP* gene sequence in pEN-L4-2-R1 with DpnI (1742 bp + 594 bp + 266 bp + 252 bp). **(C)** Enzymatic digestion of entry clone *tagRFP* gene sequence in pGEM-T Easy with PvuI (2750 bp + 1096 bp + 101 bp). **(D)** Enzymatic digestion of entry clone *SIMK* gene sequence in pDONR P2R-P3 with NdeI (3126 bp + 503 bp + 177 bp). **(E)** Enzymatic digestion of entry clone *SIMKK* gene sequence in pDONR P2R-P3 with XbaI (3806 bp). L – DNA ladder; asterisk – indicates empty entry clone as a control of enzymatic digestion and successful BP reaction.

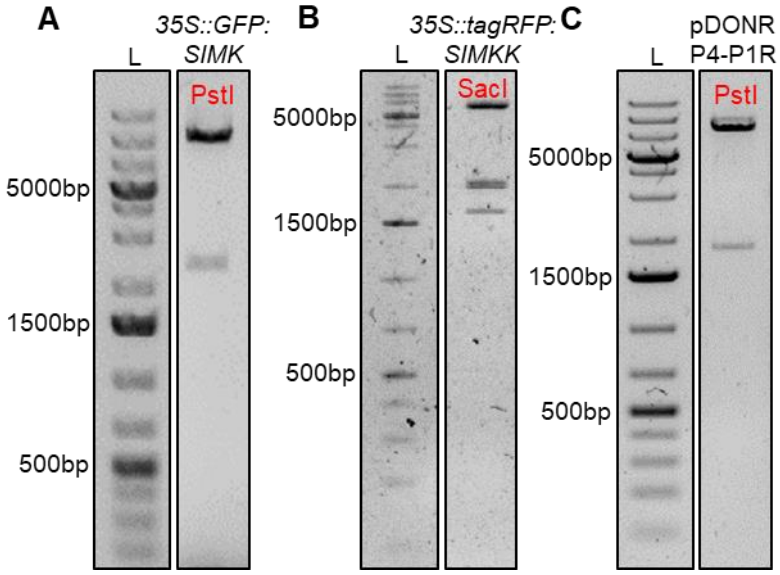


Figure 6 Representative electrophoretic images of destination vectors for *GFP*-fused *SIMK* and *tagRFP*-fused *SIMKK* genes under *35S* promoter. **(A-C)** Enzymatic digestions of destination vectors carrying *GFP*-fused *SIMK* and *tagRFP*-fused *SIMKK* genes under *35S* promoter prepared by Multisite Gateway® LR recombination reaction in pG7m34GW,0. **(A)** Destination vector *35S::GFP:SIMK* digested by PstI (8965 bp + 2287 bp). **(B)** Destination vector *35S::tagRFP:SIMKK* digested by SacI (5755 bp + 1975 bp + 1945 bp +1561 bp). **(C)** Empty destination vector pDONR P4-P1R digested by PstI (8081bp + 1864 bp) used as a control of enzymatic digestion and successful LR recombination. L – DNA ladder.

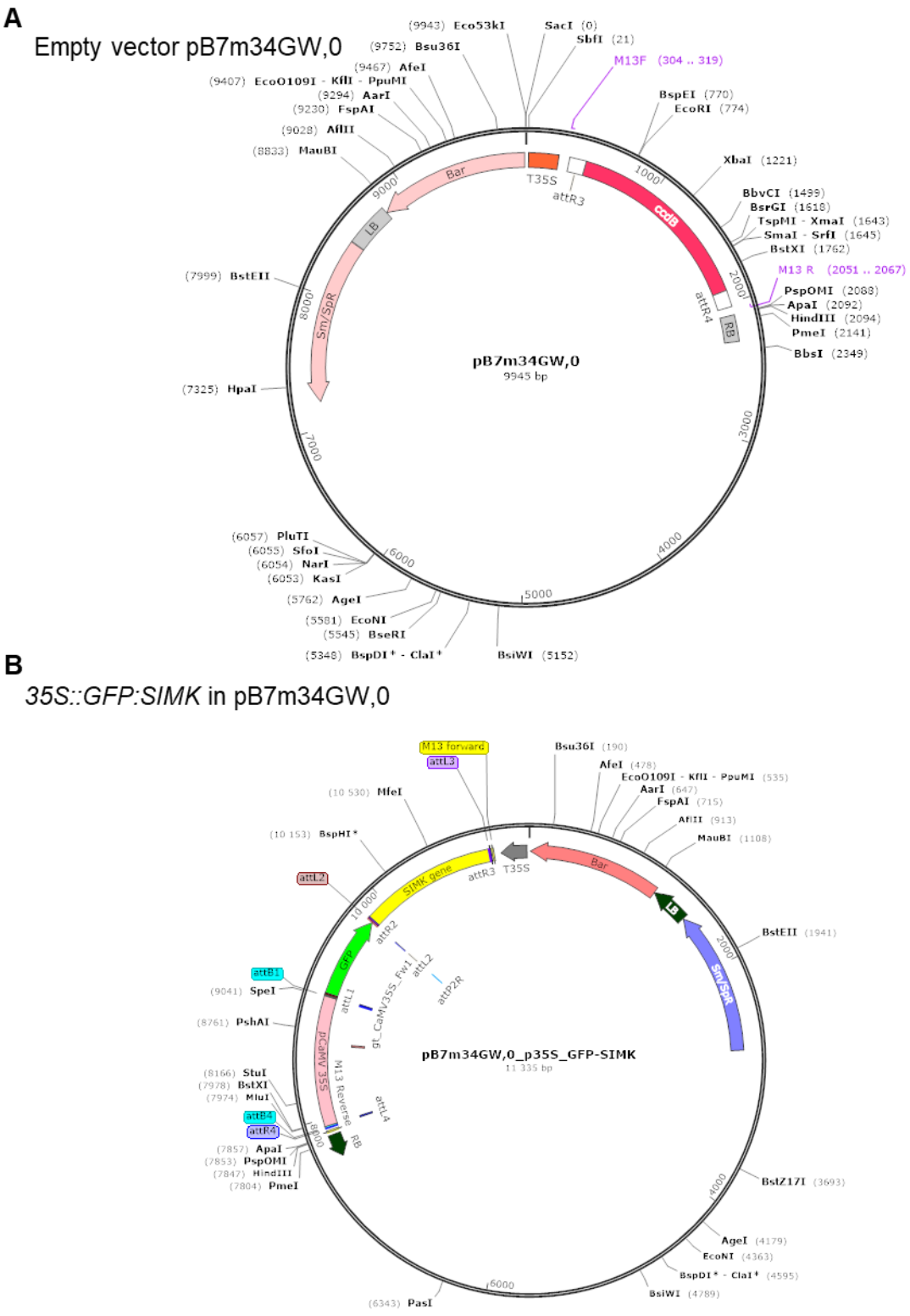


Figure 7 Construction of destination empty vector and destination vector with *35S::GFP:SIMK*. **(A)** Plasmid map of empty vector pB7m34GW,0, **(B)** vector pB7m34GW,0 with *35S::GFP:SIMK*. The SnapGene software was used for the visualization of a plasmid map.

3.2.2 Transient expression of GFP-SIMK and tagRFP-SIMKK fusion proteins in *N. benthamiana* leaves

Transient transformation of *N. benthamiana* leaves was performed in order to test *35S::GFP:SIMK* and *35S::tagRFP:SIMKK* constructs (**Figure 8,9**). This experiment proved fluorescent properties of GFP-SIMK and tagRFP-SIMKK fusion proteins. Both GFP-SIMK and tagRFP-SIMKK were preferentially localized in the nucleus (except nucleoli) and in the cytoplasm (**Figure 8,9**). The expression of the GFP-SIMK fusion protein was corroborated by immunoblot analysis of protein extracts isolated from the fluorescent areas of transformed *N. benthamiana* leaves (**Figure 8b**). The presence of *35S::tagRFP:SIMKK* construct was supported by PCR-based genotyping using transformed areas of *N. benthamiana* leaves (**Figure 9B**).

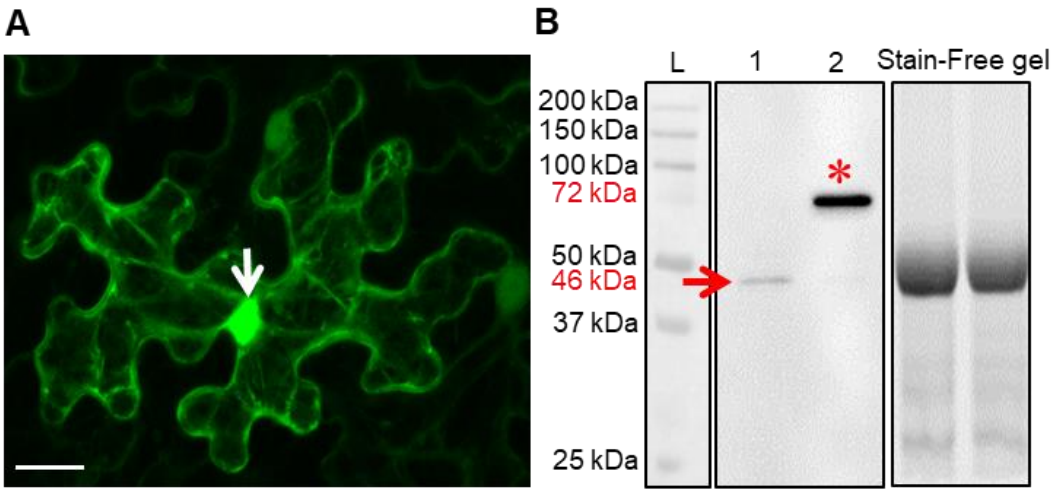


Figure 8 Representative images of transient *N. benthamiana* transformation and immunoblot showing localization and abundance of GFP-SIMK protein. **(A)** Accumulation of GFP-tagged SIMK in nuclei (white arrow) and cytoplasm in transiently transformed epidermal leaf cells of *N. benthamiana*. **(B)** Immunoblot detection of SIMK and GFP-SIMK bands using anti-SIMK antibody in transiently transformed *N. benthamiana* leaves. Endogenous SIMK has 46 kDa (red arrow) and GFP-SIMK fusion protein has 72 kDa (asterisk). Stain-free gel represents loading control for immunoblot samples. L – protein ladder, 1 – leaf sample of wild-type *M. sativa* as a control of endogenous SIMK protein, 2 – leaf sample of transiently transformed *N. benthamiana* with GFP-SIMK fusion protein. Scale bar: 20 μm.

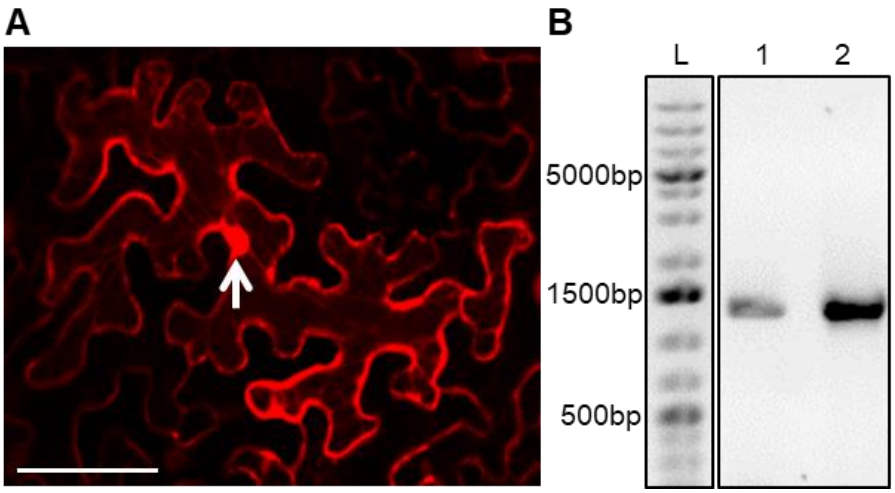


Figure 9 Representative images of transient *N. benthamiana* transformation and PCR-based genotyping showing localization of tagRFP-SIMKK and expression of *35S::tagRFP:SIMKK* construct. **(A)** Accumulation of tagRFP-tagged SIMKK in nucleus (arrow) and cytoplasm in transiently transformed epidermal leaf cells of *N. benthamiana*. **(B)** PCR-based genotyping of transiently transformed *N. benthamiana* leaves. L – DNA ladder, 1 – leaf sample of transiently transformed *N. benthamiana* with *35S::tagRFP:SIMKK* construct, 2 – destination vector pB7m34GW,0 containing *35S::tagRFP:SIMKK* as a positive control of PCR. Scale bar: 100 μm.

In order to perform colocalization studies, SIMK and SIMKK were tagged with different fluorescent proteins (GFP and tagRFP, respectively) and transiently co-expressed in epidermal cells of *N. benthamiana* leaves. Under control conditions, both GFP-tagged SIMK and tagRFP-tagged SIMKK preferentially localized to the nuclei, but they were also dispersed throughout the cytoplasm (**Figure 10**). In contrast, both GFP-SIMK and tagRFP-SIMKK relocated to cytoplasmic punctate compartments where they colocalized after 10 min of 500 mM NaCl treatment (**Figure 11**). These data suggested that salt treatment triggered tagRFP-SIMKK mediated relocation of GFP-SIMK, which was associated with colocalization of both fluorescently tagged kinases in cytoplasmic spot-like compartments. Simultaneous relocation and colocalization of both GFP-SIMK and tagRFP-SIMKK to these cytoplasmic compartments suggested that both kinases were targeted to these structures in a coordinated manner.

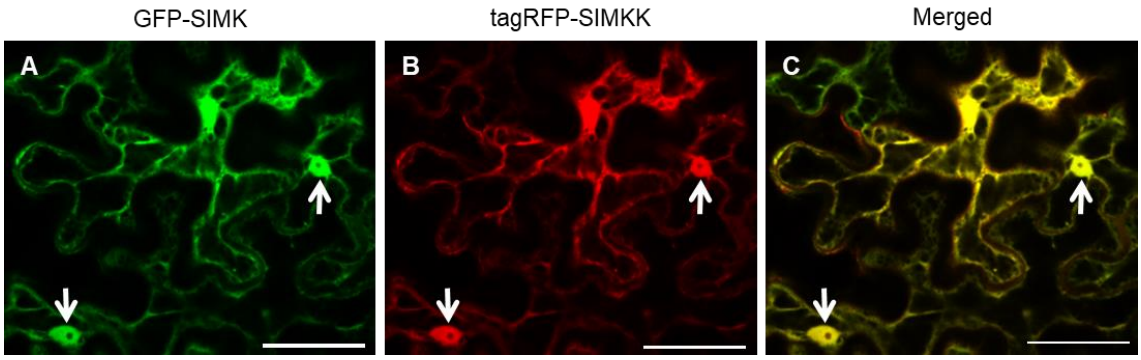


Figure 10 Co-localization of GFP-SIMK and tagRFP-SIMKK in transiently co-transformed leaf epidermal cells of *N. benthamiana*. **(A-C)** GFP-tagged SIMK **(A)** and tagRFP-SIMKK **(B)** colocalize **(C)** in *N. benthamiana* epidermal leaf cells. Both fusion proteins showed predominantly nuclear (arrows) and cytoplasmic localization (merged image shown in **C**). Scale bar: 50 µm.

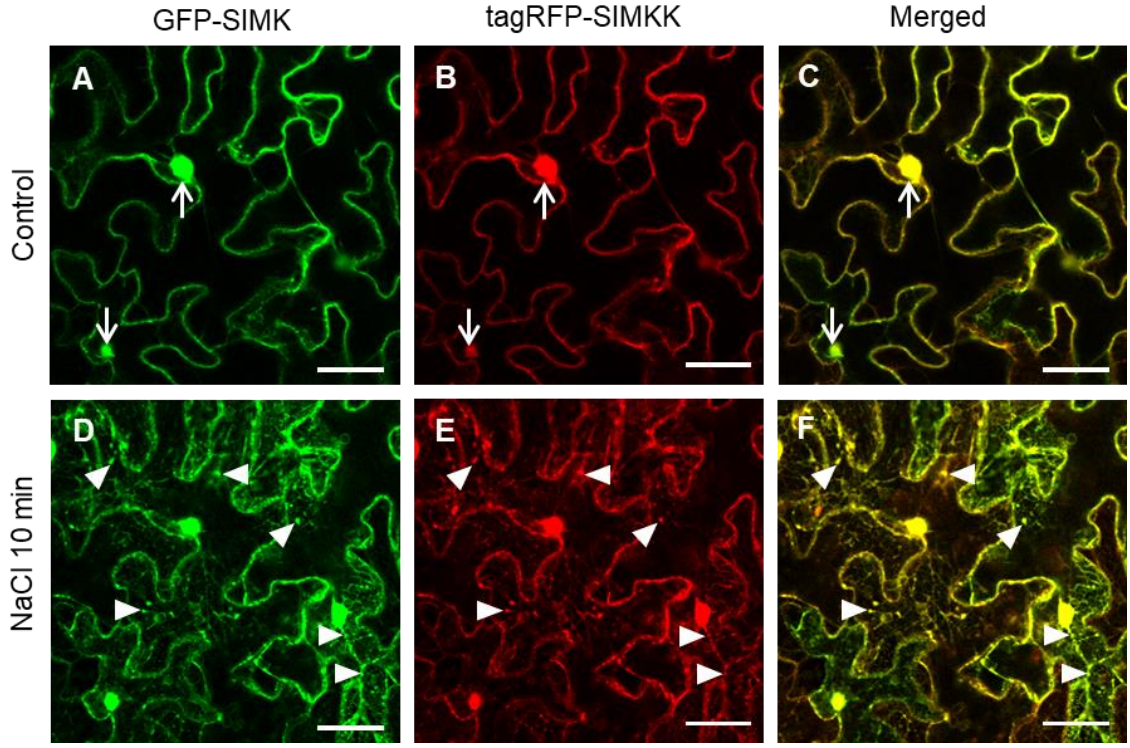


Figure 11 Co-localization of GFP-SIMK and tagRFP-SIMKK in transiently co-transformed leaf epidermal cells of *N. benthamiana* before and after salt (500 mM NaCl) treatment. **(A-C)** GFP-tagged SIMK **(A)** and tagRFP-SIMKK **(B)** colocalize **(C)** in *N. benthamiana* epidermal leaf cells. Both fusion proteins showed predominantly nuclear (arrows) and cytoplasmic localization (merged image shown in C). **(D-F)** After salt treatment (500 mM NaCl for 10 min), both GFP-SIMK **(D)** and tagRFP-SIMKK **(E)** simultaneously relocated to the cytoplasm where they colocalized on cytoplasmic punctate compartments (merged image shown in **F**, indicated by arrowheads). Scale bar: 100 µm.

3.2.3 Stable transformation of *M. sativa* leaf explants using somatic embryogenesis

Surface-sterilized leaves of alfalfa RSY L1 plants were transformed with *A. tumefaciens* strain GV3101 carrying *35S::GFP:SIMK* construct and plants regenerated by somatic embryogenesis (**Figure 12**). Indirect somatic embryogenesis was induced from young developing leaves with three leaflets (**Figure 12A**) that were gently surface-sterilized and cut into parts (**Figure 12B**). It passed through the stage of callus formation, which was induced by the application of the appropriate combination of exogenous phytohormones (**Figure 12C**). Subsequently, dedifferentiated calli were cultivated on the culture medium lacking phytohormones leading to the induction of embryogenic calli and somatic embryos (**Figure 12D**). Fully developed somatic embryos showed apical-basal polarity and were promoted to form roots and shoots (**Figure 12E**) up to the regeneration of complete plants developed and maintained *in vitro* (**Figure 12F**). Mature plants obtained by this way were fertile and able to form flowers, produce seeds and the next generation of plants from germinated seeds.

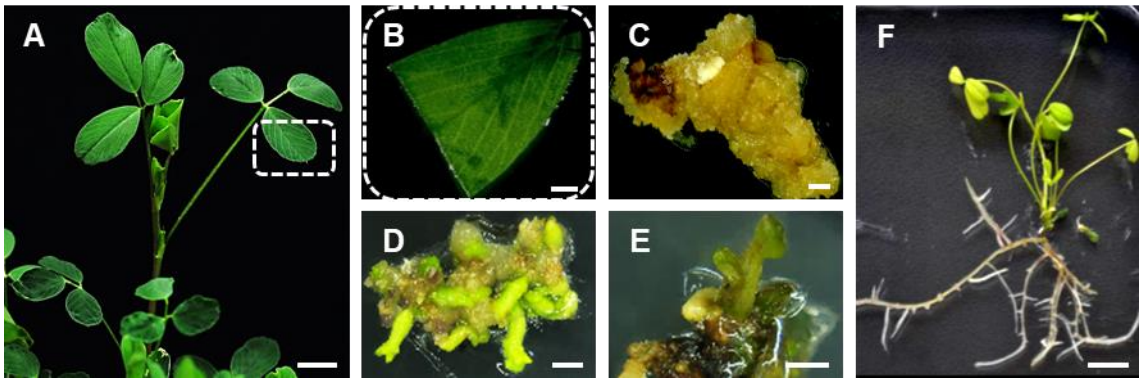


Figure 12 Representative overview showing *in vitro* propagation, multiplication and regeneration of transgenic alfalfa plants using somatic embryogenesis. **(A)** The process is induced from young leaves that are placed into *Agrobacterium* culture containing *35S::GFP:SIMK* construct and transferred to **(B)** appropriate culture medium (B5H medium). **(C)** Subsequently, the massive callogenesis occurs on the leaf explant on the selective antibiotics-containing culture medium followed by the formation of embryogenic calli (B50 medium). **(D)** The next steps of the procedure involve culture media rich in amino acids and vitamins, and for the germination of somatic embryos (MMS medium) regenerating to the **(E)** complete plants on MS medium. **(F)** Regenerated plantlets are fully viable. Scale bar: 10 cm in **(A)**, 0.5 mm in **(B)**, 1 cm in **(C, F)** and 2 cm in **(D, E)**.

3.2.4 Subcellular localization of GFP-SIMK

In order to observe subcellular localization of SIMK in alfalfa plants, SIMK was tagged with GFP marker and overexpressed under *35S* promoter. Subcellular localization

of GFP-SIMK fusion protein was performed using confocal laser scanning microscopy (CLSM) and Airyscan CLSM (**Figure 13**). Maximum intensity projection provided overview of alfalfa root tip and revealed nuclear and cytoplasmic GFP-SIMK localization (**Figure 13A**) with lower signal in nucleoli. GFP-SIMK preferentially accumulated in the nucleus and less in the cytoplasmic structures of hypocotyl cells (**Figure 13B**) in stably transformed alfalfa plants. Similar subcellular localization was found also in root hairs (**Figure 13C**), leaf epidermal cells and stomata (**Figure 13D**), and in root border cells detached from lateral root cap (**Figure 13E**). In growing root hairs, GFP-SIMK was mostly localized in nuclei and in the cytoplasm at the root hair tips (**Figure 13C**).

The pattern of subcellular localization in root cells was confirmed in GFP-SIMK transgenic line by using whole-mount immunofluorescence co-immunolabeling (Tichá *et al.*, 2020a) with GFP-specific (see Materials and Methods) and phospho-specific (anti-phospho-p44/42) antibodies (**Figure 14**). Imaging of co-immunolabeled samples with Airyscan CLSM revealed that GFP-SIMK was localized in distinct spot-like structures in the nucleoplasm and in cytoplasmic structures, preferentially in activated form (**Figure 14A-D**). Moreover, GFP-SIMK was localized in activated form in the root hair tip (**Figure 14E-H**).

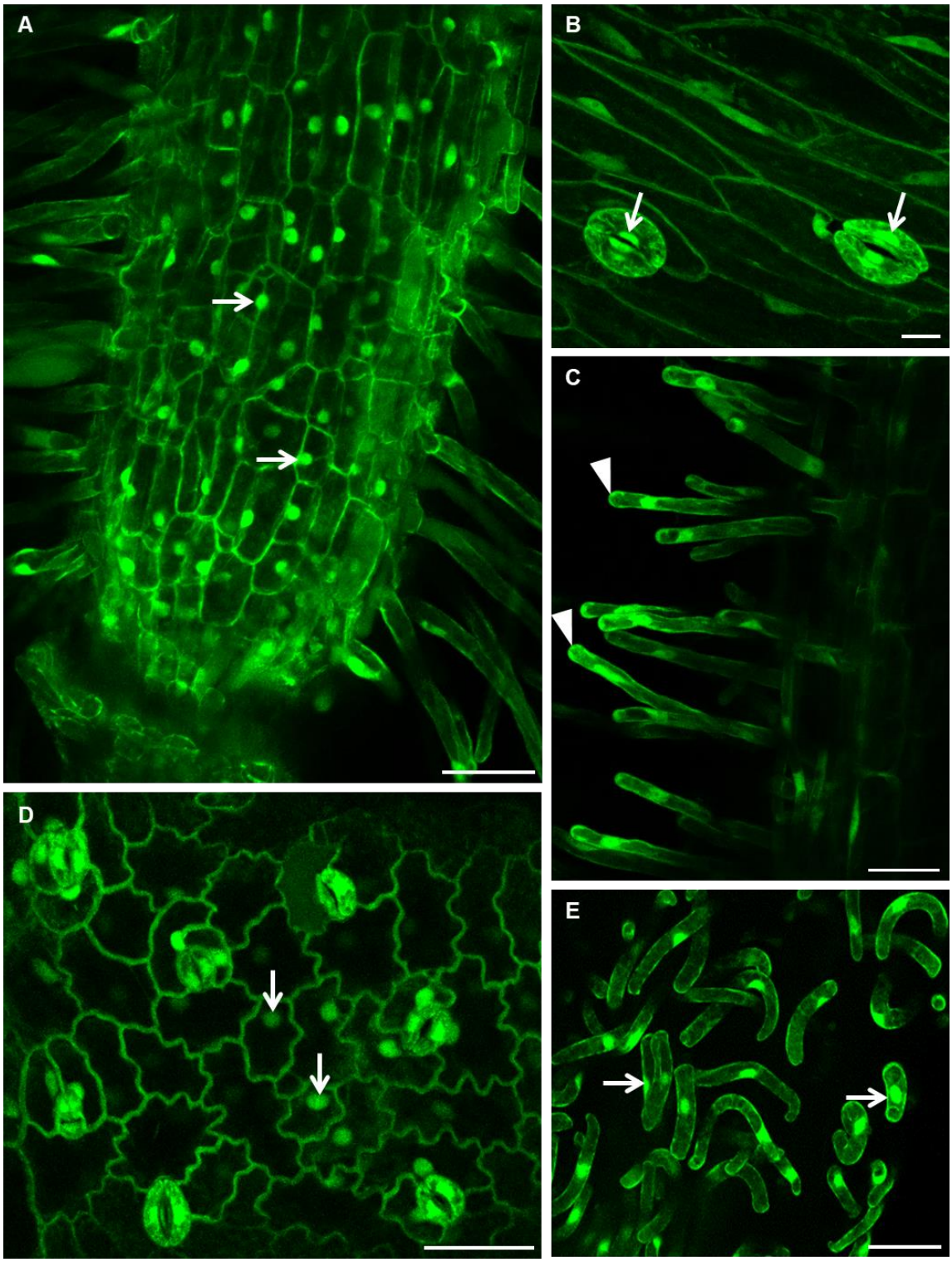


Figure 13 Subcellular localization of GFP-SIMK in stable transformed alfalfa plants using CLSM and Airyscan CLSM. **(A)** Overview of GFP-SIMK distribution pattern in the root. **(B)** Subcellular localization of GFP-SIMK in hypocotyl epidermal cells and stomata, **(C)** root hairs, **(D)** leaf epidermal cells and stomata, and **(E)** in border cells liberated from lateral root cap. Note localization of GFP-SIMK in nuclei (arrows in **A, B, D** and **E**), root hair tips (arrowheads in **C**) and in the cytoplasm. Scale bar: 50 µm in **(A, B, D, E)** and 20 µm in **(C)**.

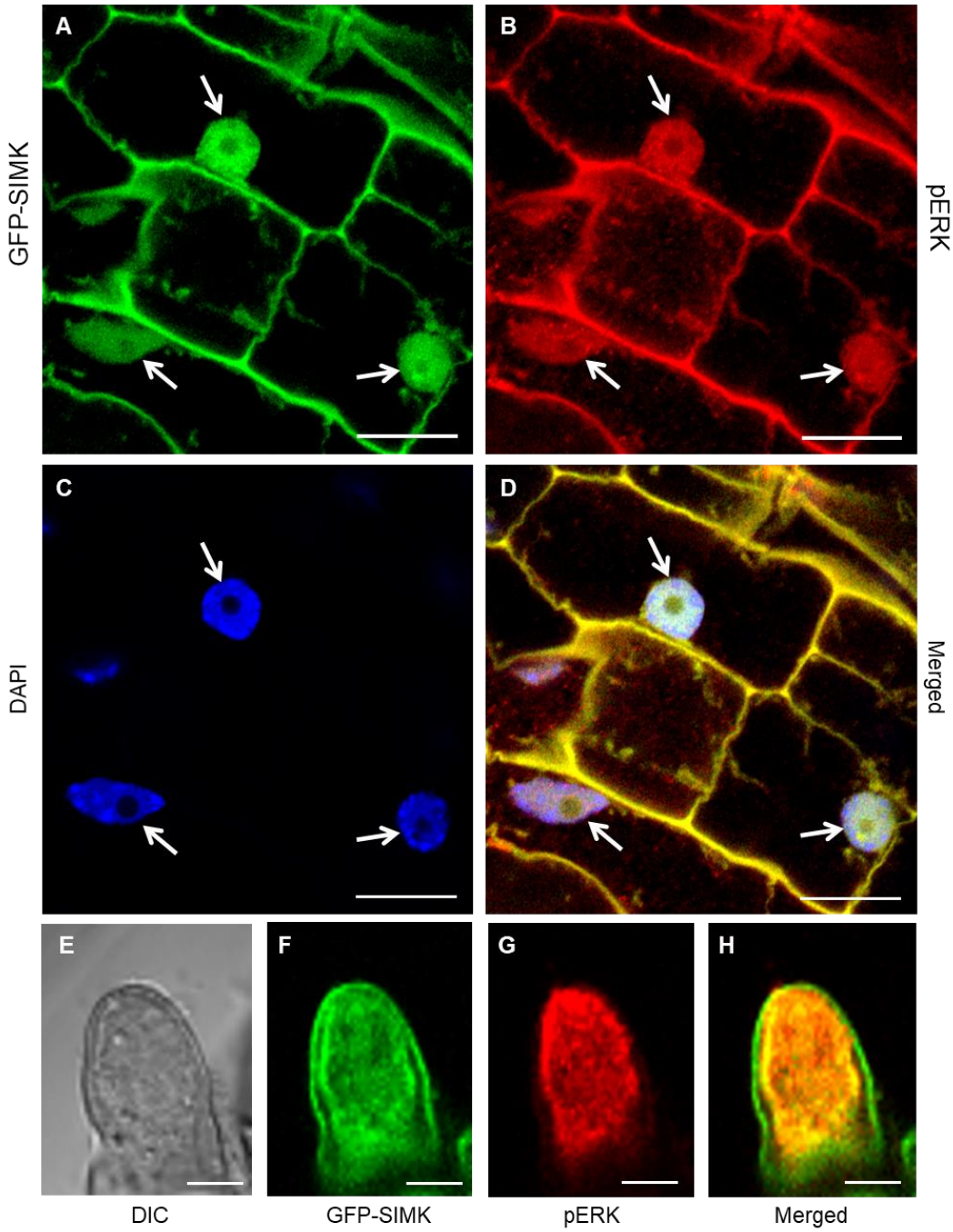


Figure 14 Whole-mount immunofluorescence localization of GFP-SIMK in epidermal cells and root hair tip of stable transformed alfalfa root using Airyscan CLSM. **(A-D)** Whole-mount immunofluorescence localization of GFP-SIMK in stable transformed root epidermal cells. **(A)** Immunolocalization of GFP-SIMK using anti-GFP antibody, **(B)** whole-mount immunolocalization of activated MAPKs (pERK) using anti-phospho-p44/42 antibody, **(C)** DAPI staining of DNA in nuclei, and **(D)** merged image of root epidermal cells. **(E-H)** Whole-mount immunofluorescence localization of GFP-SIMK in root hair tip of stable transformed alfalfa root with Airyscan CLSM. **(E)** Immunolocalization of root hair tip in DIC (differential interference contrast) microscopy, **(F)** immunolocalization of GFP-SIMK using anti-GFP antibody, **(G)** immunolocalization of activated MAPKs (pERK) using anti-phospho-p44/42 antibody, and **(H)** merged image of root hair tip. Note subcellular colocalization of SIMK and pERK in nuclei (arrows), in the cytoplasm **(D)** and in root hair tip **(H)**. Scale bar: 10 μm in **(A-D)**, 5 μm in **(E-H)**. Images provided by Olga Šamajová and Kateřina Hlaváčková. Adapted from Hrbáčková *et al.* (2021).

3.3 Discussion

The MAPK-mediated phosphorylation pathways are essential components of plant cellular signaling. They capture extracellular and developmental cues and translate them into intracellular signals. Typical MAPK cascade is composed of three functionally linked MAPKKK, MAPKK and MAPK (Komis *et al.,* 2018; Šamajová *et al.,* 2013b). Recent studies using fluorescent protein tagging technology revealed subcellular localization of MAPKs in several model organisms, such as *A. thaliana* (Jia *et al.*, 2016; Müller *et al.,* 2010), cotton (Shi *et al.,* 2011; Zhang *et al.,* 2011), *Nicotiana* species (Ishihama *et al.,* 2011), or *Medicago* species (Hrbáčková *et al.,* 2021; Ovečka *et al.,* 2014; Šamaj *et al.,* 2002). MAPK-tagged with GFP fusions are usually driven by potent constitutive promoters, such as the *35S* promoter of cauliflower mosaic virus (Palmer and Freeman, 2004; Šamajová *et al.,* 2013a) or by their own native promoter. In this work, alfalfa SIMKK and SIMK have been cloned with fluorescent marker proteins under *35S* promoter. CLSM have been applied to visualize them in different organelles and subcellular compartments in living plant organs.

Transient transformation is an efficient method for experiments aiming to visualize subcellular compartments such as nuclei, endoplasmic reticulum, endosome or cytoskeleton labeled by molecular markers containing GFP, YFP (yellow fluorescent protein) or RFP (Křenek *et al.,* 2015; Voinnet *et al.,* 2003, Yang *et al.*, 2000). In our laboratory we are routinely using *Agrobacterium*-mediated transient transformation of *N. benthamiana* leaves. First true leaves are suitable for agroinfiltration. Cloning of constructs for expression of N-terminal GFP-tagged SIMK protein and tagRFP-tagged SIMKK driven under *35S* promoter (*35S::GFP:SIMK*, *35S::tagRFP:SIMKK*) was performed in pB7m34GW,0 by MultiSite Gateway®Three-fragment vector construction kit using modified and optimized *att* sites to permit transfer of heterologous DNA sequences between vectors. Conventional cloning methods offers lower versatility in terms of cloning multiple DNA fragments because the selection of restriction sites can be difficult due to presence or absence of appropriate digestion sites within both DNA fragment and vector. Considering this, the MultiSite Gateway® cloning system was used for the preparation of constructs with *SIMK* and *SIMKK* genes. This cloning strategy is based on the bacteriophage lamda site-specific recombination system (Landy, 1989) that allows highly simultaneous and efficient way to transfer heterologous DNA into multiple vectors. Prepared vectors containing transgenes were transiently transformed into *N.*

benthamiana epidermal leaf cells for testing. Under control conditions, both GFP-tagged SIMK and tagRFP-tagged SIMKK preferentially localized to the nuclei, but they were also dispersed throughout the cytoplasm. Previously, Chen *et al.* (2017) constructed plasmids that expressed GFP-tagged MtMAPKK4 (ortholog of SIMKK) and GFP-tagged MtMAPK6 (ortholog of SIMK) fusion proteins under the control of constitutive *35S* promoter. These plasmids were delivered to *N. benthamiana* leaves. The subcellular localization patterns of GFP-tagged MtMAPKK4 and MtMAPK6 were checked by confocal microscope. Both fusion proteins were localized to the plasma membrane, cytoplasm and nucleus (Chen *et al.*, 2017). Thus, these results were consistent with localization patterns of fluorescently tagged SIMK and SIMKK in transiently transformed *N. benthamiana* leaves. Moreover, these transformed leaves with destination vector carrying *35S::GFP:SIMK* provided enough material for immunoblot assay with anti-AtMPK6 polyclonal antibody recognizing SIMK in GFP-SIMK fusion protein. Transiently transformed leaves with *35S::tagRFP:SIMKK* were used for DNA isolation and for PCR genotyping.

MAPKKs can activate their downstream MAPKs through phosphorylation. For example, AtMPK3, AtMPK6, AtMPK4 and their orthologs in plant species showed that they can be activated by several stimuli (Chen *et al.*, 2017). LjSIP2 from *L. japonicus* is orthologue of SIMKK and is important in symbiosis. LjSIP2 represents a typical plant MAPKK and interacts with AtMPK6 (orthologue of SIMK), but not with AtMPK3 in yeast cells. Recombinant LjSIP2 protein could phosphorylate casein and AtMPK6 *in vitro* (Chen *et al.*, 2012). Moreover, MtMAPKK4 (representing another ortholog of SIMKK) physically interacts with MtMAPK6 (ortholog of SIMK), which was verified in yeast and plant cells (Chen *et al.*, 2017). In alfalfa, yeast two-hybrid screen and activation studies identified SIMKK as an upstream activator of SIMK (Kiegerl *et al.*, 2000; Cardinale *et al.*, 2002). To determine whether SIMKK may be involved in mediating the salt-induced activation of SIMK, Kiegerl *et al.* (2000) performed co-expression experiments with SIMKK and SIMK in the presence and absence of salt stress. The co-expression experiments were performed in protoplasts from suspension-cultured parsley cells. SIMK showed very little kinase activity and was only slightly activated by salt stress, but co-expression with SIMKK resulted in considerably stronger SIMK activation. In order to examine colocalization of GFP-SIMK and tagRFP-SIMKK fusion proteins, *N. benthamiana* leaves were infiltrated by co-cultivated bacteria solution of these two

constructs. Ovečka *et al.* (2014) described salt-induced subcellular relocation of these two kinases. They have studied the activation and localization of the alfalfa SIMKK-SIMK module after NaCl treatment. Both SIMK and SIMKK were tagged with different fluorescent proteins, YFP and CFP, respectively. They were co-expressed in *A. thaliana* protoplasts to investigate their colocalization. Under control conditions in the inactivate state, both YFP-tagged SIMK and CFP-tagged SIMKK preferentially colocalized in the nuclei and were also dispersed throughout the cytoplasm. In contrast, SIMK-YFP and SIMKK-CFP relocated to cytoplasmic punctate compartments where they colocalized after 30 minutes of salt stress (Ovečka *et al.*, 2014). This was consistent with our results showing colocalization of GFP-SIMK and tagRFP-SIMKK fusion proteins. We showed first their colocalization in transiently transformed *N. benthamiana* epidermal leaf cells. After exposure to the salt stress, GFP-SIMK and tagRFP-SIMKK relocated to cytoplasmic compartments. Colocalization studies suggest that SIMKK functions as a scaffold protein for SIMK, or other unknown scaffold proteins can bind both SIMKK and SIMK (Ovečka *et al.*, 2014). The dynamic relocalization of the MAPK and MAPKK components from the nucleus to the cytoplasm seems to challenge the traditional view of MAPKs translocation from the cytoplasm to the nucleus after its activation. SIMK and SIMKK relocation into the cytoplasmic compartments could represent activation-dependent mechanism regulated by salt stress. Supporting evidence that the process of activation is linked with MAPK relocation to membrane targets may be related to studies dealing with salt overly sensitive (SOS) pathway (Kim *et al.*, 2012; Ovečka *et al.*, 2008, 2014). In addition, salt stress can trigger different MAPK signaling pathways in *Arabidopsis* resulting in positive or negative plant tolerance. In this respect, it is well known that AtMPK6 and AtMPK3 are activated by salt stress and it seems to be important for salt tolerance of *A. thaliana* (Ichimura *et al.*, 2000; Yu *et al.*, 2010). Another recent report showed the colocalization of *Arabidopsis* MPK6 (orthologue of SIMK) and clathrin at the plasma membrane and at trans-Golgi network (TGN) vesicles (Müller *et al.*, 2010). In this context, activated kinase modules have been identified in clathrin-coated vesicles and signaling endosomes in animals (Howe *et al.*, 2001; Sorkin and Von Zastrow, 2002).

Expression efficiency of GFP-SIMK and tagRFP-SIMKK fusion proteins was checked using transient transformation before stable transformation of alfalfa. In this thesis, a highly efficient stable transformation approach using *A. tumefaciens* was selected

for introducing foreign genes of interests into alfalfa. The stable transformation of plants mediated by *A. tumefaciens* is inheritable and persists in transformant progeny (Samac and Austin-Phillips, 2006; Sangra *et al.,* 2019). The modified protocol of Samac and Austin-Phillips (2006) for alfalfa transformation was used for this purpose. Normally, the protocol provides 80-100 % of successfully transformed plants regenerated from somatic embryos. The protocol described in this thesis utilizes highly regenerable genotype Regen-SY (Bingham, 1991) and allows a long-term maintenance of somatic embryos through subculture cycles (Samac and Austin-Phillips, 2006; Sangra *et al.,* 2019). The original protocol works with *A. tumefaciens* strain LB4404, but we tried and optimized this stable transformation with *A. tumefaciens* strain GV3101. An appropriate composition of culture media played a key role in alfalfa propagation protocol. To increase efficiency, various substances, such as vitamins, amino acids, growth regulators, or organic acids were added to the culture media (Amini *et al.,* 2016). Transgenic alfalfa plants can be regenerated within 9-14 weeks after the co-cultivation with agrobacteria. Wild type control plants and stably transformed alfalfa lines can be regenerated and propagated via somatic embryogenesis.

Using stable transgenic line, we were able to describe subcellular localization of GFP-SIMK protein in planta. Our subcellular and developmental observations using live cell CLSM imaging revealed GFP-SIMK localization predominantly to the nucleus and cytoplasm in various cell types (e.g. root border cells, root hairs, epidermis, pavement cells and stomata) of diverse organs including roots, hypocotyls and leaves. Some previous studies showed *in situ* hybridization and immunolocalization of both SIMK transcript and protein in alfalfa root hairs (Baluška *et al.,* 2000a; Munnik *et al.,* 1999; Ovečka *et al.,* 2014; Šamaj *et al.,* 2002, 2003). Later on, Chen *et al.* (2017) cloned *MtMAPKK4* and *MtMAPK6* cDNAs from *M. truncatula* with *GFP* under the *35S* promoter. GFP-tagged fusion proteins were delivered to *N. benthamiana* leaves to check their expression. They also examined mRNA transcript levels in different tissues and nodulation stages by quantitative RT-PCR. The expression of *MtMAPKK4* and *MtMAPK6* genes was detected in almost all examined tissues, including roots, stems, leaves and nodules of *M. truncatula* (Chen *et al.,* 2017). We stably transformed alfalfa to examine GFP-SIMK localization patterns using modern fluorescent microscopy methods. SIMK was strongly expressed under constitutive *35S* promoter in alfalfa root cells and it accumulated within nuclei of both meristematic and postmitotic cells of the transition

zone, consistently with previous *in situ* localization study (Baluška *et al.*, 2000a). The situation changed dramatically in growing root hairs. GFP-tagged SIMK redistributed from nuclei into the cytoplasm and accumulated at the tips of emerging and growing root hairs. This was in agreement with previously published SIMK localization pattern using immunofluorescence microscopy (Šamaj *et al.*, 2002, 2003). Concerning subnuclear localization, SIMK was less abundant in nucleoli. The pattern of subcellular localization of SIMK protein in root cells was confirmed in GFP-SIMK transgenic lines by using whole-mount immunofluorescence co-labeling with GFP-specific and phospho-specific (anti-phospho-p44/42) antibodies. Moreover, activated state of SIMK was confirmed with whole-mount immunolabeling in the root hair tip using phospho-specific antibodies. This whole-mount immunolabeling protocol is relatively fast and enables high-resolution microscopy imaging without the need to control proper physiological and environmental conditions during imaging, which are crucial for live cell imaging (Komis, *et al.*, 2018; Tichá *et al.*, 2020a). Nevertheless, it is mostly restricted only to root apices, and it is not suitable for dynamic live cell imaging provided by recombinant GFP technology used in this study.

4 Part III - Overexpression of SIMK promotes root hair growth, IT and nodule clustering and shoot biomass production in *M. sativa*

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Legumes are able to perform symbiotic interactions with nitrogen-fixing soil bacteria collectively called rhizobia (e.g. *Bradyrhizobium* or *Sinorhizobium*) which can reduce atmospheric dinitrogen (N_2) into ammonium (NH_3) in specialized organs, the so called root nodules (Wang *et al.,* 2018). Although nitrogen is one of the most abundant elements on Earth, it is very critical element for growth of plants (Rajwar *et al.,* 2013). There are approximately 700 genera and about 13,000 species of legumes and only 20 % of them are able to form nodules (Vance, 2001). This type of symbiosis plays an essential role in both natural and agronomical systems (Oldroyd *et al.,* 2011; Geurts *et al.,* 2016; Ryu *et al.,* 2017). Nodule development is a multistep process comprising mutual recognition of host plants and bacteria, attachment of bacteria to the root hair, root hair curling, bacteria internalization, formation of infection thread, and nodule development. Inside the nodules, bacteria create bacteroids, which are able to fix nitrogen (Rajwar *et al.,* 2013; Wang *et al.,* 2018).

This chapter describes production parameters of transgenic alfalfa plants with genetically manipulated SIMK after infection with *S. meliloti.* Overexpression of GFP-SIMK led to longer root hair phenotypes and promoted ITs and nodule clustering. On the other hand, we employed *SIMKK RNAi* lines showing downregulation of both *SIMKK* and *SIMK*, which was accompanied by shorter root hairs, and less ITs and nodules. Moreover, *SIMKK* and *SIMK* downregulation led to decrease, while overexpression of GFP-tagged SIMK led to increase of biomass in above ground part of plants. These data suggest that genetic manipulations causing downregulation or overexpression of SIMK affect root hair, nodule and shoot formation patterns in alfalfa, and point to the new biotechnological potential of this MAPK.

4.1 Material a methods

4.1.1 Plant and bacterial material and growth conditions

Somatic embryos of wild type plants of alfalfa RSY (two independent lines L1 and L2), transgenic lines with *SIMKK RNAi* (SIMKKi, two independent lines L3 and L4) and transgenic lines carrying *35S::GFP:SIMK* construct (GFP-SIMK, two independent lines L5 and L6) with well-developed root poles were separated, individually transferred and inserted into root and plant development medium (MMS) or Fåhreus medium without nitrogen (FAH-N_2; Fåhreus, 1957). Regenerated plants were inoculated with *S. meliloti* Sm2011 strain (Casse *et al.,* 1979). Plants were grown in an environmental chamber at 21°C and 70% humidity in a 16-h light/8-h dark cycle. Illumination intensity was 150 μmol $m^{-2}s^{-1}$.

4.1.2 Root hair phenotypic analysis and *M. sativa* plant inoculation with *S. meliloti*

Wild type plants of alfalfa RSY (lines L1 and L2), transgenic plants with *SIMKK RNAi* (SIMKKi, lines L3 and L4) and transgenic plants carrying *35S::GFP:SIMK* construct (GFP-SIMK, lines L5 and L6) were used for phenotypic analysis of root hairs. Regenerated plants (18-day-old, originating from somatic embryos) were transferred to Petri dishes with FAH-N_2 medium containing 13 g/L micro agar. These plants were used for root hair imaging with Axio Zoom.V16 (Carl Zeiss, Germany). Statistics was calculated in SigmaPlot11.0 using Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's Method) and was based on N=529-1924 with statistical significance between treatments at $p < 0.05$, indicated by lower case letters. Plants were inoculated with bacteria *S. meliloti* strain Sm2011 labeled with mRFP with OD_{600} = 0.5 (Boivin *et al.,* 1990). After 10 days post inoculation (10 dpi) infection threads were counted using Axio Zoom.V16 (Carl Zeiss, Germany) with excitation laser line 561 nm and emission filter 565 – 583 nm for mRFP and plants were scanned 5, 10, 15 and 20 dpi for visual evaluation of nodule numbers and arrangements. Statistical analysis was performed with help of Ivan Luptovčiak.

4.1.3 Shoot biomass phenotypic analysis

Images of above ground parts of RSY line L1, SIMKKi line L4 and GFP-SIMK line L5 regrown in pots 60 days after shoot cutting (Gou *et al.,* 2018) were acquired by digital camera (Nikon D5000, Japan). Individual shoots were detached from the plants and shoot length (in cm), shoot weight (in g), number of shoots per plant, and biomass

weight per plant (in g) were recorded. Quantitative analysis was performed in SigmaPlot11.0 using Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's Method) or using One Way Analysis of Variance (Holm-Sidak method) and was based on N=94-196 shoots (for shoot length and weight) and N=4-13 plants (for number of shoots per plant and biomass weight). Images of fresh shoots and first three developed leaves beneath the shoot apex from RSY line L1, SIMKKi line L4 and GFP-SIMK line L5 (plant lines grown in pots 15 days after cutting of the above ground part) were taken by digital camera (Nikon D5000, Japan). Quantitative analysis of total leaf areas (area of left, right and apical leaflet together) and full lengths of petioles were performed on 1st, 2nd and 3rd leaf of one shoot. In total, leaves of 5 independent shoots from 5 independent plants (maximal N=25 for each 1st, 2nd and 3rd trifoliate leaf) of each line were analyzed. Total leaf areas and lengths of the petioles were measured using measurement functions of ImageJ (http://rsb.info.nih.gov/ij/) and statistically evaluated in SigmaPlot11.0 using Two Way Analysis of Variance (Holm-Sidak method) based on N=11-25 (leaf area) and N=25 (petiole length). Different lower case letters in graphs indicate statistical significance between treatments ($p < 0.05$). Ivan Luptovčiak performed statistical analysis. Images of above ground parts were taken with help of Miroslav Ovečka and Dominik Novák.

4.1.4 Quantitative analysis of transcript levels by RT-qPCR

Total RNA was isolated from the roots of wild type RSY line L1, transgenic line with *SIMKK RNAi* (SIMKKi L4) and transgenic line carrying *35S::GFP:SIMK* construct (GFP-SIMK L5) powdered in liquid nitrogen, and using phenol-chlorophorm extraction (Sigma-Aldrich, USA). RNA concentration and purity were determined before DNaseI digestion with a NanoDrop Lite (ThermoScientific, USA). The template-primer mix for reverse transcription was composed of 1 µl oligo(dT) primers, 1 µl RNasin Plus RNase inhibitor (Promega, USA), 1 µg RNA and PCR H_2O in a total volume of 14 µl. The mixture was denatured at 70°C for 10 min. The following components were added: 4 µl M-MLV reverse transcriptase 5x reaction buffer (Promega, USA), 1 µl deoxynucleotide mix (10 mM each), 1 µl M-MLV reverse transcriptase (Promega, USA) in a total volume of 6 µl. Reverse transcription reactions were performed under the following conditions: 42°C for 50 min and 65°C for 15 min for inactivation of the reverse transcriptase. qRT-PCR was performed in a 96-well plate with the StepOnePlus Real-Time PCR system (Applied Biosystems, USA) using SYBR Green to monitor dsDNA synthesis. The

reaction contained 5 µl Power SYBR Green PCR Master mix (Life Technologies, USA), 2.5 µl cDNA and 2.5 µl gene-specific primers (0.5 µM). The following standard thermal profile was used for all PCRs: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Experiments were performed in three biological replicates. The transcription data were normalized to the transcription of *ACT2* as a reference gene, and relative gene transcription was calculated by the 2^(−ΔΔCt) method. Relative transcript levels were calculated as a ratio to control RSY L1, thus RSY level was always one without dispersion of variation. Statistics was calculated in Microsoft Excel using t-Test and was based on N=3. Error bars represent ± standard deviation (SD). Asterisks indicated statistical significance between treatments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n. s. means no statistical significance. The primers are listed in **Table 3**. Primers for detection of total (endogenous native *SIMKe* + GFP-tagged) *SIMK* transcript were specific for third exon of *SIMK* gene. Primers for endogenous native *SIMKe* transcript were specific for 5' UTR sequence and first exon of *SIMK* gene. Quantitative analysis of transcript levels by RT-qPCR was performed with help of Ivan Luptovčiak.

Table 3 List of forward and reverse primers used for the quantitative real-time PCR analysis.

Primer description	**Primer sequence 5'-3'**
qACT2m_F2	GGATAAGAGGTGAGATCGGAGGG
qACT2m_R2	GCAACCAACCTACAGACATCCAG
qSIMKK_F2	AACCACCAGAAGCTCCAACGA
qSIMKK_R2	ACCTCGAAGCAGTCCATCTCC
Total (endogenous native *SIMKe* + GFP-tagged *SIMK*) specific for 3rd exon	
qSIMK_F1	AATGGACACCGACCTTCACCA
qSIMK_R1	CAGTGCTCCTCCGATAGTGCT
Endogenous native *SIMKe* specific for 5UTR and 1st exon	
qSIMK_5UTR_F1	CAGAGCTTGAAGAAGAGAAACAACA
qSIMK_E1_R1	GATCCCCATCTGCGGCG

4.1.5 Immunoblotting analysis

Immunoblotting analysis was performed as described in Takáč *et al.* (2017). Plants of 20-day-old alfalfa RSY L1, transgenic line with *SIMKK RNAi* construct

(SIMKKi L4) and transgenic line carrying *35S::GFP:SIMK* construct (GFP-SIMK L5) were used for immunoblotting analysis. Roots from 20-day-old alfalfa plants were homogenized using liquid nitrogen to fine powder and the proteins were extracted in E-buffer [50 mM HEPES (pH 7.5), 75 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM NaF, 10 % (v/v) glycerol, Complete™ EDTA-free protease inhibitor and PhosSTOP™ phosphatase inhibitor cocktails (both from Roche, Basel, Switzerland)]. After centrifugation, supernatants were mixed with Laemmli buffer [final concentration 62.5 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 10 % (v/v) glycerol, 300 mM 2-mercaptoethanol]. After protein concentration measurement using Bradford assay equal protein amounts (10 ng) were separated on 12% TGX Stain-Free™ (Bio-Rad) gels (Biorad). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes in a wet tank unit (Bio-Rad) overnight at 24 V and 4°C using the Tris-glycin-methanol transfer buffer. Membranes were blocked in 4% (w/v) bovine serum albumin in Tris-buffered-saline (TBS, 100 mM Tris-HCl; 150 mM NaCl; pH 7.4) at 4°C overnight. Following washing step with TBS-T (TBS, 0.1 % Tween 20) membranes were incubated with polyclonal anti-AtMPK6 antibody (Sigma, Life Science, USA), highly specific for SIMK detection (Bekešová *et al.*, 2015), diluted 1:15000 in TBST-T containing 1% (w/v) BSA or with anti-phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204) antibody (Cell Signaling, Netherlands) diluted 1:1000 in TBS-T containing 1% (w/v) BSA at 4°C overnight. After five washing steps in TBS-T blots were incubated with a horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody (diluted 1:5000) in the case of both anti-AtMPK6 and anti-phospho-p44/42 primary antibodies. The signals were developed using Clarity Western ECL substrate (Biorad, Hercules, CA) and detected on Chemidoc MP documentation system (Biorad). In total nine immunoblots were performed from three biological samples representing different lines. Arbitrary units measured from immunoblotting using software ImageLab (Biorad) were normalized according to stain-free gels for corrections of imbalanced loading. After normalization, relative protein levels were calculated as a ratio to control RSY L1, thus RSY level is one (zero in $\log_2$ graphs) without dispersion of variation. Statistics was calculated in Microsoft Excel using t-Test and was based on N=3-8. Error bars represent SD. Asterisks indicated statistical significance between treatments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n. s. means no statistical significance. Statistical analysis was performed with help of Ivan Luptovčiak.

4.1.6 Statistical analysis

All statistical parameters of the performed experiments are included in the figures or figure legends, number of samples (N), type of statistical tests and methods used, statistical significance denoted by lowercase letters or stars. Statistics was calculated in SigmaPlot11.0 using Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's method) if normality and/or equal variance failed or using One Way Analysis of Variance (Duncan's method) or Two Way Analysis of Variance (Holm-Sidak method) if normality and equal variance passed. Different lowercase letters indicate statistical significance between treatments ($p < 0.05$). Statistical analysis using t-Test was done in Microsoft Excel and statistical significance between treatments is indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.2 Results

4.2.1 Root hair phenotypes in stable transformed *M. sativa* plants

Root hair phenotypes were examined in stable transformed alfalfa lines with downregulated or upregulated *SIMK,* using *SIMKK RNAi* or overexpression (both under constitutive *35S* promoter) approaches, respectively. The appropriate parameter of root hair length in mature parts of the root was selected and measured in these lines for evaluation of root hair growth efficiency. In control wild type lines (RSY, lines L1 and L2) root hair length median value in both of them was 471 μm (**Figure 15A,B,G**). In transgenic lines carrying *SIMKK RNAi* construct (annotated as SIMKKi, lines L3 and L4), showing strong downregulation of *SIMKK* and *SIMK* transcripts (**Figure 17**) and SIMK protein (**Figure 18C**), root hair length median decreased to 345 μm and 311 μm, respectively (**Figure 15C,D,G**). In contrast, overexpressor transgenic lines carrying *35S::GFP:SIMK* construct in wild type RSY background (annotated as GFP-SIMK, lines L5 and L6) showed an increase of root hair length median to 527 μm and 504 μm, respectively (**Figure 15E,F,G**).

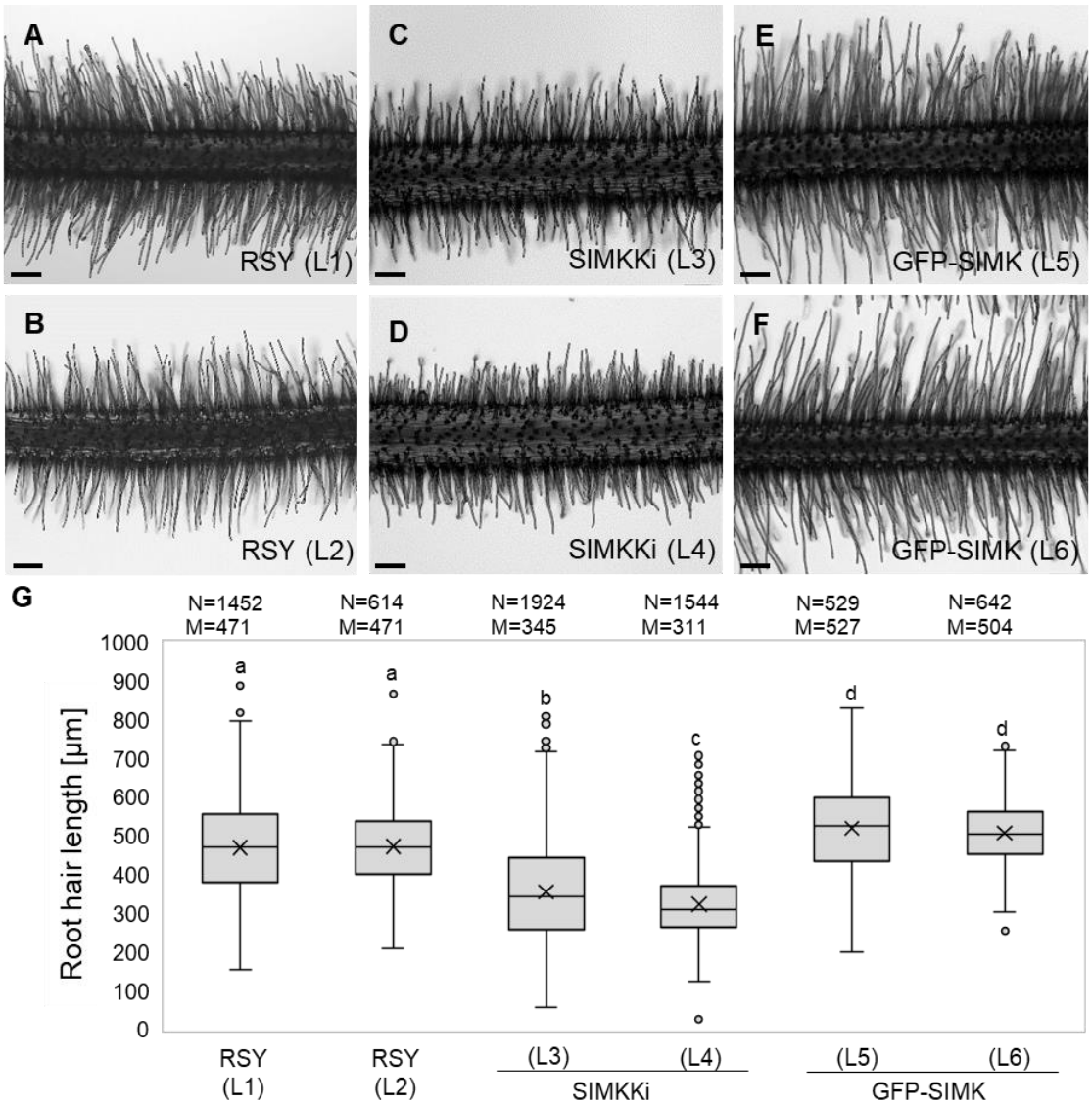

Figure 15 Root hair phenotypes in alfalfa RSY, SIMKK RNAi (SIMKKi) lines and lines overexpressing GFP-SIMK. **(A,B)** Representative images of root hair phenotypes of plants from two independent lines (L1, L2) of control wild type RSY, **(C,D)** two independent transgenic lines with SIMKK RNAi construct (SIMKKi L3, L4), and **(E,F)** two independent transgenic lines expressing *35S::GFP:SIMK* in wild type RSY background (GFP-SIMK L5, L6). **(G)** Box plot graph depicting comparison of root hair lengths of indicated lines, number of observations N and median value M. Statistics was calculated in SigmaPlot11.0 using Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's Method) and is based on N=529-1924. The numbers of root hairs observed were 1452 (RSY L1), 614 (RSY L2), 1924 (SIMKKi L3), 1544 (SIMKKi L4), 529 (GFP-SIMK L5), 642 (GFP-SIMK L6). Different lower case letters indicate statistical significance between treatments ($p < 0.05$). Scale bar: 200 µm in **(A-F)**. Adapted from Hrbáčková *et al.* (2021).

Root hair phenotypes of alfalfa lines were depicted in the form of contingency graph with 25 µm intervals (**Figure 16**). Graph showed a relative root hair number (%) found within each root hair length interval. In SIMKKi lines (L3, L4), the root hair distribution pattern was shifted to the left (**Figure 16**) in comparison to RSY (L1, L2),

which means an earlier cessation of root hair tip growth. In contrast, the distribution of root hairs in GFP-SIMK lines (L5, L6) was shifted to the right while distribution curves showed higher values in the range of longer root hairs (**Figure 16**), which means later cessation of the tip growth and higher proportion of longer root hairs.

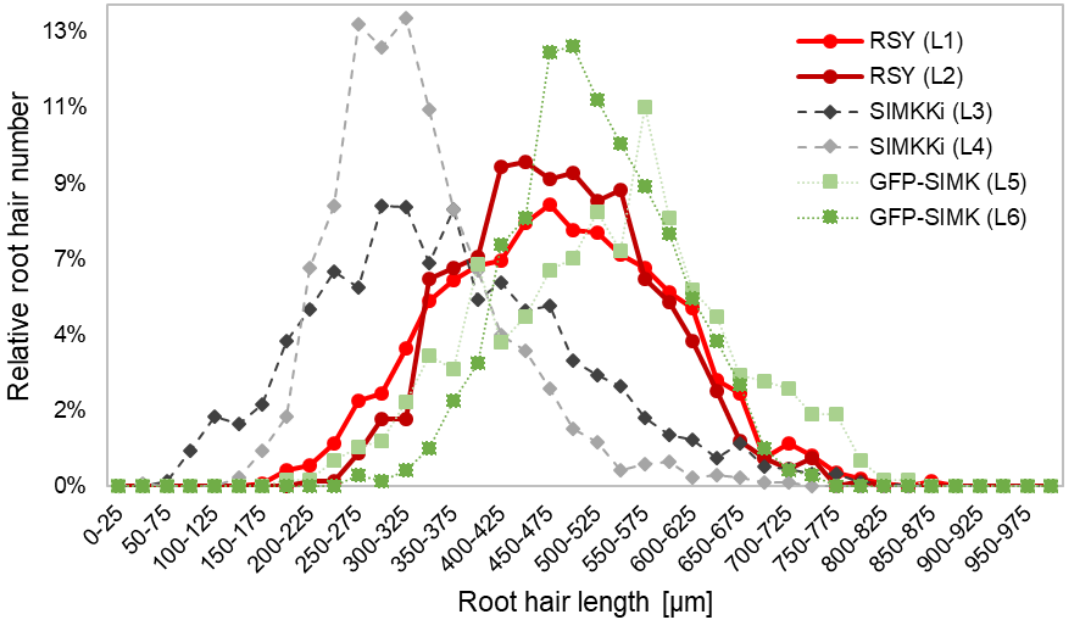

Figure 16 Relative distribution of root hair lengths in indicated alfalfa lines. Normalized root hair number was evaluated using 25 µm intervals distribution. Transgenic lines show different distribution pattern of root hair lengths as compared to RSY wild type lines. Adapted from Hrbáčková *et al.* (2021).

Quantitative RT-PCR of native *SIMKK*, native *SIMK* (*SIMKe*) and total *SIMK* (meaning the sum of *GFP-SIMK* and native *SIMKe* levels) transcripts was performed in order to gain insight in transcriptional regulation of these selected genes in transgenic lines as compared to RSY. This analysis revealed downregulation of *SIMKK* gene in SIMKKi line L4 to approximately 63 % compared to *SIMKK* transcript level in RSY L1. Simultaneously, SIMKKi line L4 showed downregulation of native *SIMKe* transcript level to approximately 30-40 % (screened by two different set of primers) compared to *SIMK* transcript level in RSY L1 (**Figure 17**). In GFP-SIMK line L5 total *SIMK* transcript level was upregulated approximately 3.14 times due to overexpression of *GFP-SIMK*, while native *SIMKe* transcript level was downregulated to approximately 65 % compared to *SIMK* transcript level in RSY L1 (**Figure 17**).

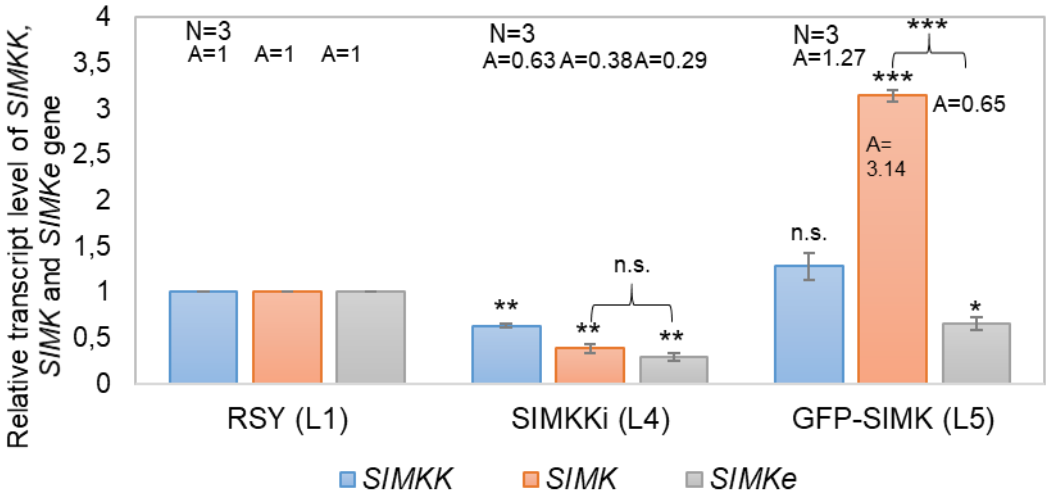

Figure 17 Expression analysis of *SIMKK* and *SIMK* genes by quantitative real time (qRT-PCR). Deregulated transcript levels of *SIMKK*, total (endogenous native *SIMKe* + GFP-tagged) *SIMK* and endogenous native *SIMKe* gene in SIMKKi L4 and GFP-SIMK L5 transgenic lines of *M. sativa*. Statistics was calculated in Microsoft Excel using t-Test and is based on N=3-8. Error bars show ± SD. Asterisks indicate statistical significance between treatments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n. s. indicates no statistical significance.

Immunoblot analysis for semiquantive evaluation of SIMK protein level (**Figure 18A,C**) and phosphorylated SIMK (pSIMK) protein level (**Figure 18B,D**) was performed in order to explain previously obtained phenotypical results at the level of protein abundance and activity. Endogenous SIMK protein with molecular mass around 46 kDa and recombinant GFP-SIMK protein with molecular mass around 72 kDa (**Figure 18A**) were quantified (**Figure 18C**). Relative SIMK abundance was strongly decreased in SIMKKi line L4 to approximately 1 % (**Figure 18A,C**). Relative GFP-SIMK abundance was strongly increased in *35S::GFP:SIMK* line L5 to approximately 6.48 times (**Figure 18A,C**) showing upregulation similarly to relative transcript level (**Figure 17**), while relative abundance of endogenous SIMK showed a decrease to approximately 49 % (**Figure 18A,C**) similarly to reduced relative transcript level (**Figure 17**). These results are consistent with the root hair length phenotypes and indicate that relative SIMK abundance in above-mentioned lines correlates with effectiveness of the root hair tip growth. Phospho-specific pERK antibody was used to check out activity status of respective proteins. Endogenous phosphorylated pSIMK protein with molecular mass around 46 kDa and also phosphorylated GFP-pSIMK with molecular mass around 72 kDa (**Figure 18B**) were quantified (**Figure 18D**). Relative level of pSIMK was considerably decreased in SIMKKi line L4 to approximately 12 % (**Figure 18B,D**) while relative level

of GFP-pSIMK was strongly increased in *35S::GFP:SIMK* line L5 to approximately 8.21 times and relative level of endogenous pSIMK level showed non-significant change compared to RSY line L1 (**Figure 18B,D**). These results are also consistent with root hair length phenotypic results.

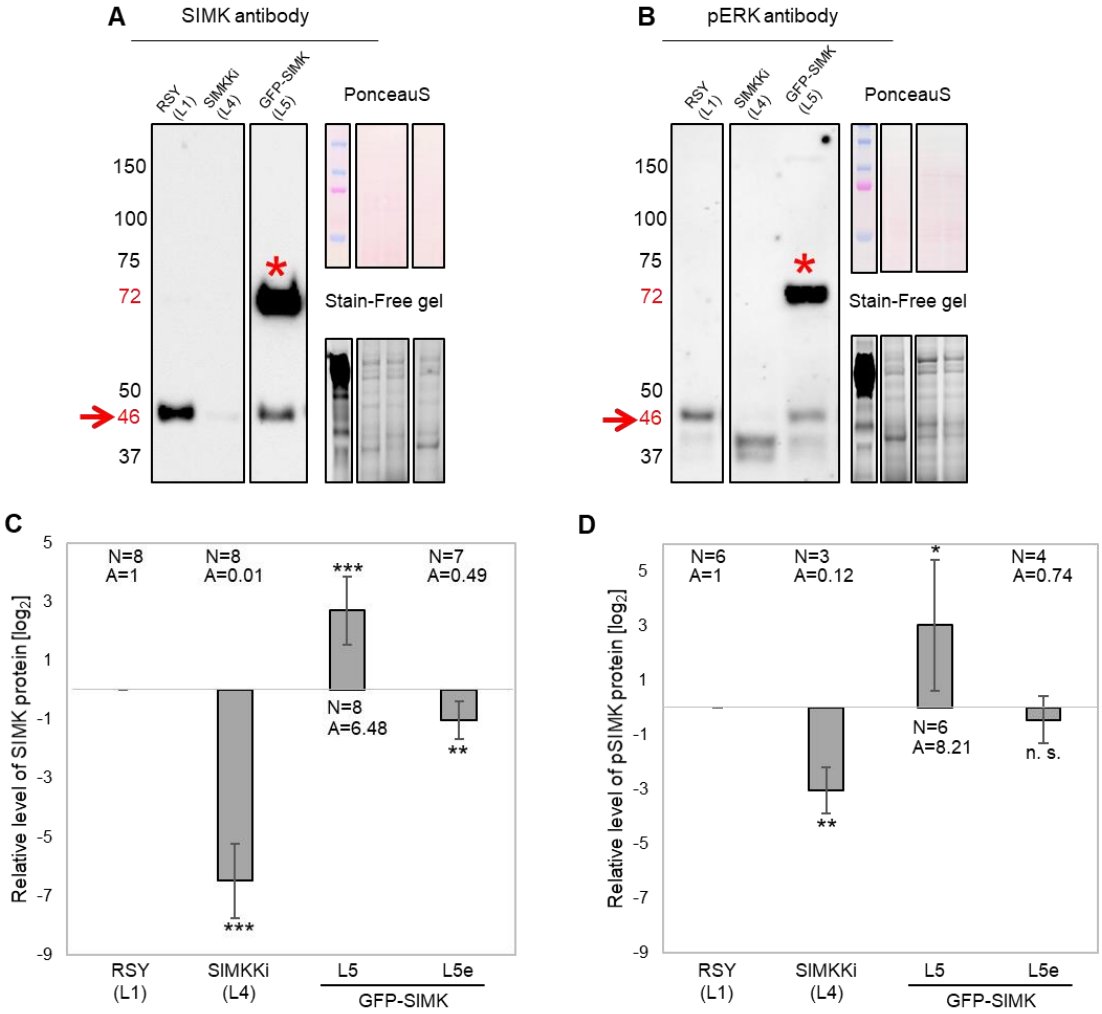

Figure 18 Immunoblotting analysis of total endogenous SIMK, active endogenous SIMK and both total and active GFP-SIMK. **(A)** Western blot detection of SIMK and GFP-SIMK bands using anti-SIMK antibody and **(B)** detection of phosphorylated proteins pSIMK and GFP-pSIMK bands using anti-pERK antibody in root tissue of control and transgenic alfalfa plants of SIMKKi (L4) and expressing *35S::GFP:SIMK* (L5). Arrows point to the 46 kDa bands that corresponds to **(A)** endogenous SIMK and **(B)** endogenous pSIMK, while asterisks show bands around 72 kDa that corresponds to **(A)** GFP-SIMK and **(B)** GFP-pSIMK. **(C,D)** Log_2 graphs depicting comparison of protein levels in respective lines (SIMKKi L4, GFP-SIMK L5) relative to RSY L1, number of observations N and average value A (presented as inversed log_2 values). GFP-SIMK L5e refer to endogenous level of protein, while GFP-SIMK L5 refer to GFP-SIMK level. **(C)** Relative SIMK protein level in roots of control and transgenic plants (RSY1 L1, SIMKKi L4, GFP-SIMK L5). **(D)** Relative pSIMK protein level in roots of control and transgenic plants (RSY1 L1, SIMKKi L4, GFP-SIMK L5). **(C,D)** Statistics was calculated in Microsoft Excel using t-Test and is based on N=3-8. Error bars show ± SD. Asterisks indicate statistical significance between treatments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n. s. indicates no statistical significance. Adapted from Hrbáčková *et al.* (2021).

4.2.2 Impact of overexpressed GFP-SIMK on infection thread formation

Possible function of GFP-SIMK in infection thread (IT) formation was examined after inoculation with *S. meliloti* (Sm2011 strain) labelled with monomeric red fluorescent protein (mRFP). Evaluation of ITs was performed 10 day-post-inoculation (10 dpi) per whole root system in alfalfa RSY plants L1 (**Figure 19A**), transgenic SIMKKi plants L4 (**Figure 19B**) and GFP-SIMK plants L5 (**Figure 19C**). Transgenic lines were compared to RSY and between each other. GFP-SIMK line L5 showed IT clustering (**Figure 19C-E**). ITs were also longer, which was consistent with longer root hairs. Quantitative analysis showed that most of ITs in RSY L1 (76.34 %) and SIMKKi L4 (83.61 %) developed individually, while only 45.74 % of ITs was spatially separated in GFP-SIMK L5 (**Figure 19D**). The rest, 54.26 % of ITs in GFP-SIMK L5 line was present in clusters. Portion of ITs in clusters was only 23.66 % in RSY L1 and 16.39 % in SIMKKi L4 (**Figure 19D**). In RSY L1 and SIMKKi L4 most of the clusters contained two or three ITs, while in GFP-SIMK L5 there was a significant amount of clusters possessing also four or five ITs. In 5.7 % of clusters in GFP-SIMK L5 we found five and more ITs; it occurred in only 4.5 % of clusters in RSY L1, while it was absent completely in SIMKKi L4 (**Figure 19E**).

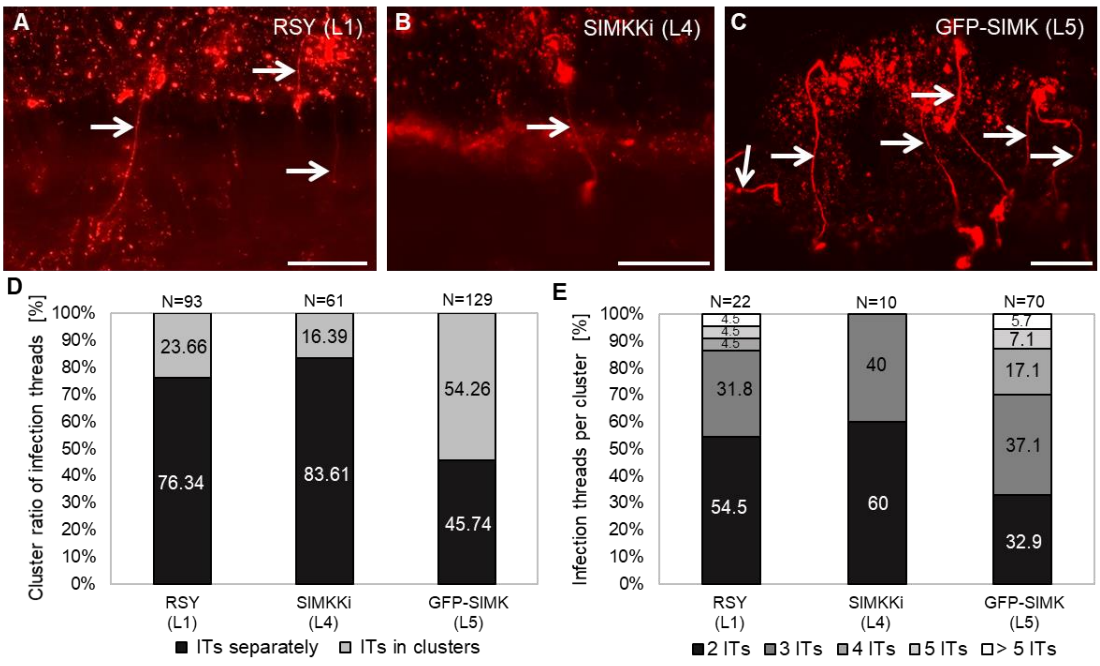

Figure 19 Infection thread and nodule formation in alfalfa roots inoculated with *Sinorhizobium meliloti* labelled with mRFP. **(A-C)** Overview of the infection threads containing *S. meliloti* labelled with mRFP (white arrows) in roots of **(A)** wild type RSY line L1, **(B)** in transgenic SIMKKi line L4 and **(C)** in transgenic GFP-SIMK line L5 at 10 dpi. **(D)** Ratio of individual/clustered infection threads (in %) at 10 dpi. **(E)** Number of infection threads per cluster (in %) at 10 dpi. N = number of observations. Scale bar: 100 µm in (**A-C**). Adapted from Hrbáčková *et al.* (2021).

Live cell microscopic observation of GFP-SIMK, as well as immunolocalization of SIMK in root hairs of alfalfa plants inoculated with *S. meliloti* revealed accumulation of SIMK in infection pockets (**Figure 20**) and its presence along ITs (**Figure 21**). Moreover, the expression level and localization of GFP-SIMK was examined in root nodules of L5 line (**Figure 22**) inoculated with mRFP-marked *S. meliloti*. Nodules were harvested 10 dpi (**Figure 22A**) and 20 dpi (**Figure 22B**) and analyzed by CLSM live-cell imaging with the appropriate settings of lasers for GFP and mRFP channels. GFP-SIMK was expressed in young nodules (**Figure 22A**) as well as in mature nodules, including meristematic (I), infection (II), and symbiotic (III) zones (**Figure 22B**).

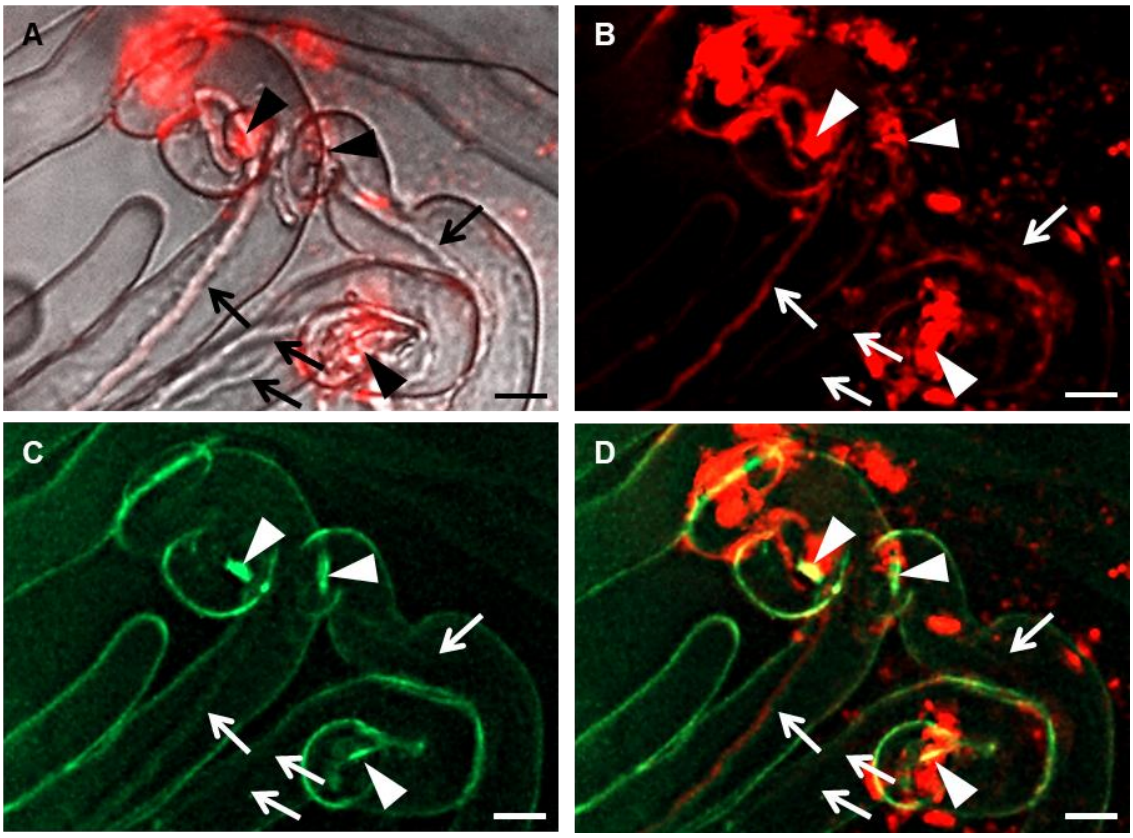

Figure 20 Localization of SIMK during infection thread formation. **(A-D)** Live cell imaging of **(A)** root hairs with ITs, **(B)** *S. meliloti* labelled with mRFP, **(C)** GFP-SIMK and **(D)** merged image using epifluorescence microscope. Arrowheads point to GFP-SIMK accumulation in infection pockets of curled root hairs, arrows point to infection threads. Scale bar: 10 μm in **(A-D)**. Adapted from Hrbáčková *et al.* (2021).

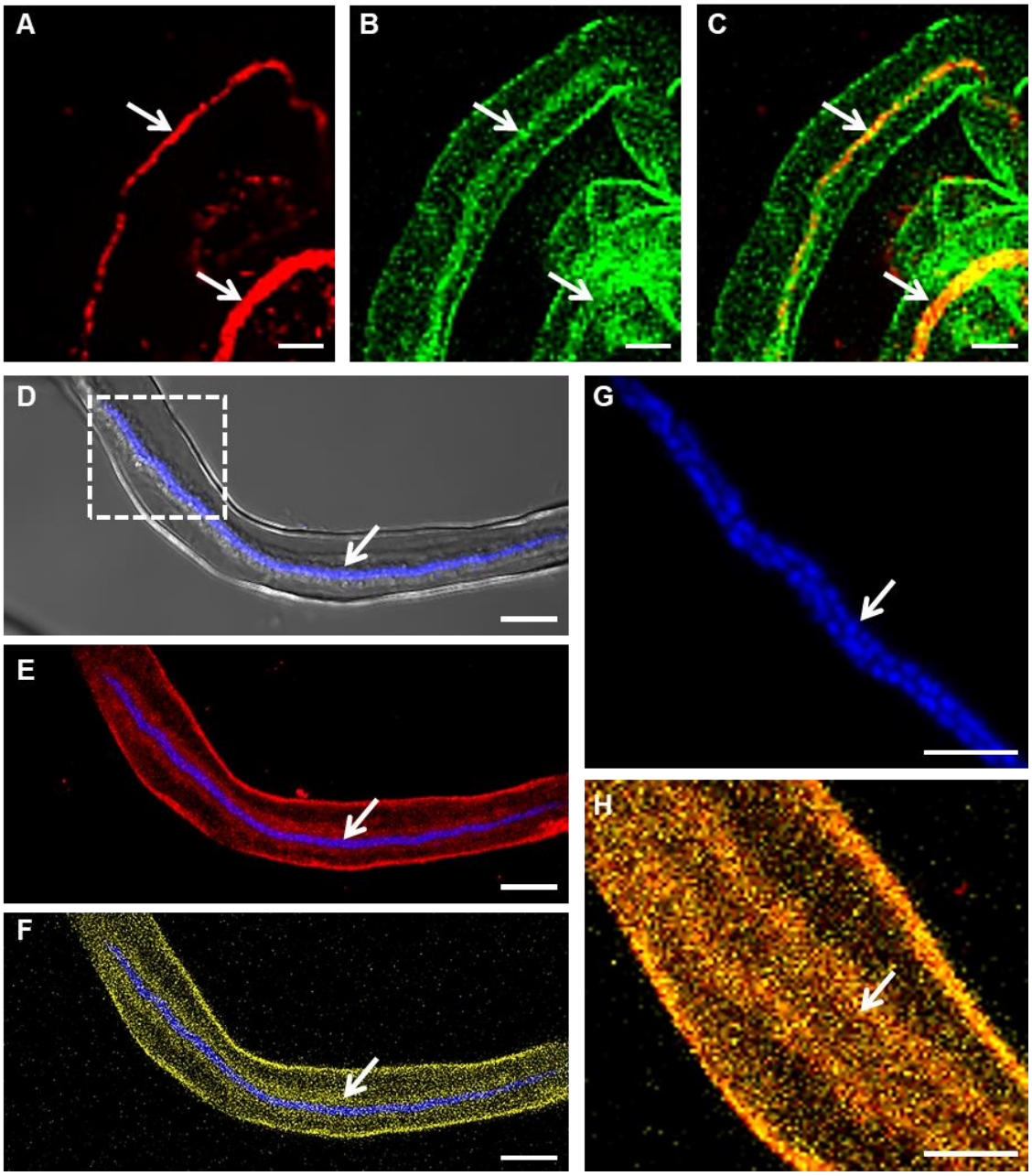

Figure 21 Localization of SIMK during infection thread formation. **(A-C)** CLSM live cell imaging of **(A)** *S. meliloti* labelled with mRFP, **(B)** GFP-SIMK and **(C)** merged image. **(D-H)** CLSM whole-mount immunofluorescence localization of **(D)** DAPI-stained bacteria in IT within root hair, **(E)** SIMK, **(F)** activated MAPKs (pERK), **(G)** DAPI-stained bacteria, and **(H)** merged image of SIMK and activated MAPKs (pERK) in a close-up view of the white marked box in **(D)**. Arrows point to ITs. Scale bar: 10 μm in **(A-F)**; 5 μm in **(G-H)**. Images **D-H** were provided by Kateřina Hlaváčková. Adapted from Hrbáčková *et al.* (2021).

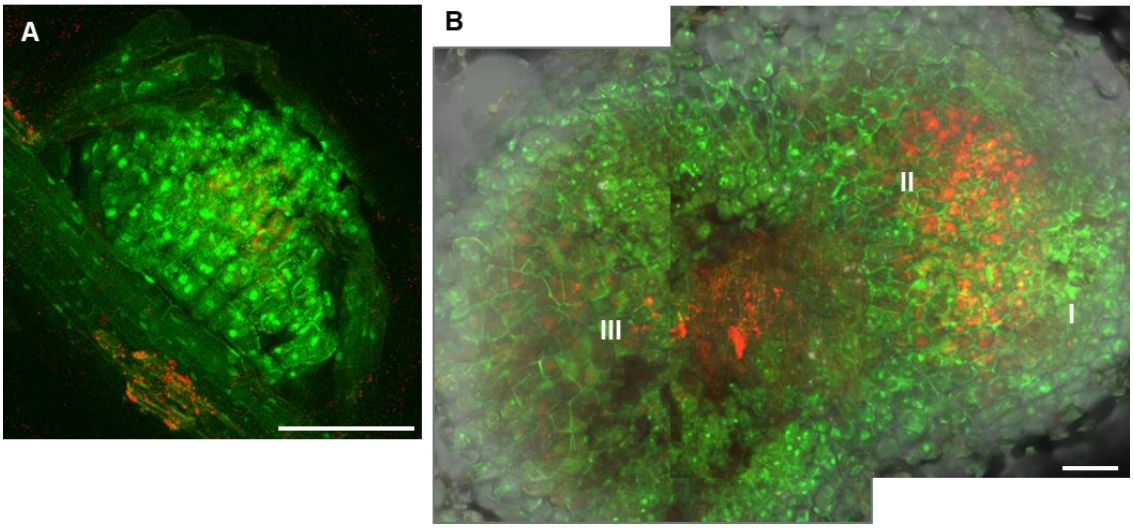

Figure 22 Localization of GFP-SIMK in alfalfa root nodules. Examples of nodule **(A)** at the early stage of development and **(B)** at the late stage of development observed by CLSM. Localization of GFP-SIMK fusion protein (green) in root nodules developed after inoculation of GFP-SIMK line L5 with *S. meliloti* marked with mRFP (red) at 10 dpi **(A)** and 20 dpi **(B)**. Tissue organization of the late nodule: I, meristematic zone; II, infection and differentiation zone; III, symbiotic zone. Scale bar: 100 μm in **(A)**; 200 μm in **(B)**. Adapted from Hrbáčková *et al.* (2021).

4.2.3 Impact of overexpressed GFP-SIMK on nodule formation

When ITs reach the nodule primordium, rhizobia are released into host cells by an endocytosis, which allows to form functional nitrogen-fixing bacteroids within infected plant cells of the root nodule. Possible function of GFP-SIMK in nodule formation was examined after alfalfa inoculation with *S. meliloti* (Sm2011 strain) labelled with monomeric red fluorescent protein (mRFP). Evaluation of nodules was performed 15 days-post-inoculation (15 dpi) per whole root system in alfalfa RSY plants L1 (**Figure 23A, 24A,B**), transgenic SIMKKi plants L4 (**Figure 23B, 24C,D**) and GFP-SIMK plants L5 (**Figure 23C, 24E,F**). GFP-SIMK line L5 often produced nodules in clusters (**Figure 23C, 24E,F**), which was consistent with previous results in infection thread formation. This was less frequent in RSY line L1 and in SIMKKi line L4 (**Figure 23B, 24C,D**). Analysis of nodule clustering showed that 89.8 % of nodules in RSY L1, 95 % in SIMKKi L4 and 87.4 % in GFP-SIMK L5 developed individually (**Figure 23D**). However, clusters in transgenic GFP-SIMK L5 line contained much higher number of nodules in comparison to RSY L1 and SIMKKi L4 (**Figure 23E**). Detailed analysis revealed that 27.3 % of clusters in GFP-SIMK line L5 possessed five and more nodules while in RSY L1 it was only in 2.9 % of clusters (**Figure 23E**) and SIMKKi line L4 did not form clusters with five or more nodules (**Figure 23E**). On the contrary, RSY line L1 and SIMKKi line

L4 had 62.9 % and 61.5 % of clusters formed from two nodules only, respectively, as compared to 18.2 % of such clusters in GFP-SIMK line L5 (**Figure 23E**). It is resembling IT clustering where the ratio of clusters with two ITs represented 54.5 % and 60 % in RSY line L1 and SIMKKi line L4, respectively, but it was only 32.9 % in GFP-SIMK line L5 (**Figure 19E**). GFP-SIMK line L5 was able to very effectively produce ITs and nodules spatially organized in bigger clusters (**Figure 19C, 23C**).

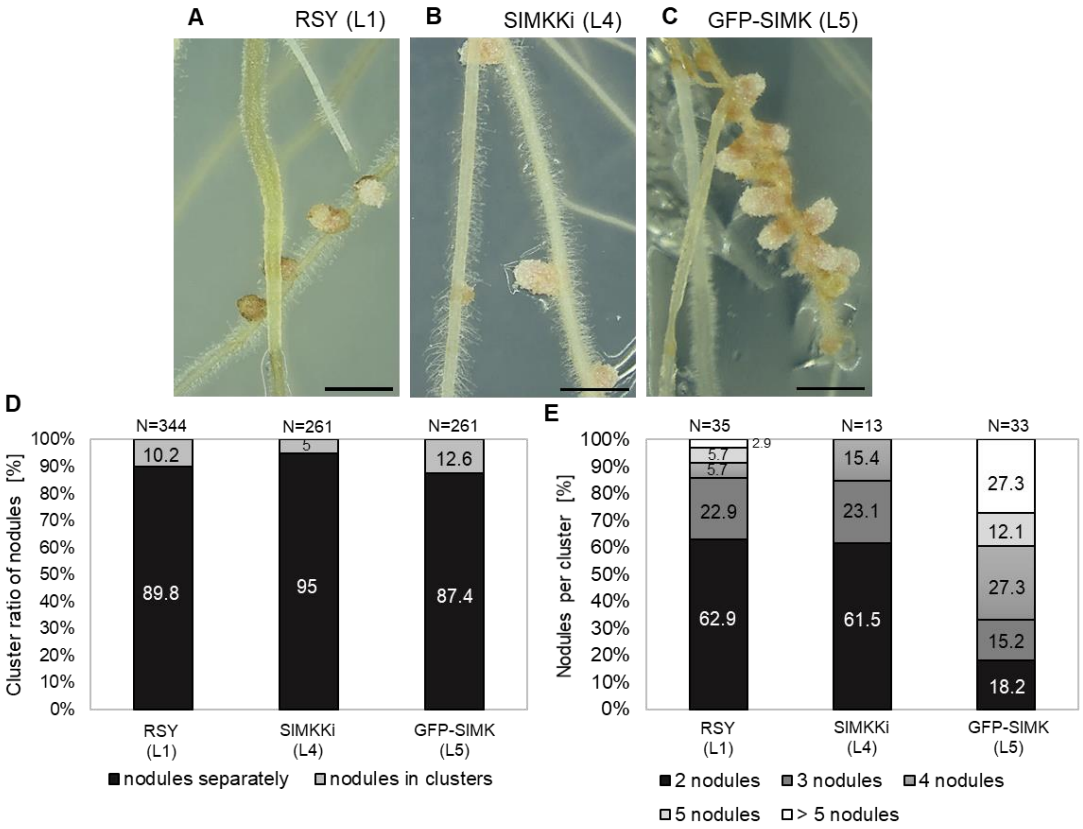

Figure 23 Nodule formation in alfalfa roots inoculated with *Sinorhizobium meliloti*. **(A-C)** Representative images of root nodules formed in respective alfalfa lines, **(A)** control RSY line L1, **(B)** SIMKKi line L4 and **(C)** GFP-SIMK line L5 inoculated with *S. meliloti* labelled with mRFP on Fåhreus medium at 15 dpi. **(D)** Ratio of individual/clustered nodules (in %) at 15 dpi. **(E)** Number of nodules per cluster (in %) at 15 dpi. N = number of observations. Scale bar: 1 cm in **(A-C)**. Adapted from Hrbáčková *et al.* (2021).

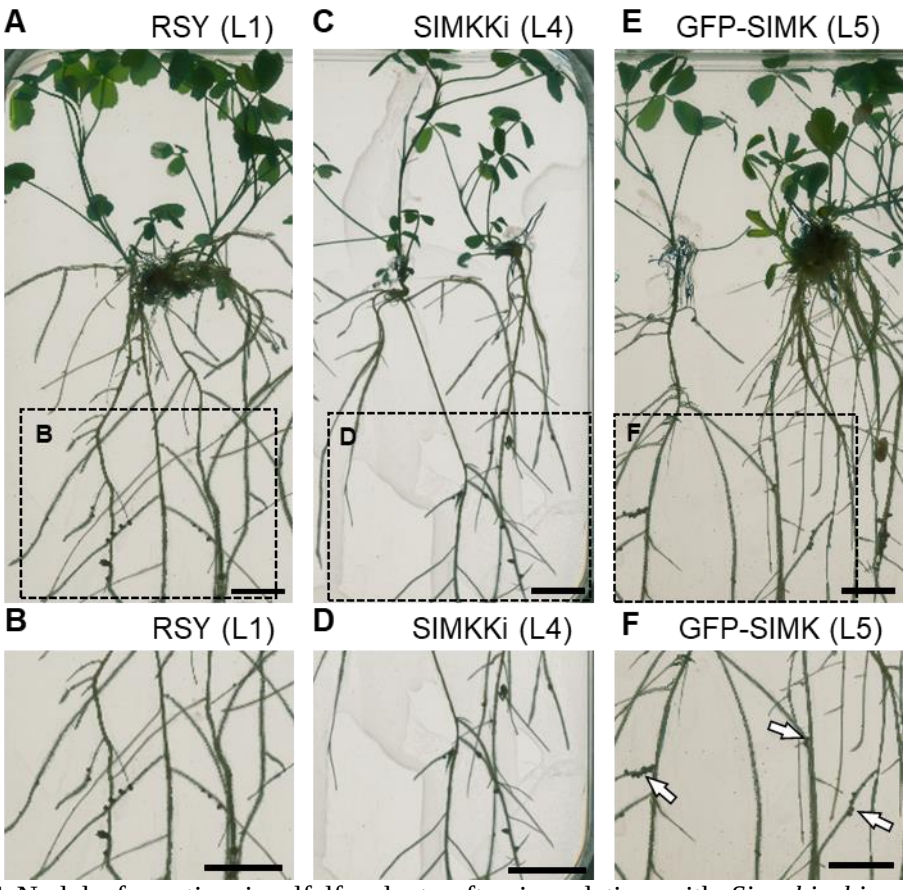

Figure 24 Nodule formation in alfalfa plants after inoculation with *Sinorhizobium meliloti*. **(A,C,E)** Representative images of alfalfa **(A)** RSY line L1, **(C)** SIMKKi line L4 and **(E)** GFP-SIMK line L5 plants 15 days after inoculation with *S. meliloti* labelled with mRFP on Fåhreus medium at 15 dpi. **(B,D,F)** A close-up view of the images in marked boxes **(A,C,E)** showing nodules on alfalfa roots of **(B)** RSY line L1, **(D)** SIMKKi line L4 and **(F)** GFP-SIMK line L5. Note formation of nodules in clusters in GFP-SIMK line L5 (**E,F**, white arrows in **F**). Adapted from Hrbáčková *et al.* (2021).

4.2.4 Impact of overexpressed GFP-SIMK on shoot biomass and leaf formation

In addition to root hair phenotypes and symbiotic interaction with *S. meliloti*, the role of overexpressed GFP-SIMK was also examined in above ground biomass production (**Figure 25**) as well as on leaf development (**Figure 26**). Formation and regrowth of new individual alfalfa shoots was induced and synchronized by cutting off the green part. Documentation was taken after 60 days plant growth in pots with soil. SIMKKi line L4 showed smaller and thinner habitus of shoots (**Figure 25B**) in comparison to RSY line L1 (**Figure 25A**). Above ground parts in GFP-SIMK line L5 showed more robust and bushy habitus (**Figure 25C**). In quantitative terms, GFP-SIMK line L5 produced significantly longer shoots (**Figure 25D**) with significantly higher weight (**Figure 25E**) in comparison to both RSY line L1 and SIMKKi L4 plants. SIMKKi line L4 developed significantly lower number of shoots per plant in comparison to both

RSY L1 and GFP-SIMK L5 (**Figure 25F**). Consequently, the biomass weight per plant was significantly decreased in SIMKKi L4 plants, but significantly increased in GFP-SIMK L5 plants in comparison to control RSY L1 (**Figure 25G**).

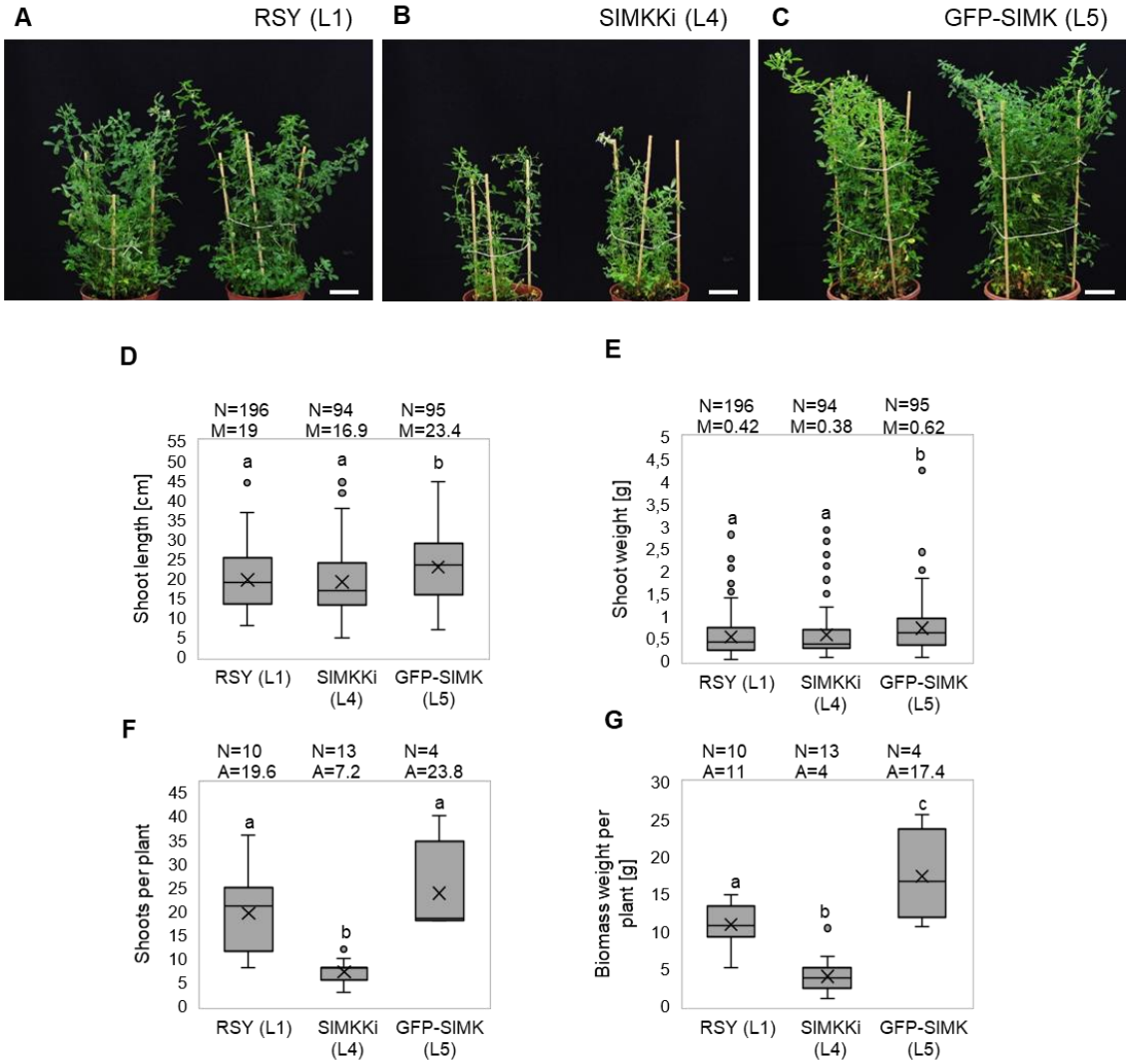

Figure 25 Shoot biomass production in transgenic alfalfa plants grown *in vivo*. **(A-C)** Representative images of above ground parts of mature plants grown in pots in control RSY L1 **(A)**, SIMKKi L4 **(B)** and GFP-SIMK L5 **(C).** Regrown plants were documented 60 days after cutting the shoots. **(D)** Box plot graph depicting comparison of shoot length of indicated lines, number of observations N and median value M. **(E)** Box plot graph depicting comparison of shoot weight of indicated lines, number of observations N and median value M. **(F)** Box blot graph depicting comparison of shoots number per plant of indicated lines, number of observations N and average value A. **(G)** Box plot graph depicting comparison of biomass weight per plant of indicated lines, number of observations N and average value A. Statistics was calculated in SigmaPlot11.0 using Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's method) **(D,E)** or using One Way Analysis of Variance (Holm-Sidak method) **(F,G)** and is based on **(D,E)** N=94-196 and **(F,G)** N=4-13. Different lower case letters indicate statistical significance between treatments ($p < 0.05$). Scale bar: 4 cm in **(A-C)**. Images were provided by Miroslav Ovečka. Statistics was provided by Ivan Luptovčiak. Adapted from Hrbáčková *et al.* (2021).

Besides documentation of above ground part of alfalfa plants, individual shoots of RSY line L1, SIMKKi line L4 and GFP-SIMK line L5 were examined (**Figure 26**). Above ground part of plants was cut off and the green part contained shoot apical meristem with leaf primordia and already formed trifoliate compound leaves after 15 days. They developed from nodes, interconnected by elongated internodes (**Figure 26A**). The size and the shape of leaves of SIMKKi line L4 were considerably affected (**Figure 26B,E**). Leaves of SIMKKi line L4 were smaller, narrower and slightly curled in comparison to RSY line L1 (**Figure 26A,D**). SIMK overexpression in GFP-SIMK line L5 led to the enhanced development of shoots, in accordance with formation of large leaves with long petioles (**Figure 26C,F**). Regarding the shape, SIMKKi line L4 showed more narrow leaves and less notched at their apices, while GFP-SIMK line L5 showed longer and broader leaves in comparison to more oval ones in RSY line L1 (**Figure 26D-F**).

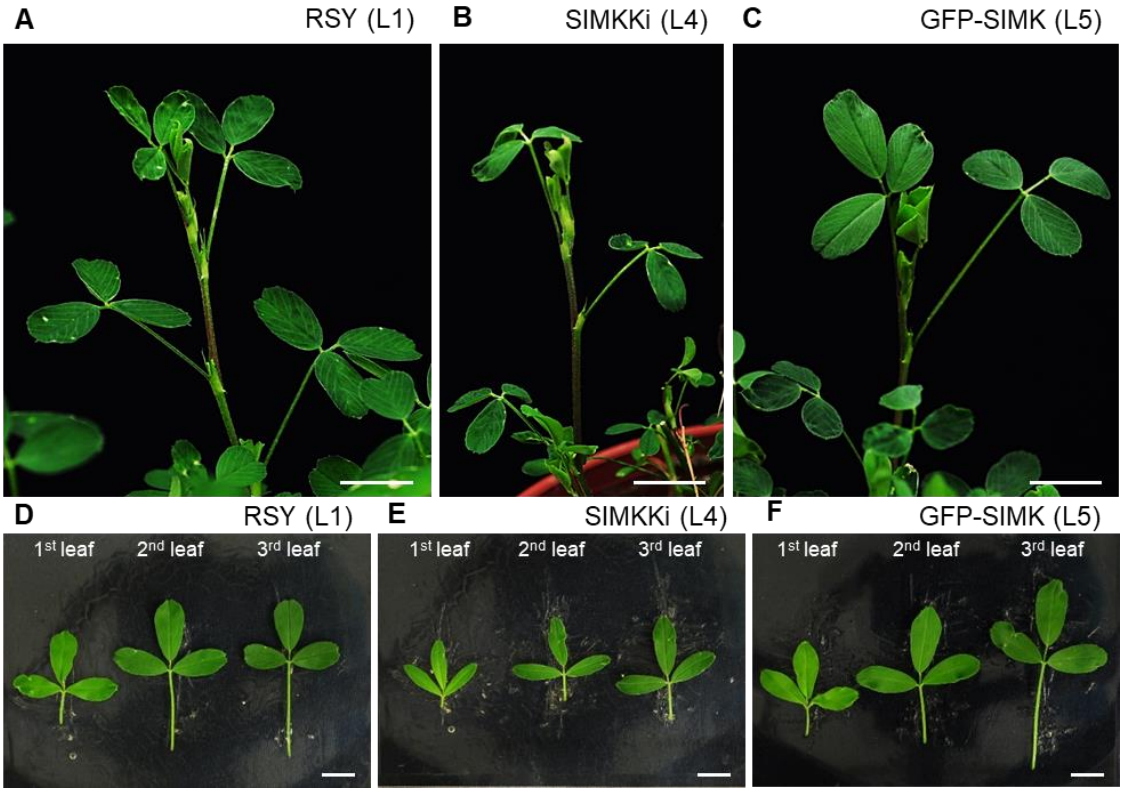

Figure 26 Shoot and leaf phenotypes in plants of alfalfa lines grown in soil. **(A-C)** Representative images of fresh axillary shoots developed on mature plants of **(A)** control RSY line L1, **(B)** SIMKKi line L4 and **(C)** GFP-SIMK line L5 grown in pots 15 days after cutting of the above ground part. **(D-F)** Phenotype of first three developed leaves beneath of the shoot apex from individual shoot of **(D)** control RSY line L1, **(E)** SIMKKi line L4 and **(F)** GFP-SIMK line L5. Scale bar: 10 mm in **(A-F)**. Images were provided by Miroslav Ovečka. Adapted from Hrbáčková *et al.* (2021).

Size and shape of leaves were analyzed in more details. Five representative individual shoots from examined plants (5 plants per line) were selected and first three fully developed leaves beneath the shoot apex were dissected, photographed (**Figure 26C-D**) and measured (**Figure 27**). Regarding the size of analyzed leaves, SIMKKi line L4 contained always the smallest leaves, which appeared in all leaf types (first, second and third leaf, **Figure 27A**). Interestingly, the third leaves of GFP-SIMK line L5 showed the largest leaf area (**Figure 27A**). Leaves of SIMKKi line L4 showed significantly shorter petioles in all leaf types (**Figure 27B**), and petioles were significantly longer in the third leaves of GFP-SIMK line L5 (**Figure 27B**).

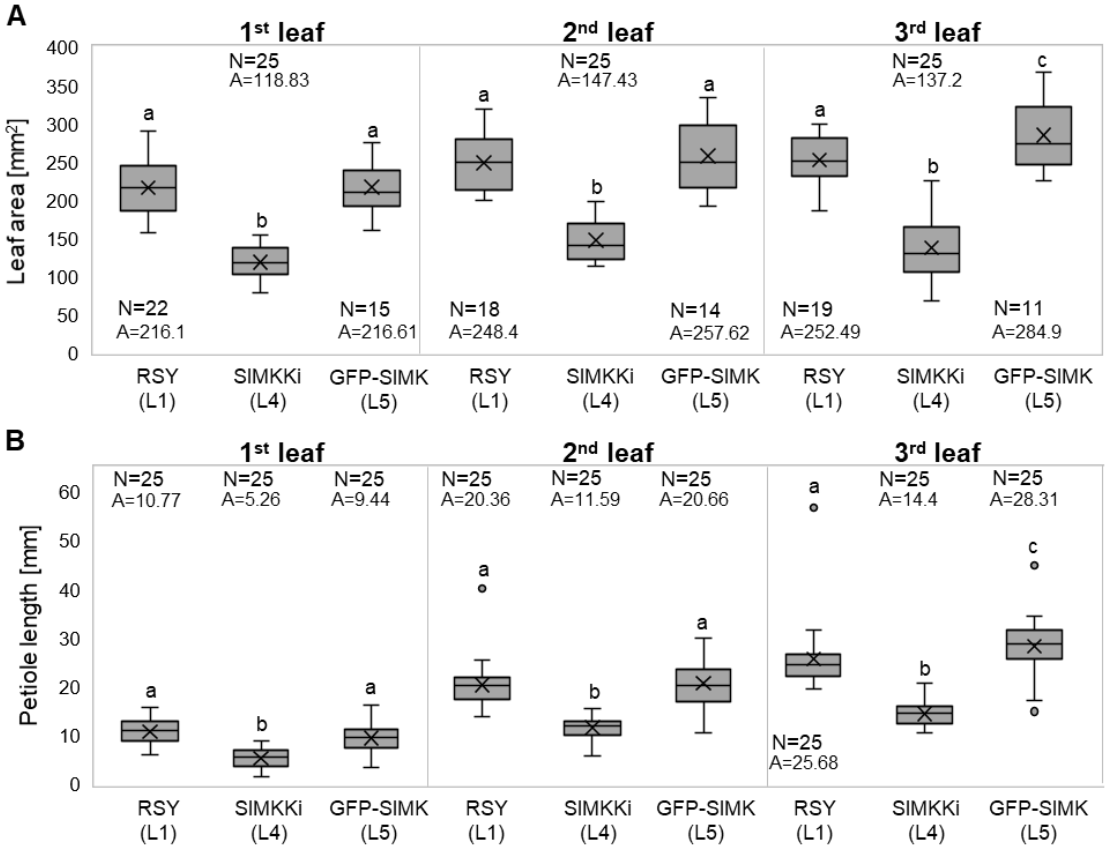

Figure 27 Shoot and leaf phenotypes in alfalfa lines grown in soil. **(A)** Box plot graph of 1st, 2nd and 3rd leaf area of control RSY line L1, SIMKKi line L4 and GFP-SIMK line L5, number of observations N and average value A. **(B)** Box plot graph of petiole length of 1st, 2nd and 3rd leaf of control RSY line L1, SIMKKi line L4 and GFP-SIMK line L5, number of observations N and average value A. Statistics was calculated in SigmaPlot11.0 using Two Way Analysis of Variance (Holm-Sidak method) and is based on **(A)** N=11-25 and **(B)** N=25. Different lower case letters indicate statistical significance between treatments ($p < 0.05$). Statistics was provided by Ivan Luptovčiak. Adapted from Hrbáčková *et al.* (2021).

4.3 Discussion

Leguminous plant species are important members of the agricultural ecosystems and are widely utilized also in nutritional production industry. They are able to grow in soils deficient for nitrogen due to the symbiotic interaction with rhizobia in root nodules, specialized organs for atmospheric nitrogen fixation. Alfalfa is an important legume crop in agronomy, especially for forage or silage production. Both initial and later interactions between legumes and rhizobia require exchange of different signals and activation of signal transduction pathways. Protein phosphorylation in general is one of the major signaling mechanisms controlling cellular responses to external stimuli. In particular, MAPK-dependent signal transduction cascades regulate many developmental and cellular processes in plants (Komis *et al.,* 2018). Systemic approaches indicated that legume-rhizobia interactions and subsequent root nodule development involve activity of various protein kinases (Grimsrud *et al.,* 2010; Roy *et al.,* 2020). Main effort in research of symbiotic nitrogen fixation is conducted in legumes important for food production like bean (Ge *et al.,* 2016), soybean (Lee *et al.,* 2008), pea (Stracke *et al.,* 2002), or non-crop model *M. truncatula* (Ryu *et al.,* 2017). However, the regulation of symbiotic interactions, nodule development and nitrogen fixation, including possible involvement of MAPK signaling, is much less clear in alfalfa.

In this chapter, we studied effects of SIMK downregulation and overexpression in alfalfa using genetically modified transgenic lines. We characterized parameters like length of root hairs, phenotype of above ground plant parts and size of leaves, but also addressed possible involvement of SIMK in the efficiency of root nodulation, through determination of clustering of ITs and nodules. In order to decrease SIMK functions, we prepared two independent RNAi transgenic lines downregulating SIMKK, an upstream activator of SIMK (SIMKKi lines). We confirmed that SIMK expression was strongly downregulated in these lines.

Alfalfa nodulation requires attachment of *S. meliloti* to root hairs. In general, root hairs are long tubular outgrowths of root epidermal cells that are morphologically similar to pollen tubes (Pei *et al.,* 2012). They provide easier water and nutrient uptake from the soil into the plant and help to keep the plant body stable in the soil (Šamaj *et al.,* 2004). To gain further insight into the importance of SIMK in root hair development, independent transgenic lines of alfalfa were compared. The question remained whether genetically-based downregulation or overexpression of SIMK might have an effect on the

root hair growth in homologous alfalfa species. We addressed this question and report here about earlier cessation of root hair tip growth leading to short root hairs in SIMKKi lines with strongly downregulated SIMK, while GFP-SIMK overexpressing lines showed an opposite phenotype manifested by later cessation of the tip growth and longer root hairs. Crucial components of the tip-growth machinery are: a tip-focused cytoplasmic Ca^{2+} gradient, polarly targeted vesicular traffic and the actin cytoskeleton (Carol *et al.,* 2002; Hepler *et al.,* 2001; Šamaj *et al.,* 2002). From the results showed in previous chapter of this thesis, the SIMK kinase is strongly expressed in growing root hairs. SIMK protein is accumulated in nuclei of root epidermal cells. The situation start to change during root hairs formation. SIMK redistributes from nuclei into the cytoplasm and into the tips of growing root hairs (Šamaj *et al.,* 2002, 2004). During this redistribution, SIMK is active (Šamaj *et al.,* 2002). This selective enrichment of active SIMK in tips of growing root hairs coincides with dynamic F-actin meshworks (Braun *et al.,* 1999; Baluška *et al.,* 2000b; Baluška and Volkmann, 2002). Depolymerization and stabilization of F-actin activates SIMK, indicating that MAPK activity is directly affected by F-actin dynamics (Šamaj *et al.,* 2002). After treatment with actin disruptors such as LB or cytochalasin D, tip-focused localization of SIMK disappeared and resulted in nuclear accumulation of SIMK. On the other side, an inducer of actin polymerization, jasplakinolide, also activated SIMK (Šamaj *et al.,* 2002). Moreover, Šamaj *et al.* (2002) by exchanging the homologous amino acid in SIMK observed higher MAPK activity in transformed protoplasts and plants of tobacco line SR1. This gain-of-function construct of SIMK induced longer root hairs, which is consistent with results in the case of overexpressed GFP-SIMK in alfalfa. On the other hand, loss-of-function construct of SIMK in tobacco showed no visible root hair phenotype. Participation of SIMK and other MAPKs in the regulation of root hair tip growth in general is part of complex signal transduction pathways. MAPKs can participate in the transcriptional regulation of genes involved in root hair development. Previously, Bekešová *et al.* (2015) showed decreased accumulation of phosphorylated SIMK in SIMKKi lines, which is confirmed also in this thesis. SIMKKi transgenic line exhibited strong downregulation of *SIMKK* and *SIMK* transcripts and SIMK protein, and showed shorter root hairs. Such decreases in root hair growth in the SIMKKi line and increase growth in the overexpressed transgenic line support a positive role of SIMK in the root hair formation.

Effectivity in the root hair tip growth is an important aspect affecting early stages of plant-rhizobia interaction. Root nodule symbiosis allows conversion of atmospheric N_2 into NH_3 absorbed by plants (Yan *et al.,* 2020). MAPK cascades play central roles in various intracellular signal transduction processes through sequential phosphorylation of three-linked kinases (MAPKKK, MAPKK, MAPK) (Lewis *et al.,* 1998; Madhani and Fink, 1998; Schaeffer and Weber, 1999; Colcombet and Hirt, 2008; Raja *et al.,* 2017; Šamajová *et al.,* 2013b). Publications reporting involvement of MAPK signaling cascades in nodule development are rather scarce. A comparative study confirmed that MAPK signaling cascade and stress-related responses are activated early upon plant infection with symbiotic rhizobia (Lopez-Gomez *et al.,* 2012).

Most of the advances in the area originate from the studies of two model legumes, *M. truncatula* and *L. japonicus* (Clúa *et al.,* 2018). It has been shown that the legume SIP2 is a MAPKK in *L. japonicus* directly interacting with receptor-like kinase SymRK (Chen *et al.,* 2012) and have an essential role in the early symbiosis signaling and nodule organogenesis (Chen *et al.,* 2012). Recent study demonstrated that phosphorylation target of LjSIP2 is LjMPK6 (orthologue of SIMK) (Yin *et al.,* 2019). SIP2/SymRK interaction inhibits the kinase activity of LjSIP2 on the LjMPK6 substrate. Thus, signaling module SymRK-SIP2-MPK6 is required for nodulation, playing a positive role in nodule formation and organogenesis in *L. japonicus* (Yan *et al.,* 2020). Moreover, Yan *et al.* (2020) also showed that LjPP2C, a PP2C-type phosphatase, specifically interacts with LjMPK6 *in vitro* and dephosphorylates LjMPK6. Phosphatase assay demonstrated that LjPP2C is a genuine protein phosphorylating the LjMPK6 in cellular signal transduction as reported for other plant PP2C (Yan *et al.,* 2020). Meskiene *et al.* (2003) showed, that MP2C, a wound-induced alfalfa PP2C, negatively regulate and also directly inactivates SIMK.

In alfalfa, SIMKK is specific upstream activator of SIMK under salt stress (Kiegerl *et al.,* 2000). Interestingly, SIMKK shares 88% amino acid similarity with LjSIP2 (Chen *et al.,* 2012). The orthologue of LjSIP2 has been identified in *M. truncatula* as MtMAPKK4 (Chen *et al.,* 2017). It is involved in the regulation of different plant developmental processes and also mediates root nodule formation. Chen *et al.* (2012) prepared *SIP2 RNAi* line and after inoculation with *Mesorhizobium loti,* average nodule number was lower in comparison to control line. In another study, Chen *et al.* (2017) prepared heterozygous mutant *mapkk4*/+. This heterozygote mutant showed decreased

expression levels of *MtMAPKK4*, which is consistent with our SIMKKi transgenic line exhibited with strong downregulation of *SIMKK* and *SIMK* transcripts and SIMK protein, and showed less numbers of ITs and nodules. The *mapkk4/+* heterozygotes showed also reduced numbers of ITs and nodules (Chen *et al.,* 2017). Downstream interacting partners of MtMKK4 are MtMPK3 and MtMPK6 (Chen *et al.,* 2017). Moreover, another *M. truncatula* MAPKK, namely MtMKK5, also interacts with MtMPK3 and MtMPK6 in alternative signaling pathway, having a negative role in the symbiotic nodule formation (Ryu *et al.,* 2017). On the other side, overexpressed GFP-SIMK transgenic line showed higher numbers of ITs and nodules. Yin *et al.* (2019) prepared overexpressed *LjMPK6-ox* transgenic lines, which showed significantly higher numbers of ITs and nodules compared to control plants after inoculation with *M. loti* bacteria strain. Such decreases in formation of ITs and nodules in the SIMKKi line but enhanced amounts of ITs and nodules in overexpression GFP-SIMK line support a positive role of SIMK in the alfalfa nodulation.

Clustering of ITs after inoculation with *S. meliloti* in overexpressor GFP-SIMK line is another interesting finding. Moreover, clustered development of ITs correlated well with clustered formation of fully developed and equally growing root nodules. This may represent important aspect of root nodule formation, since appropriate number of nodules developed in whole root system is tightly regulated by the plant and depends on overall physiological conditions. It has been observed that legumes tend to maintain development of the minimal number of nodules that are required for optimal growth at given growth conditions (Mortier *et al.,* 2012). This mechanism is regulated by local and systemic endogenous signals. Locally, the number of developing nodules is controlled through ethylene signaling pathway, restricting the initiation of nodule primordia to cortical cells close to xylem poles (Heidstra *et al.,* 1997), and through nitrate-induced signaling peptides of the CLAVATA family (Mortier *et al.,* 2010; Saur *et al.,* 2011). A particular class of these small signaling CLE peptides, induced by rhizobia infection, controls also systemic regulation of nodulation (Mortier *et al.,* 2010; Djordjevic *et al.,* 2015; Concha and Doerner, 2020). CLE peptides move as a long-distance signals from roots to shoots where specifically interact with shoot receptors, like leucine-rich-repeat receptor SUNN in *M. truncatula* (Schnabel *et al.,* 2005), and negatively autoregulate the nodule number. On the other hand, root competence for nodulation is controlled also by the microRNAs like miR2111, which is produced upon activity of the receptor CRA2 in

shoots, and affecting positively root nodulation as systemic regulation signal (Gautrat *et al.*, 2020). This feedback mechanism is controlled by the number, the activity and the age of early-developed nodules (Pierce and Bauer, 1983; Caetano-Anollés *et al.,* 1991). Formation of IT and nodule clusters in GFP-SIMK lines may indicate a new SIMK role in spatial control of nodule formation on the root system. Nodules developing close to each other and forming clusters might require SIMK involvement in IT formation and viability, as less infection events might abort in early stages of development. This unique aspect of nodulation process and mode of its regulation within the whole root system certainly deserves further detailed study.

Possible scenario how SIMK may be involved in alfalfa nodulation and symbiosis with *S. meliloti* can be anticipated from its subcellular localization pattern. Originally, it was observed during root hair tip growth that SIMK relocates from nucleus to the tip of growing root hairs (Šamaj *et al.,* 2002). Upon salt stress, both SIMKK and SIMK became activated and relocated from nucleus to cytoplasm, where they accumulated in spot-like structures (Ovečka *et al.*, 2014). Overexpressor GFP-SIMK was also observed at the root hair tip and spot-like structures of GFP-SIMK and tagRFP-SIMKK after salt treatment. Presence of activated SIMK in root hair tips was confirmed by whole-mount immunofluorescence localization with phospho-specific antibodies. Importantly, live cell imaging of GFP-tagged SIMK, as well as SIMK immunolocalization in root hairs of alfalfa plants inoculated with *S. meliloti* revealed presence of SIMK along ITs and its accumulation in infection pockets.

Another aspect interesting from the biotechnological point of view, is the development and production of above ground plant parts. Particularly in alfalfa, an important forage crop, total leaf surface area and shoot biomass are agronomical parameters of interest. In this respect, genetic manipulation of SIMK brought interesting and potentially promising results. Downregulation of *SIMKK* and *SIMK* genes led to decreased root hair growth, numbers of ITs, or nodules. Moreover, downregulation of *SIMKK* and *SIMK* genes in SIMKKi lines influenced negatively the development of above ground plant parts, leading to formation of smaller leaves and production of less shoot biomass per plant. SIMK overexpression in GFP-SIMK lines, on the other hand, resulted in higher shoot biomass per plant, production of bigger size of analyzed leaves and their better distribution in shoots due to the longer petioles. This result may support a general effort of alfalfa biotechnological improvement as a forage crop. Nowadays,

genetic, genomic and recombinant DNA technology approaches are widely utilized in alfalfa improvements, including leaf production parameters and biomass yield (Biazzi *et al.,* 2017; Lei *et al.,* 2017). Compared to other crops, alfalfa shows relatively high drought tolerance (Arshad *et al.,* 2017; Lei *et al.,* 2017). Also salinity is a big threat for alfalfa production (Arshad *et al.,* 2017). Previous studies provided some interesting information. For example, overexpression of *WXP1,* a gene encoding AP2 domain-containing putative transcription factor from *M. truncatula* under the control of the *35S* promoter in alfalfa resulted in excessive formation of cuticular wax layer on leaves. Such leaves were more resistant to water loss making these plants more resistant against drought stress (Zhang *et al.,* 2005). A number of microRNAs have been used to improve various crop species via genetic engineering. For example, miR156 is a plant microRNA characterized in many plants (Aung *et al.,* 2015). Overexpression of miR156 in alfalfa caused silencing of seven *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes, and thereby enhanced biomass production and shoot branching (Aung *et al.,* 2017; Gao *et al.,* 2016). In another study, genetic modification of the *MsSPL8* gene in alfalfa significantly altered shoot architecture. Knockdown of *MsSPL8* significantly increased shoot branching and biomass yield, however, shoot branching was suppressed and biomass yield was reduced by *MsSPL8* overexpression (Gou *et al.,* 2018). We observed increase in total leaf surface area and shoot biomass production in SIMK-overexpressing lines.

5 General conclusions

This Ph.D. thesis describes preparation and characterization of SIMK and SIMKK transgenic alfalfa lines expressed under the *35S* promoter. We have studied in detail the role of overexpressed SIMK in root hair growth, IT and nodule clustering and shoot biomass production.

The thesis consists of three parts. The first part summarizes current knowledge on biotechnological potential of alfalfa, including genetic transformation methods as well as genomic and transcriptomic perspectives focused on alfalfa responses to abiotic and biotic stress. This section also includes important information on legume-rhizobia interactions. The main functions of Nod factors and flavonoids as symbiotic signals as well as structural aspects of nodule development are described. The last subsection is dedicated to the large number of MAPKs identified in alfalfa and in the model plant organism *A. thaliana*.

In the second part, the process of cloning of fluorescently-tagged *SIMK* and *SIMKK* genes is described. Expression of GFP-tagged SIMK and tagRFP-tagged SIMKK was checked using transient transformation of *N. benthamiana* leaves before stable transformation of alfalfa. Our results supported localization of GFP-SIMK and tagRFP-SIMKK in nuclei and dispersed in cytoplasm. Moreover, we also studied colocalization of SIMK and SIMKK and showed formation of *SIMKK-SIMK* spot-like structures in the cytoplasm after salt stress. New transgenic alfalfa lines have been prepared by improved and optimized protocol for stable transformation by *Agrobacterium tumefaciens* with high efficiency. Up to now, localization experiments were performed solely on fixed plant material, such as root protoplasts or root tips. For the first time, we were able to perform live cell imaging of GFP-SIMK in the whole alfalfa plant including different cell types and organs. A systemic study of GFP-SIMK localization patterns at whole organ, tissue and cellular levels can help to better understand its role in alfalfa. Modern microscopic methods such as CLSM and Airyscan CLSM were used for subcellular localization of GFP-SIMK and for colocalization with activated MAPKs in alfalfa using live cell imaging or immunolabelling. The investigation of GFP-SIMK localization during development of alfalfa roots revealed its strong accumulation in the nuclei of root tip and epidermal cells but signal was weaker in nucleoli. GFP-SIMK regularly accumulated in tips of growing root hairs. It was also localized in root border cells detached from lateral root cap. In the above ground plant parts, GFP-SIMK accumulated in the nuclei and less

in the cytoplasm of hypocotyl cells. Similar subcellular localization was found also in leaf epidermal cells and stomata. Immunolabeling method confirmed the pattern of subcellular localization of GFP-SIMK in root cells by using whole-mount immunofluorescence co-immunolabeling with GFP-specific and phospho-specific (anti-phospho-p44/42) antibodies. Imaging of co-immunolabeled samples with Airyscan CLSM revealed that GFP-SIMK is localized in distinct spot-like structures in the nucleoplasm and in cytoplasmic structures, preferentially in activated form. Moreover, GFP-SIMK was localized in activated form in the root hair tips.

The last part of the thesis deals with genetic manipulation (overexpression and suppression) of *SIMK* and *SIMKK*. In more detail, we generated stable transgenic alfalfa lines overexpressing GFP-tagged SIMK as well as transgenic SIMKK RNAi (SIMKKi) line with downregulated *SIMKK* and *SIMK* genes. We confirmed that SIMK expression was strongly downregulated in these lines. Next, we quantitatively characterized parameters like root hair growth, possible involvement of SIMK in the efficiency of IT and nodule formation and shoot biomass production in these alfalfa transgenic lines. We showed that downregulation or overexpression of *SIMK* affected root hair formation and growth in alfalfa. GFP-SIMK overexpressor line revealed longer root hairs phenotype manifested also by later cessation of their tip growth as compared to the control and the SIMKKi lines. Clustering of ITs and nodules after inoculation with *S. meliloti* in overexpressor GFP-SIMK line is an interesting finding. It is possible that nodules developing close to each other and forming clusters might require SIMK involvement in IT formation and viability. Besides a new role of SIMK in alfalfa nodulation process, we also showed positive impacts of SIMK overexpression on some other important agronomical factors, such as shoot biomass production, petiole and leaf development, leading to the formation of bigger leaves and enhanced production of green biomass. This shows a biotechnological potential of SIMK for genetic improvement of alfalfa as a forage crop.

6 References

Abdelrahman, M., Jogaiah, S., Burritt, D. J., Tran, L. S. P. (2018). Legume genetic resources and transcriptome dynamics under abiotic stress conditions. *Plant Cell Environ.* 41, 1972–1983. doi: 10.1111/pce.13123

Adhikari, L., Missaoui, A. M. (2019). Quantitative trait loci mapping of leaf rust resistance in tetraploid alfalfa. *Physiol. Mol. Plant Pathol*. 106, 238–245. doi: 10.1016/j.pmpp.2019.02.006

Ahlfors, R., Macioszek, V., Rudd, J., Brosché, M., Schlichting, R., Scheel, D., et al. (2004). Stress hormone-independent activation and nuclear translocation of mitogen-activated protein kinases in *Arabidopsis thaliana* during ozone exposure. *Plant J*. 40, 512–522. doi: 10.1111/j.1365-313X.2004.02229.x

Albrecht, K. A., Beauchemin, K. A. (2003). Alfalfa and other perennial legume silage. *Sil. Sci. Tech.*, 42, 633–664. doi: 10.2134/agronmonogr42.c14

Amini, M., Deljou, A., Nabiabad, H. S. (2016). Improvement of in vitro embryo maturation, plantlet regeneration and transformation efficiency from alfalfa (*Medicago sativa* L.) somatic embryos using *Cuscuta campestris* extract. *Physiol. Mol. Biol. Plants* 22, 321–330. doi: 10.1007/s12298-016-0374-y

Ammirato, P.V. (1983). The regulation of somatic embryo development in plant cell cultures: suspension culture techniques and hormone requirements. *Nat. Biotechnol.* 1, 68–73. doi: 10.1038/nbt0383-68

An, Y. M., Song, L. L., Liu, Y. R., Shu, Y. J., Guo, C. H. (2016). De novo transcriptional analysis of alfalfa in response to saline-alkaline stress. *Front. Plant Sci.* 7, 931. doi: 10.3389/fpls.2016.00931

Anderson, N. G., Maller, J. L., Tonks, N. K., and Sturgill, T. W. (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* 343, 651–653. doi: 10.1038/343651a0

Andreasson, E., Ellis, B. (2010). Convergence and specificity in the Arabidopsis MAPK nexus. *Trends Plant Sci.* 15, 106–113. doi: 10.1016/j.tplants.2009.12.001

Annicchiarico, P., Barrett, B., Brummer, E. C., Julier, B., Marshall A. H. (2015). Achievements and challenges in improving temperate perennial forage legumes. *Crit Rev Plant Sci.* 34, 327–380. doi: 10.1080/07352689.2014.898462

Annicchiarico, P., Nazzicari, N., Brummer, E. C. (2016). "Alfalfa genomic selection: Challenges, strategies, transnational Cooperation", in Breeding in a World of Scarcity, eds. I. Roldán-Ruiz, J. Baert, D. Reheul (Cham: Springer), 145–149. doi: 10.1007/978-3-319-28932-8_22

Aranjuelo, I., Molero, G., Erice, G., Avice, J. C., Nogués, S. (2011). Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.). *J. Exp. Bot.* 62, 111–123. doi: 10.1093/jxb/erq249

Ari, Ş., Arikan, M. (2016). "Next-generation sequencing: advantages, disadvantages, and future," in Plant Omics: Trends and Applications, eds. K. Hakeem, H. Tombuloglu, G. Tombuloglu (Cham: Springer). 109–135. doi: 10.1007/978-3-319-31703-8_5

Arshad, M., Feyissa, B. A., Amyot, L., Aung, B., Hannoufa, A. (2017). MicroRNA156 improves drought stress tolerance in alfalfa (*Medicago sativa*) by silencing SPL13. *Plant Sci.* 258, 122–136. doi: 10.1016/j.plantsci.2017.01.018

Arshad, M., Gruber, M. Y., Hannoufa, A. (2018). Transcriptome analysis of microRNA156 overexpression alfalfa roots under drought stress. *Sci. Rep.* 8, 9363. doi: 10.1038/s41598-018-27088-8

Asamizu, E., Nakamura, Y., Sato, S., Tabata, S. (2004). Characteristics of the *Lotus japonicus* gene repertoire deduced from large-scale expressed sequence tag (EST) analysis. *Plant Mol. Biol.* 54, 405–414. doi: 10.1023/B:PLAN.0000036372.46942.b8

Atanassov, A., Brown, D.C. (1984). Plant regeneration from suspension culture and mesophyll protoplasts of *Medicago sativa* L. *Plant Cell Tiss. Organ Cult.* 3, 149–162. doi: 10.1007/BF00033736.

Aung, B., Gao, R., Gruber, M. Y., Yuan, Z. C., Sumarah, M., Hannoufa, A. (2017). MsmiR156 affects global gene expression and promotes root regenerative capacity and nitrogen fixation activity in alfalfa. *Transgenic Res.* 26, 541–557. doi: 10.1007/s11248-017-0024-3

Aung, B., Gruber, M. Y., Hannoufa, A. (2015). The microRNA156 system: a tool in plant biotechnology. *Biocatal. Agric. Biotechnol.* 4, 432–442. doi: 10.1016/j.bcab.2015.08.002

Austin, S., Bingham, E. T., Koegel, R. G., Mathews, D. E., Shahan, M. N., Straub, R. J., et al. (1994). An overview of a feasibility study for the production of industrial enzymes in transgenic alfalfa. *Ann. N. Y. Acad. Sci.* 721, 234–244. doi: 10.1111/j.1749-6632.1994.tb47395.x

Austin, S., Bingham, E. T., Mathews, D. E., Shahan, M. N., Will, J., Burgess, R. R. (1995). Production and field performance of transgenic alfalfa (*Medicago sativa* L.) expressing alpha-amylase and manganese-dependent lignin peroxidase. *Euphytica* 85, 381–393. doi: 10.1007/BF00023971

Austin, S., Ziegelhoffer, T. (2001). "The production of value-added proteins in transgenic alfalfa," in: Molecular Breeding of Forage Crops. eds. G. Spangenburg, (Kluwer Academic Publishers, Dordrecht, The Netherlands), 285–301. doi: 10.1007/978-94-015-9700-5_18

Avery, O. T., Macleod, C. M., McCarty, M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a

desoxyribonucleic acid fraction isolated from pneumococcus type III. *J. Exp. Med.* 79, 137–158. doi: 10.1084/jem.79.2.137

Azevedo, L. B., van Zelm, R., Leuven, R. S., Hendriks, A. J., Huijbregts, M. A. (2015). Combined ecological risks of nitrogen and phosphorus in European freshwaters. *Environ. Pollut.* 200, 85–92. doi: 10.1016/j.envpol.2015.02.011

Aziz, N., Paiva, N. L., May, G. D., Dixon, R. A. (2005). Transcriptome analysis of alfalfa glandular trichomes. *Planta* 221, 28–38. doi: 10.1007/s00425-004-1424-1

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57, 233–266. doi: 10.1146/annurev.arplant.57.032905.105159

Baker, C. M., Burns, A., Wetzstein, H. Y. (1994). Influence of photoperiod and medium formulation on peanut somatic embryogenesis. *Plant Cell Rep.* 13, 159–163. doi: 10.1007/BF00239884

Baluška, F., Ovečka, M., Hirt, H. (2000a). Salt stress induces changes in amounts and localization of the mitogen-activated protein kinase SIMK in alfalfa roots. *Protoplasma* 212, 262–267. doi: 10.1007/BF01282926

Baluška, F., Salaj, J., Mathur, J., Braun, M., Jasper, F., Šamaj, J., Volkmann, D. (2000b). Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Develop. Biol.* 227, 618–632. doi: 10.1006/dbio.2000.9908

Baluška, F., Volkmann, D. (2002). Actin-driven polar growth of plant cells. *Trends Cell Biol.* 12, 14. doi: 10.1007/s004250050746

Barabaschi, D., Guerra, D., Lacrima, K., Laino, P., Michelotti, V., Urso, S., et al. (2012). Emerging knowledge from genome sequencing of crop species. *Mol. Biotech.* 50, 250–266. doi: 10.1007%2Fs12033-011-9443-1

Barnett, A., Weaver, J. C. (1991). Electroporation: a unified, quantitative theory of reversible electrical breakdown and mechanical rupture in artificial planar bilayer membranes. *Bioelectrochem. Bioenerg.* 25, 163–182. doi: 10.1016/0302-4598(91)87001-W

Barnett, M. J., Fisher, R. F. (2006). Global gene expression in the rhizobial–legume symbiosis. *Symbiosis*. 42, 1–24. doi: 10.1111/j.1574-6968.1999.tb13650.x

Bartels, S., González Besteiro, M. A., Lang, D., Ulm, R. (2010). Emerging functions for plant MAP kinase phosphatases. *Trends Plant Sci.* 15, 322–329. doi: 10.1016/j.tplants.2010.04.003

Bates, G. W., Gaynor, J. J., Shekhawat, N. S. (1983). Fusion of plant protoplasts by electric fields. *Plant Physiol.* 72, 1110–1113. doi: 10.1104/pp.72.4.1110

Beck, M., Komis, G., Ziemann, A., Menzel, D., Šamaj, J. (2011). Mitogen-activated protein kinase 4 is involved in the regulation of mitotic and cytokinetic microtubule transitions

in *Arabidopsis thaliana*. *New Phytol.* 189, 1069–1083. doi: 10.1111/j.1469-8137.2010.03565.x

Beever, D.E., Thorp, C. (1996). "Advances in the understanding of factors influencing the nutritive value of legumes," in: Legumes in sustainable agriculture, eds. D. Yonie. (Occasional Symp. 30. British Grassland Society, Reading, UK). 194–207

Bekešová, S., Komis, G., Křenek, P., Vyplelová, P., Ovečka, M., Luptovčiak, I., Šamaj, J. (2015). Monitoring protein phosphorylation by acrylamide pendant Phos-Tag™ in various plants. *Front. Plant Sci.* 6, 336. doi: 10.3389/fpls.2015.00336

Berger, A., Boscari, A., Frendo, P., Brouquisse, R. (2019). Nitric oxide signaling, metabolism and toxicity in nitrogen-fixing symbiosis. *J. Exp. Bot.* 70, 4505–4520. doi: 10.1093/jxb/erz159

Berrocal-Lobo, M., Segura, A., Moreno, M., López, G., García-Olmedo, F., Molina, A. (2002). Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection. *Plant Physiol.* 128, 951–961. doi: 10.1104/pp.010685

Bevan, M. W., Uauy, C., Wulff, B. B., Zhou, J., Krasileva, K., Clark, M. D. (2017). Genomic innovation for crop improvement. *Nature* 543, 346–354. doi: 10.1038/nature22011

Bhattacharyya, S. N., Habermacher, R., Martine, U., Closs, E. I., Filipowicz, W. (2006). "Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells," in: Cold Spring Harbor symposia on quantitative biology (Cold Spring Harbor Laboratory Pres), 71, 513–521. doi: 10.1101/sqb.2006.71.038

Biazzi, E., Nazzicari, N., Pecetti, L., Brummer, E. C., Palmonari, A., Tava, A., et al. (2017). Genome-wide association mapping and genomic selection for alfalfa (*Medicago sativa*) forage quality traits. *PLoS ONE* 12, e0169234. doi: 10.1371/journal.pone.0169234

Bigeard, J., Hirt, H. (2018). Nuclear signaling of plant MAPKs. *Front. Plant Sci.* 9, 469. doi: 10.3389/fpls.2018.00469

Bingham, E.T., Hurley L.V., Kaatz, D.M., Saunders, J.W. (1975). Breeding alfalfa which regenerates from callus tissue in culture. *Crop Sci.* 15, 719–721. doi: 10.2135/cropsci1975.0011183X001500050032x

Bingham, E.T. (1991). Registration of alfalfa hybrid Regen-SY germplasm for tissue culture and transformation research. *Crop Sci.* 31, 1098. doi: 10.2135/cropsci1991.0011183X003100040075x

Biondi, R. M., Nebreda, A. R. (2003). Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. *Biochem. J.* 372, 1–13. doi: 10.1042/bj20021641

Birch, R. G., Franks, T. (1991). Development and optimisation of microprojectile systems for plant genetic transformation. *Funct. Plant Biol.* 18, 453–469. doi: 10.1071/PP9910453

Blaydes, D. F. (1966). Interaction of kinetin and various inhibitors in the growth of soybean tissue. *Physiol. Plant.* 19, 748–753. doi: 10.1111/j.1399-3054.1966.tb07060.x

Blondon, F., Marie, D., Brown, S., Kondorosi, A. (1994). Genome size and base composition in *Medicago sativa* and *M. truncatula* species. *Genome* 37, 264–270. doi: 10.1139/g94-037

Bögre, L., Calderini, O., Binarova, P., Mattauch, M., Till, S., Kiegerl, S., et al. (1999). A MAP kinase is activated late in plant mitosis and becomes localized to the plane of cell division. *Plant Cell* 11, 101–113. doi: 10.1105/tpc.11.1.101

Bögre, L., Ligterink, W., Meskiene, I., Barker, P. J., Heberle-Bors, E., Huskisson, N. S., et al. (1997). Wounding induces the rapid and transient activation of a specific MAP kinase pathway. *Plant Cell* 9, 75–83. doi: 10.1105/tpc.9.1.75

Bohnert, H. J., Jensen, R. G. (1996). Strategies for engineering water-stress tolerance in plants. *Trends Biotechnol.* 14, 89–97. doi: 10.1016/0167-7799(96)80929-2

Boivin, C., Camut, S., Malpica, C. A., Truchet, G., Rosenberg, C. (1990). *Rhizobium meliloti* genes encoding catabolism of trigonelline are induced under symbiotic conditions. *Plant Cell* 2, 1157–1170. doi: 10.1105/tpc.2.12.1157

Bolaños-Vásquez, M. C., Werner, D. (1997). Effects of *Rhizobium tropici*, *R. etli*, and *R. leguminosarum* bv. phaseoli on nod gene-inducing flavonoids in root exudates of *Phaseolus vulgaris*. *Mol. Plant-Micr. Interact.* 10, 339–346. doi: 10.1094/MPMI.1997.10.3.339

Bonaldi, K., Gourion, B., Fardoux, J., Hannibal, L., Cartieaux, F., Boursot, M., et al. (2010). Large-scale transposon mutagenesis of photosynthetic *Bradyrhizobium* sp. strain ORS278 reveals new genetic loci putatively important for nod-independent symbiosis with *Aeschynomene indica*. *Mol Plant-Micr. Interact.* 23, 760–770. doi: 10.1094/MPMI-23-6-0760

Bora, K. S., Sharma, A. (2011). Phytochemical and pharmacological potential of *Medicago sativa*: a review. *Pharm. Biol.* 49, 211–220. 10.3109/13880209.2010.504732

Bourras, S., Rouxel, T., Meyer, M. (2015). *Agrobacterium tumefaciens* gene transfer: how a plant pathogen hacks the nuclei of plant and nonplant organisms. *Phytopathology* 105, 1288–1301. doi: 10.1094/PHYTO-12-14-0380-RVW

Boscari, A., Meilhoc, E., Castella, C., Bruand, C., Puppo, A., Brouquisse, R. (2013). Which role for nitric oxide in symbiotic N_2-fixing nodules: toxic by-product or useful signaling/metabolic intermediate? *Front. Plant Sci.* 4, 384. doi: 10.3389/fpls.2013.00384

Braun, M., Baluška, F., von Witsch, M. Menzel, D. (1999). Redistribution of actin, profilin and phosphatidylinositol-4, 5-bisphosphate in growing and maturing root hairs. *Planta* 209, 435–443. doi: 10.1007/s004250050746

Breakspear, C. Liu, S. Roy, N. Stacey, C. Rogers, M. Trick, G., et al. (2014). The root hair "infectome" of *Medicago truncatula* uncovers changes in cell cycle genes and reveals a requirement for auxin signaling in rhizobial infection. *Plant Cell* 26, 4680–4701. doi: 10.1105/tpc.114.133496

Brencic, A., Winans, S. C. (2005). Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria detection of and response to signals involved in hostmicrobe interactions by plant-associated bacteria. *Microbiol. Mol. Biol. Rev.* 69,155–194. doi: 10.1128/MMBR.69.1.155

Brock, A. K., Willmann, R., Kolb, D., Grefen, L., Lajunen, H. M., Bethke, G., et al. (2010). The Arabidopsis mitogen-activated protein kinase phosphatase PP2C5 affects seed germination, stomatal aperture, and abscisic acid-inducible gene expression. *Plant Physiol.* 153, 1098–1111. doi: 10.1104/pp.110.156109

Broghammer, A., Krusell, L., Blaise, M., Sauer, J., Sullivan, J. T., Maolanon, N., et al. (2012). Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. *Proc. Natl. Acad. Sci. U.S.A.* 109, 13859–13864. doi: 10.1073/pnas.1205171109

Brouwer, D. J., Osborn, T. C. (1999). A molecular marker linkage map of tetraploid alfalfa (*Medicago sativa* L.). *Theor. Appl. Gen.* 99, 1194–1200. doi: 10.1007/s001220051324

Brummer, E. C., Bouton, J. H., Kochert, G. (1993). Development of an RFLP map in diploid alfalfa. *Theor. Appl. Gen.* 86, 329–332. doi: 10.1007/BF00222097

Buchanan, R. E. (1926). What names should be used for the organisms producing nodules on the roots of leguminous plants? *Proc. Iowa Acad. Sci.* 33, 81–90. doi: scholarworks.uni.edu/pias/vol33/iss1/9

Budak, H., Kantar, M., Bulut, R., Akpinar, B. A. (2015). Stress responsive miRNAs and isomiRs in cereals. *Plant Sci.* 235, 1–13. doi: 10.1016/j.plantsci.2015.02.008

Caetano-Anollés, G., Paparozzi, E. T., Gresshoff, P. M. (1991). Mature nodules and root tips control nodulation in soybean. *J. Plant. Physiol.* 137, 389–396. 10.1016/S0176-1617(11)80306-8

Calderini, O., Bogre, L., Vicente, O., Binarová, P., Heberle-Bors, E., Wilson, C. (1998). A cell cycle regulated MAP kinase with a possible role in cytokinesis in tobacco cells. *J. Cell Sci.* 111, 3091–3100.

Calvert, H. E., Pence, M. K., Pierce, M., Malik, N. S., Bauer, W. D. (1984). Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. *Can. J. Bot.* 62, 2375–2384. doi: 10.1139/b84-324

Camps, M., Nichols, A., Arkinstall, S. (2000). Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J.* 14, 6–16. doi: 10.1096/fasebj.14.1.6

Cárdenas, L., Thomas-Oates, J. E., Nava, N., López-Lara, I. M., Hepler, P. K., Quinto, C. (2003). The role of nod factor substituents in actin cytoskeleton rearrangements in *Phaseolus vulgaris*. *Mol. Plant-Micr. Interact.* 16, 326–334. doi: 10.1094/MPMI.2003.16.4.326

Cardinale, F., Jonak, C., Ligterink, W., Niehaus, K., Boller, T., Hirt, H. (2000). Differential activation of four specific MAPK pathways by distinct elicitors. *J. Biol. Chem.* 275, 36734–36740. doi: 10.1074/jbc.M007418200

Cardinale, F., Meskiene, I., Ouaked, F., Hirt, H. (2002). Convergence and divergence of stress-induced mitogen-activated protein kinase signaling pathways at the level of two distinct mitogen-activated protein kinase kinases. *Plant Cell* 14, 703–711. doi: 10.1105/tpc.010256

Carol, R. J., Dolan, L. (2002). Building a hair: tip growth in *Arabidopsis thaliana* root hairs. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 357, 815–821. doi: 10.1098/rstb.2002.1092

Carrasco, J. L., Castelló, M. J., Naumann, K., Lassowskat, I., Navarrete-Gómez, M., Scheel, D., et al. (2014). Arabidopsis protein phosphatase DBP1 nucleates a protein network with a role in regulating plant defense. *PLoS ONE* 9:e90734. doi: 10.1371/journal.pone.0090734

Casse, F., Boucher, C., Julliot, J. S., Michel, M., Denarie, J. (1979). Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. *Microbiol.* 113, 229–242. doi: 10.1099/00221287-113-2-229

Chabaud, M., Passiatore, J. E., Cannon, F., Buchanan-Wollaston, V. (1988). Parameters affecting the frequency of kanamycin resistant alfalfa obtained by Agrobacterium tumefaciens mediated transformation. *Plant Cell Reports* 7, 512–516. doi: 10.1007/BF00272744

Chandra, A. (2009). Screening global *Medicago* germplasm for weevil (Hypera postica Gyll.) tolerance and estimation of genetic variability using molecular markers. *Euphytica* 169, 363-374. doi: 10.1007/s10681-009-9969-5

Chao, Y., Yuan, J., Guo, T., Xu, L., Mu, Z., Han, L. (2019). Analysis of transcripts and splice isoforms in *Medicago sativa* L. by single-molecule long-read sequencing. *Plant Mol. Biol.* 99, 219–235. doi: 10.1007/s11103-018-0813-y

Charron, D., Pingret, J. L., Chabaud, M., Journet, E. P., Barker, D. G. (2004). Pharmacological evidence that multiple phospholipid signaling pathways link *Rhizobium* nodulation factor perception in *Medicago truncatula* root hairs to intracellular responses, including Ca^{2+} spiking and specific ENOD gene expression. *Plant Physiol.* 136, 3582–3593. doi: 10.1104/pp.104.051110

Chen, H., Zeng, Y., Yang, Y., Huang, L., Tang, B., Zhang, H., Qiu, Q. (2020). Allele-aware chromosome-level genome assembly and efficient transgene-free genome editing for the autotetraploid cultivated alfalfa. *Nature Comm.* 11, 1–11. doi: 10.1038/s41467-020-16338-x

Chen, T. H., Murata, N. (2002). Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol*. 5, 250–257. doi: 10.1016/s1369-5266(02)00255-8

Chen, T. H., Murata, N. (2008). Glycinebetaine: an effective protectant against abiotic stress in plants. *Trends Plant Sci.* 13, 499–505. doi: 10.1016/j.tplants.2008.06.007

Chen, T., Zhou, B., Duan, L., Zhu, H., Zhang, Z. (2017). MtMAPKK4 is an essential gene for growth and reproduction of *Medicago truncatula*. *Physiol. Plant.* 159, 492–503. doi: 10.1111/ppl.12533

Chen, T., Zhu, H., Ke, D., Cai, K., Wang, C., Gou, H., et al. (2012). A MAP kinase kinase interacts with SymRK and regulates nodule organogenesis in *Lotus japonicus. Plant Cell* 24, 823–838. doi: 10.1105/tpc.112.095984

Chen, W. X., Yan, G. H., Li, J. L. (1988). Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *Int. J. Syst. Evol. Microbiol.* 38, 392–397. doi: 10.1099/00207713-38-4-392

Cheung, F., Haas, B. J., Goldberg, S. M. D., May, G. D., Xiao, Y., Town C. D. (2006). Sequencing *Medicago truncatula* expressed sequenced tags using 454 Life Sciences technology. *BMC Genom.* 7, 272. doi: 10.1186/1471-2164-7-272

Choudhary, A. D., Kessmann, H., Lamb, C. J., Dixon, R. A. (1990). Stress responses in alfalfa (*Medicago sativa* L.) IV. Expression of defense gene constructs in electroporated suspension cell protoplasts. *Plant Cell Rep.* 9, 42–46. doi: 10.1007/BF00232133

Chovanec, P., Novák, K. (2005). Visualization of nodulation gene activity on the early stages of *Rhizobium leguminosarum* bv. *viciae* symbiosis. *Folia Microbiol.* 50, 323–331. doi: 10.1007/BF02931413

Christou, P. (1992). Genetic transformation of crop plants using microprojectile bombardment. *Plant J.* 2, 275–281. doi: 10.1111/j.1365-313X.1992.00275.x

Chuderland, D., Konson, A., Seger, R. (2008). Identification and characterization of a general nuclear translocation signal in signaling proteins. *Mol. Cell* 31, 850–861. doi: 10.1016/j.molcel.2008.08.007

Clúa, J., Roda, C., Zanetti, M. E., Blanco, F. A. (2018). Compatibility between Legumes and Rhizobia for the Establishment of a Successful Nitrogen-Fixing Symbiosis. *Genes* 9, 125. doi: 10.3390/genes9030125

Colcombet, J., Hirt, H. (2008). *Arabidopsis* MAPKs, a complex signalling network involved in multiple biological processes. *Biochem. J.* 413, 217–226. doi: 10.1042/BJ20080625

Concha, C., Doerner, P. (2020). The impact of the rhizobia–legume symbiosis on host root system architecture. *J. Exp. Bot.* 71, 3902–3921. doi: 10.1093/jxb/eraa198

Conn, H. J. (1942). Validity of the genus *Alcaligenes. J. Bact.* 44, 353. doi: 10.1128/JB.44.3.353-360.1942

Conrad, H.R., Klopfenstein, T. J. (1988). "Role in livestock feeding- Greenchop. silage, hay, and dehy," in: Alfalfa and alfalfa improvement, eds. A. A. Hanson et al. (Agron. Monogr. 29. ASA, CSSA, and SSSA, Madison). 539–551. doi: 10.2134/agronmonogr29.c17

Cooper, J. E. (2004). “Multiple responses of rhizobia to flavonoids during legume root infection,” in: Incorporating advances in plant pathology, eds. J. A. Callow (Academic, Amsterdam), 1–62. doi: 10.1016/S0065-2296(04)41001-5

Cooper, J. E. (2007). Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *J. Appl. Microbiol.* 103, 1355–1365. doi: 10.1111/j.1365-2672.2007.03366.x

Cren, M., Kondorosi, A., Kondorosi, E. (1995). NolR controls expression of the *Rhizobium meliloti* nodulation genes involved in the core Nod factor synthesis. *Mol. Microbiol.* 15, 733–747. doi: 10.1111/j.1365-2958.1995.tb02381.x

D'Halluin, K., Botterman, J., De Greef, W. (1990). Engineering of herbicide-resistant alfalfa and evaluation under field conditions. *Crop Sci.* 30, 866–871. doi: 10.2135/cropsci1990.0011183X003000040020x

Dahan, J., Wendehenne, D., Ranjeva, R., Pugin, A., Bourque, S. (2010). Nuclear protein kinases: still enigmatic components in plant cell signalling. *New Phytol.* 185, 355–368. doi: 10.1111/j.1469-8137.2009.03085.x

Dangeard, P. A. (1926) Recherches sur les tubercules radicaux des Légumineuses. Botaniste (Paris) 16, 1–275.

Danquah, A., Zélicourt, A., Boudsocq, M., Neubauer, J., Frei Dit, F. N., Leonhardt, N., et al. (2015). Identification and characterization of an ABA-activated MAP kinase cascade in *Arabidopsis thaliana*. *Plant J.* 82, 232–244. doi: 10.1111/tpj.12808

Delves, A. C., Mathews, A., Day, D. A., Carter, A. S., Carroll, B. J., Gresshoff, P. M. (1986). Regulation of the soybean-*Rhizobium* nodule symbiosis by shoot and root factors. *Plant Physiol.* 82, 588–590. doi: 10.1104/pp.82.2.588

de Ruijter, N. C. A., Bisseling, T., Emons, A. M. C. (1999). *Rhizobium* Nod factors induce an increase in sub-apical fine bundles of actin filaments in *Vicia sativa* root hairs within minutes. *Mol. Plant-Micr. Interact.* 12, 829–832. doi: 10.1094/MPMI.1999.12.9.829

de Zélicourt, A., Diet, A., Marion, J., Laffont, C., Ariel, F., Moison, M., et al. (2011). Dual involvement of a Medicago truncatula NAC transcription factor in root abiotic stress response and symbiotic nodule senescence. *Plant J.* 70, 220–230. doi: 10.1111/j.1365-313X.2011.04859.x

Debellé, F., Moulin, L., Mangin, B., Dénarié, J., Boivin, C. (2001). Nod genes and Nod signals and the evolution of the *Rhizobium* legume symbiosis. *Acta Biochim. Pol.* 48, 359–365. doi: 10.18388/abp.2001_3921

Denchev, P., Velcheva, M., Atanassov, A. (1991). A new approach to direct somatic embryogenesis in *Medicag*o. *Plant Cell Rep.* 10, 338–341. doi: 10.1007/BF00193154

Deokar, A. A., Kondawar, V., Jain, P. K., Karuppayil, S. M., Raju, N. L., Vadez, V., et al. (2011). Comparative analysis of expressed sequence tags (ESTs) between drought-tolerant

and -susceptible genotypes of chickpea under terminal drought stress. *BMC Plant Biol*. 11, 70. doi: 10.1186/1471-2229-11-70

Dijak, M., Brown, D. C. W. (1987). Patterns of direct and indirect embryogenesis from mesophyll protoplasts of *Medicago sativa*. *Plant Cell Tissue Organ Cult.* 1987; 9: 121 – 130. doi: 10.1007/BF00044247

Dixon, R., Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. *Nat. Rev. Microbiol.* 2, 621–631. doi: 10.1038/nrmicro954

Dixon, R.A., Steele, C.L. (1999). Flavonoids and isoflavonoids – a gold mine for metabolic engineering. *Trends Plant Sci.* 4, 394–400. doi: 10.1016/S1360-1385(99)01471-5

Djordjevic, M. A., Mohd-Radzman, N. A., Imin, N. (2015). Small-peptide signals that control root nodule number, development, and symbiosis. *J. Exp. Bot.* 66, 5171–5181. doi: 10.1093/jxb/erv357

Dóczi, R., Okrész, L., Romero, A. E., Paccanaro, A., Bögre, L. (2012). Exploring the evolutionary path of plant MAPK networks. *Trends Plant Sci.* 17, 518–525. doi: 10.1016/j.tplants.2012.05.009

Dong, L., Liu, H., Zhang, J., Yang, S., Kong, G., Chu, J. S., et al. (2015). Single-molecule real-time transcript sequencing facilitates common wheat genome annotation and grain transcriptome research. *BMC Genom.* 16, 1039. doi: 10.1186/s12864-015-2257-y

Downie, J. A., González, J. E. (2008). Cell-to-cell communication in rhizobia: quorum sensing and plant signalling. Chemical communication among bacteria. *ASM Press, Washington, DC*, 213–232. doi: 10.1128/9781555815578

Doyle, J. J. (2016). Chasing unicorns: Nodulation origins and the paradox of novelty. *Am. J. Bot.* 103,1865–1868. doi:10.3732/ajb.1600260

Doyle, J. J., Luckow, M. A. (2003). The rest of the iceberg. Legume diversity and evolution in a phylogenetic context. *Plant Physiol.* 131, 900–910. doi: 10.1104/pp.102.018150

Du, S., Erickson, L., Bowley, S. (1994). Effect of plant genotype on the transformation of cultivated alfalfa (*Medicago sativa*) by *Agrobacterium tumefaciens*. *Plant cell Rep.* 13, 330–334. doi: 10.1007/BF00232631

Dupont, L., Hérouart, D., Alloing, G., Hopkins, J., Pierre, O., Frendo, P., et al. (2012). The legume root nodule: from symbiotic nitrogen fixation to senescence. London: INTECH Open Access Publisher. *IntechOpen.* doi: 10.5772/34438

Echt, C. S., Kidwell, K. K., Knapp, S. J., Osborn, T. C., McCoy, T. J. (1994). Linkage mapping in diploid alfalfa (*Medicago sativa*). *Genome* 37, 61–71. doi: 10.1139/g94-008

Edwards, D., Batley, J. (2010). Plant genome sequencing: applications for crop improvement. *Plant Biotechnol. J*. 8, 2–9. doi: 10.1111/j.1467-7652.2009.00459.x

Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., et al. (2009). Real-time DNA sequencing from single polymerase molecules. *Science* 323, 133–138. doi: 10.1126/science

Elgin, Jr. J. H., Welty, R. E., Gilchrist, D. B. (1988). Breeding for disease and nematode resistance. *Alfalfa Alfalfa Impr.* 29, 827–858. doi: 10.2134/agronmonogr29.c27

El-Showk, S., Ruonala, R., Helariutta, Y. (2013). Crossing paths: cytokinin signalling and crosstalk. *Development* 140, 1373–1383. doi: 10.1242/dev.086371

Esseling, J. J., Lhuissier, F. G., Emons, A. M. (2003). Nod factor-induced root hair curling: continuous polar growth towards the point of nod factor application. *Plant Physiol.* 132, 1982–1988. doi: 10.1104/pp.103.021634

Fåhreus, G. (1957). The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *Microbiol.* 16, 374–381. doi: 10.1099/00221287-16-2-374

Ferguson, B. J., Mathesius, U. (2014). Phytohormone regulation of legume-rhizobia interactions. *J. Chem. Ecol.* 40, 770–790. doi: 10.1007/s10886-014-0472-7

Feyissa, B. A., Arshad, M., Gruber, M. Y., Kohalmi, S. E., Hannoufa, A. (2019). The interplay between miR156/SPL13 and DFR/WD40–1 regulate drought tolerance in alfalfa. *BMC plant Biol.* 19, 1–19. doi: 10.1186/s12870-019-2059-5

Fisher, R. F., Egelhoff, T. T., Mulligan, J. T., Long, S. R. (1988). Specific binding of proteins from *Rhizobium meliloti* cell-free extracts containing NodD to DNA sequences upstream of inducible nodulation genes. *Genes Dev.* 2, 282–293. doi: 10.1101/gad.2.3.282

Fleming, M. B., Patterson, E. L., Reeves, P. A., Richards, C. M., Gaines, T. A., Walters, C. (2018). Exploring the fate of mRNA in aging seeds: protection, destruction, or slow decay? *J. Exp. Bot.* 69, 4309–4321. doi: 10.1093/jxb/ery215

Foucher, F., Kondorosi, E. (2000). Cell cycle regulation in the course of nodule organogenesis in *Medicago*. *Plant Mol. Biol.* 43, 773–786. doi: 10.1023/a:1006405029600

Franche, C., Lindström, K., Elmerich, C. (2009). Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant Soil* 321, 35–59. doi: 10.1007/s11104-008-9833-8

Fukuda, A., Nakamura, A., Tanaka, Y. (1999). Molecular cloning and expression of the Na^+/H^+ exchanger gene in *Oryza sativa*. *Biochem. Biophys. Acta* 1446, 149–155. doi: 10.1016/s0167-4781(99)00065-2

Fürstenberg-Hägg, J., Zagrobelny, M., Bak, S. (2013). Plant defense against insect herbivores. *Int. J. Mol. Sci.* 14, 10242–10297. doi: 10.3390/ijms140510242

Gage, D. J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol Biol Rev.* 68, 280–300. doi: 10.1128/MMBR.68.2.280-300.2004

Gamborg, O. L., Miller, R. A., Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50, 151–158. doi: 10.1016/0014-4827(68)90403-5

Gao, M., Liu, J., Bi, D., Zhang, Z., Cheng, F., Chen, S., et al. (2008). MEKK1, MKK1/MKK2 and MPK4 function together in a mitogen-activated protein kinase cascade to regulate innate immunity in plants. *Cell Res.* 18, 1190–1198. doi: 10.1038/cr.2008.300

Gao, R., Austin, R. S., Amyot, L., Hannoufa, A. (2016). Comparative transcriptome investigation of global gene expression changes caused by miR156 overexpression in *Medicago sativa*. *BMC Genom.* 17, 1–15. doi: 10.1186/s12864-016-3014-6

Gao, Z., Luo, W., Liu, H., Zeng, C., Liu, X., Yi, S., et al. (2012). Transcriptome analysis and SSR/SNP markers information of the blunt snout bream (*Megalobrama amblycephala*). *PloS One* 7. doi: 10.1371/journal.pone.0042637

García, A. N., Ayub, N. D., Fox, A. R., Gómez, M. C., Diéguez, M. J., Pagano, E. M., et al. (2014). Alfalfa snakin-1 prevents fungal colonization and probably coevolved with rhizobia. *BMC Plant Biol.* 14, 248. doi: 10.1186/s12870-014-0248-9

Gautrat, P., Laffont, C., Frugier, F. (2020). Compact root architecture 2 promotes root competence for nodulation through the miR2111 systemic effector. *Curr. Biol.* 30, 1339–1345. doi: 10.1016/j.cub.2020.01.084

Ge, Y. Y., Xiang, Q. W., Wagner, C., Zhang, D., Xie, Z. P., Staehelin, C. (2016). The type 3 effector NopL of *Sinorhizobium* sp. strain NGR234 is a mitogen-activated protein kinase substrate. *J. Exp. Biol*. 67, 2483–2494. doi: 10.1093/jxb/erw065

Gelvin, S. B. (2003). Agrobacterium-Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool. *Microbiol. Mol. Biol. Rev.* 67, 16–37. doi: 10.1128/MMBR.67.1.16-37.2003

Gelvin, S. B. (2017). Integration of *Agrobacterium* T-DNA into the Plant Genome. *Ann. Rev. Gen.* 51, 195–217. doi: 10.1146/annurev-genet-120215-035320

Genre, A., Chabaud, M., Timmers, T., Bonfante, P., Barker, D. G. (2005). Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. *Plant Cell* 17, 3489–3499. doi: 10.1105/tpc.105.035410

Geurts, R., Xiao, T. T., Reinhold-Hurek, B. (2016). What does it take to evolve a nitrogen-fixing endosymbiosis? *Trends Plant Sci*. 21, 199–208. doi: 10.1016/j.tplants.2016.01.012

Gibson, K. E., Kobayashi, H., Walker, G. C. (2008). Molecular determinants of a symbiotic chronic infection. *Ann. Rev. Gen.* 42, 413–441. doi. 10.1146/annurev. genet.42.110807.091427

Glazebrook, J., Walker, G. C. (1989). A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. *Cell* 56, 661–672. doi: 10.1016/0092-8674(89)90588-6

Gómez-Gómez, L., Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell* 5, 1003–1011. doi: 10.1016/S1097-2765(00)80265-8

Gong, B., Li, X., Bloszies, S., Wen, D., Sun, S., Wei, M. (2014). Sodic alkaline stress mitigation by interaction of nitric oxide and polyamines involves antioxidants and physiological strategies in *Solanum lycopersicum*. *Free Radic. Biol. Med.* 71, 36–48. doi: 10.1016/j.freeradbiomed.2014.02.018

González Besteiro, M. A., Ulm, R. (2013). Phosphorylation and stabilization of Arabidopsis MAP kinase phosphatase 1 in response to UV-B stress. *J. Biol. Chem.* 288, 480–486. doi: 10.1074/jbc.M112.434654

Good, M. C., Zalatan, J. G., Lim, W. A. (2011). Scaffold proteins: hubs for controlling the flow of cellular information. *Science* 332, 680–686. doi: 10.1126/science.1198701

Gou, J., Debnath, S., Sun, L., Flanagan, A., Tang, Y., Jiang, Q., et al. (2018). From model to crop: functional characterization of SPL 8 in *M. truncatula* led to genetic improvement of biomass yield and abiotic stress tolerance in alfalfa. *Plant Biotechnol. J.* 16, 951–962. doi: 10.1111/pbi.12841

Gourion, B., Berrabah, F., Ratet, P., Stacey, G. (2015). *Rhizobium*-legume symbioses: the crucial role of plant immunity. *Trends Plant Sci.* 20, 186–194. doi: 10.1016/j. tplants.2014.11.008

Graham, P. H. (1963). Antigenic affinities of the root-nodule bacteria of legumes. *Antonie van Leeuwenhoek* 29, 281–291. doi: 10.1007/BF02046070

Griffin, G. D. (1990). "Diseases caused by nematodes," in: Compendium of Alfalfa Diseases, second edition, eds. D.L. Stuteville and D.C. Erwin (APS Press. St. Paul, MN). 46–51.

Griffith, F. (1928). The Significance of Pneumococcal Types. *J. Hyg.* 27, 113–159. doi: 10.1017/s0022172400031879

Grimsrud, P. A., den Os, D., Wenger, C. D., Swaney, D. L., Schwartz, D., Sussman, M. R., et al. (2010). Large-scale phosphoprotein analysis in *Medicago truncatula* roots provides insight into in vivo kinase activity in legumes. *Plant Physiol.* 152, 19–28. doi: 10.1104/pp.109.149625

Gull, A., Lone, A. A., Wani, N. U. I. (2019). Biotic and abiotic stresses in plants. *Ab. Biot. Stress Plants* 1–19. doi: 10.5772/intechopen.85832

Gupta, R., Chakrabarty, S. K. (2013). Gibberellic acid in plant, still a mystery unresolved. *Plant Signal. Behav.* 8:e25504. doi: 10.4161/psb.25504

Ha, C.V., Watanabe, Y., Tran, U. T., Le, D. T., Tanaka, M., Nguyen, K. H., et al. (2015). Comparative analysis of root transcriptomes from two contrasting drought-responsive Williams 82 and DT2008 soybean cultivars under normal and dehydration conditions. *Front. Plant Sci.* 6, 551. doi: 10.3389/fpls.2015.00551

Hafez, S. L., Sundararaj, P. (2009). “Nematodes of alfalfa and their management,” in: Proceedings, Western Alfalfa & Forage Conference, Reno, Nevada, 2–4.

Halbleib, C. M., Ludden, P. W. (2000). Regulation of biological nitrogen fixation. *J. Nutr.* 130, 1081–1084. doi: 10.1093/jn/130.5.1081

Han, Y., Kang, Y., Torres-Jerez, I., Cheung, F., Town, C. D., Zhao, P. X., et al. (2011). Genome-wide SNP discovery in tetraploid alfalfa using 454 sequencing and high resolution melting analysis. *BMC genom.* 12, 350. doi: 10.1186/1471-2164-12-350

Harrison, M. J., Choudhary, A. D., Dubery, I., Lamb, C. J., Dixon, R. A. (1991). Stress responses in alfalfa (*Medicago sativa* L.). 8. Cis-elements and trans-acting factors for the quantitative expression of a bean chalcone synthase gene promoter in electroporated alfalfa protoplasts. *Plant Mol. Biol.* 16, 877–890. doi: 10.1007/BF00015079

Hawkins, C., Yu, L. X. (2018). Recent progress in alfalfa (*Medicago sativa* L.) genomics and genomic selection. *Crop J.* 6, 565–575. doi: 10.1016/j.cj.2018.01.006

He, X. Z., Dixon, R. A. (2000). Genetic manipulation of isoflavone 7-O-methyltransferase enhances biosynthesis of 4'-O-methylated isoflavonoid phytoalexins and disease resistance in alfalfa. *Plant Cell* 12, 1689–1702. doi: 10.1105/tpc.12.9.1689

Heidstra, R., Geurts, R., Franssen, H., Spaink, H. P., Van Kammen, A. B., Bisseling, T. (1994). Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. *Plant Physiol.* 105, 787–797. doi: 10.1104/pp.105.3.787

Heidstra, R., Yang, W. C., Yalcin, Y., Peck, S., Emons, A. M., Kammen van, A., et al. (1997). Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in *Rhizobium*–legume interaction. *Development* 124, 1781–1787.

Henderson, I.R., Jacobsen, S.E. (2007). Epigenetic inheritance in plants. *Nature* 447, 418–424. doi: 10.1038/nature05917.

Hepler, P. K., Vidali, L., Cheung, A. Y. (2001). Polarized cell growth in higher plants. *Annu. Rev. Cell Dev. Biol.* 17, 159–187. doi: 10.1146/annurev.cellbio.17.1.159

Herrmann, D., Flajoulot, S., Barre, P., Huyghe, C., Ronfort, J., Julier, B. (2018). Comparison of morphological traits and molecular markers to analyse diversity and structure of alfalfa (Medicago sativa L.) cultivars. *Gen. Res. Crop Evol.* 65, 527–540. doi: 10.1007/s10722-017-0551-z

Hettenhausen, C., Baldwin, I. T., Wu, J. (2012). Silencing MPK4 in *Nicotiana attenuata* enhances photosynthesis and seed production but compromises abscisic acid-induced stomatal closure and guard cell-mediated resistance to *Pseudomonas syringae* pv tomato DC3000. *Plant Physiol.* 158, 759–776. doi: 10.1104/pp.111.190074

Hettenhausen, C., Schuman, M. C., Wu, J. (2014). MAPK signaling - a key element in plant defense response to insects. *Insect Sci.* 22, 157–164. doi: 10.1111/1744-7917.12128

Hipskind, J. D., Paiva, N. L. (2000). Constitutive accumulation of a resveratrol-glucoside in transgenic alfalfa increases resistance to *Phoma medicaginis*. *Mol. Plant Microbe In.* 13, 551–556. doi: 10.1094/MPMI.2000.13.5.551

Hirakawa, Y., Sawa, S. (2019). Diverse function of plant peptide hormones in local signaling and development. *Curr. Opin. Plant Biol.* 51, 81–87. doi: 10.1016/j.pbi.2019.04.005

Hirsch, A. M., Bhuvaneswari, T. V., Torrey, J. G., Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 86, 1244–1248. doi: 10.1073/pnas.86.4.1244

Howe, C. L., Valletta, J. S., Rusnak, A. S., Mobley, W. C. (2001). NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway. *Neuron* 32, 801–814. doi: 10.1016/S0896-6273(01)00526-8

Hrbáčková, M., Dvořák, P., Takáč, T., Tichá, M., Luptovčiak, I., Šamajová, O., et al. (2020). Biotechnological Perspectives of Omics and Genetic Engineering Methods in Alfalfa. *Front. Plant Sci.* 11, 592. doi: 10.3389/fpls.2020.00592

Hrbáčková, M., Luptovčiak, I., Hlaváčková, K., Dvořák, P., Tichá, M., Šamajová, O., et al. (2021). Overexpression of alfalfa SIMK promotes root hair growth, nodule clustering and shoot biomass production. *Plant Biotechnol. J.* 19, 767-784. doi: 10.1111/pbi.13503

Huault, E., Laffont, C., Wen, J., Mysore, K. S., Ratet, P., Duc, G., et al. (2014). Local and systemic regulation of plant root system architecture and symbiotic nodulation by a receptor-like kinase. *PLoS Genet,* 10, e1004891. doi: 10.1371/journal.pgen.1004891

Huang, X., Kurata, N., Wang, Z. X., Wang, A., Zhao, Q., Zhao, Y., et al. (2012). A map of rice genome variation reveals the origin of cultivated rice. *Nature* 490, 497–501. doi: 10.1038/nature11532

Hwang, E. Y., Song, Q., Jia, G., Specht, J. E., Hyten, D. L., Costa, J., Cregan, P. B. (2014). A genome-wide association study of seed protein and oil content in soybean. *BMC genom.* 15. doi: 10.1186/1471-2164-15-1

Ichimura, K., Mizoguchi, T., Irie, K., Morris, P., Giraudat, J., Matsumoto, K., et al. (1998). Isolation of ATMEKK1 (a MAP kinase kinase kinase)-interacting proteins and analysis of a MAP kinase cascade in *Arabidopsis*. *Biochem. Biophys. Res. Commun.* 253, 532–543. doi: 10.1006/bbrc.1998.9796

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T., Shinozaki, K. (2000). Various abiotic stresses rapidly activate Arabidopsis MAP kinases ATMPK4 and ATMPK6. *Plant J.* 24, 655–665. doi: 10.1046/j.1365-313x.2000.00913.x

Ichimura, K., Shinozaki, K., Tena, G., Sheen, J., Henry, Y., Champion, A., Heberle-Bors, E. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends Plant Sci.* 7, 301–308. doi: 10.1016/s1360-1385(02)02302-6

Imin, N., Mohd-Radzman, N. A., Ogilvie, H. A., Djordjevic, M. A. (2013). The peptide-encoding CEP1 gene modulates lateral root and nodule numbers in *Medicago truncatula*. *J. Exp. Bot.* 64, 5395–5409. doi: 10.1093/jxb/ert369

Ishihama, N., Yamada, R., Yoshioka, M., Katou, S., and Yoshioka, H. (2011). Phosphorylation of the *Nicotiana benthamiana* WRKY8 transcription factor by MAPK functions in the defense response. *Plant Cell* 23, 1153–1170. doi: 10.1105/tpc.110.081794

Ito, Y., Nakanomyo, I., Motose, H., Iwamoto, K., Sawa, S., Dohmae, N., Fukuda, H. (2006). Dodeca-CLE peptides as suppressors of plant stem cell differentiation. *Science* 313, 842–845. doi: 10.1126/science.1128436

Jagodzik, P., Tajdel-Zielinska, M., Ciesla, A., Marczak, M., Ludwikow, A. (2018). Mitogen-activated protein kinase cascades in plant hormone signaling. *Front. Plant Sci.* 9, 1387. doi: 10.3389/fpls.2018.01387

Jain, M., Olsen, H. E., Paten, B., Akeson, M. (2016). The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. *Gen. Biol.* 17, 239. doi: 10.1186/s13059-016-1103-0

Jammes, F., Song, C., Shin, D., Munemasa, S., Takeda, K., Gu, D., et al. (2009). MAP kinases MPK9 and MPK12 are preferentially expressed in guard cells and positively regulate ROS-mediated ABA signaling. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20520–20525. doi: 10.1073/pnas.0907205106

Janczarek, M., Rachwał, K., Marzec, A., Grzadziel, J., Palusińska-Szysz, M. (2014). Signal molecules and cell-surface components involved in early stages of the legume-rhizobium interactions. *Appl. Soil Ecol.* 85, 94–113. doi: 10.1016/j.apsoil.2014.08.010

Janczarek, M., Rachwal, K., Marzec, A., Grzadziel, J., and Palusinska-Szysz, M. (2015). Signal molecules and cell-surface components involved in early stages of the legume-*Rhizobium* interactions. *Appl. Soil Ecol.* 85, 94–113. doi: 10.1016/j.apsoil.2014.08.010

Jia, R. Z., Zhang, R. J., Wei, Q., Chen, W. F., Cho, I. K., Chen, W. X., et al. (2015). Identification and classification of rhizobia by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Proteomics Bioinform.* 8, 98–107. doi: 10.4172/jpb.1000357

Jia, W., Li, B., Li, S., Liang, Y., Wu, X., Ma, M., Wang, Y. (2016). Mitogen-activated protein kinase cascade MKK7-MPK6 plays important roles in plant development and regulates shoot branching by phosphorylating PIN1 in *Arabidopsis*. *PLoS Biol.* 14, e1002550. doi: 10.1371/journal.pbio.1002550

Jiang, Q., Fu, C., Wang, Z. Y. (2019). A Unified *Agrobacterium*-Mediated Transformation Protocol for Alfalfa (*Medicago sativa* L.) and *Medicago truncatula*. *Methods Mol. Biol.* 1864, 153–163. doi: 10.1007/978-1-4939-8778-8_11

Jin, T., Chang, Q., Li, W., Yin, D., Li, Z., Wang, D., et al. (2010). Stress-inducible expression of GmDREB1 conferred salt tolerance in transgenic alfalfa. *Plant Cell T. Organ Cult.* 100, 219–227. doi: 10.1007/s11240-009-9628-5

Joersbo, M., Brunstedt, J. (1990). Direct gene transfer to plant protoplasts by electroporation by alternating, rectangular and exponentially decaying pulses. *Plant Cell Rep.* 8, 701–705. doi: 10.1007/BF00272098

Jonak, C., Kiegerl, S., Ligterink, W., Barker, P. J., Huskisson, N. S., Hirt, H. (1996). Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Natl. Acad. Sci. USA.* 93, 11274–11279. doi: 10.1073/pnas.93.20.11274

Jonak, C., Kiegerl, S., Lloyd, C., Chan, J., Hirt, H. (1995). MMK2, a novel alfalfa MAP kinase, specifically complements the yeast MPK1 function. *Mol. Gen Genet.* 248, 686–694. doi: 10.1007/BF02191708

Jones, J. D. G., Dangl, J. L. (2006). The plant immune system. *Nature* 444**,** 323–329. doi: 10.1038/nature05286

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., Walker, G. C. (2007). How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nature Rev. Microbiol.* 5, 619–633. doi: 10.1038/nrmicro1705

Jordan, D. C. (1984). Family III. *Rhizobiaceae* Conn 1938. Bergey's manual of systematic bacteriology, 1, 234–244.

Julier, B., Flajoulot, S., Barre, P., Cardinet, G., Santoni, S., Huguet, T., et al. (2003). Construction of two genetic linkage maps in cultivated tetraploid alfalfa (*Medicago sativa*) using microsatellite and AFLP markers. *BMC Plant B.* 3, 9. doi: 10.1186/1471-2229-3-9

Kang, Y., Han, Y., Torres-Jerez, I., Wang, M., Tang, Y., Monteros, M., et al. (2011). System responses to long-term drought and re-watering of two contrasting alfalfa varieties. *Plant J.* 68, 871–889. doi: 10.1111/j.1365-313X.2011.04738.x

Kang, Y., Sakiroglu, M., Krom, N., Stanton-Geddes, J., Wang, M., Lee, Y. C., et al. (2015). Genome-wide association of drought-related and biomass traits with HapMap SNPs in *Medicago truncatula*. *Plant Cell Env.* 38, 1997–2011. doi: 10.1111/pce.12520

Kannenberg, E., Perzl, M., Härtner, T. (1995). The occurrence of hopanoid lipids in *Bradyrhizobium* bacteria. *FEMS Microbiol. Let.* 127, 255–261. doi: 10.1111/j.1574-6968.1995.tb07482.x

Kao, K. N., Michayluk, M. R. (1980). Plant regeneration from mesophyll protoplasts of alfalfa. *Zeit. Pflanzenphysiol.* 96, 135–141. doi: 10.1016/S0044-328X(80)80051-1

Kao, K.N., Michayluk, M.R. (1981). Embryoid formation in alfalfa cell suspension cultures from different plants. *In Vitro* 17, 645–648. doi: 10.1007/BF02618463.

Kape, R., Parniske, M., Brandt, S., Werner, D. (1992). Isoliquiritigenin, a strong nod gene-inducing and glyceollin resistance-inducing flavonoid from soybean root exudate. *Appl. Environ. Microbiol*. 1992, 1705–1710. doi: 10.1128/AEM.58.5.1705-1710.1992

Karimi, M., Bleys, A., Vanderhaeghen, R., Hilson, P. (2007). Building blocks for plant gene assembly. *Plant Physiol.* 145, 1183–1191. doi: 10.1104/pp.107.110411

Karin, M. **(**1998). Mitogen-activated protein kinase cascades as regulators of stress responses. *Ann. NY Acad. Sci.* 851**,** 139–146. doi: 10.1111/j.1749-6632.1998.tb08987.x

Kersey, P. J. (2019). Plant genome sequences: past, present, future. *Curr. Opin. Plant Biol.* 48, 1–8. doi: 10.1016/j.pbi.2018.11.001

Khan, M. S., Zaidi, A., Musarrat, J. (2010). "Microbes for legume improvement," in: Vienna: Springer, eds. M. Saghir Khan, A. Zaidi and J. Musarrat (Springer Vienna). 535. doi: 10.1007/978-3-211-99753-6

Khu, D. M., Reyno, R., Han, Y., Zhao, P. X., Bouton, J. H., Brummer, E. C., et al. (2013). Identification of aluminum tolerance quantitative trait loci in tetraploid alfalfa. *Crop Sci.* 53, 148–163. doi: 10.2135/cropsci2012.03.0181

Kiegerl, S., Cardinale, F., Siligan, C., Gross, A., Baudouin, E., Liwosz, A., et al. (2000). SIMKK, a mitogen-activated protein kinase (MAPK) kinase, is a specific activator of the salt stress–induced MAPK, SIMK. *Plant Cell* 12, 2247–2258. doi: 10.1105/tpc.12.11.2247

Kim, J. M., Woo, D. H., Kim, S. H., Lee, S. Y., Park, H. Y., Seok, H. Y., et al. (2012). *Arabidopsis* MKKK20 is involved in osmotic stress response via regulation of MPK6 activity. *Plant Cell Rep.* 31, 217–224. doi: 10.1007/s00299-011-1157-0

Kim, K. Y., Kwon, S. Y., Lee, H. S., Hur, Y., Bang, J. W., Kwak, S. S. (2003). A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. *Plant Mol. Biol.* 51, 831–838. doi: 10.1023/a:1023045218815

Kineman, B.D., Brummer, E.C., Paiva, N.L., Birt, D.F. (2010): Resveratrol from transgenic alfalfa for prevention of aberrant crypt foci in mice. *Nutr. Canc.* 623, 351–361. doi: 10.1080/01635580903407213

Kiss, G. B., Csanádi, G., Kálmán, K., Kaló, P., Ökrész, L. (1993). Construction of a basic genetic map for alfalfa using RFLP, RAPD, isozyme and morphological markers. *Mol. Gen. Gen.* 238, 129–137. doi: 10.1007/BF00279539

Komis G, Šamajová O, Ovečka M, Šamaj J (2018) Cell and developmental biology of plant mitogen-activated protein kinases. *Ann. Rev. Plant Biol.* 69, 237–265. doi: 10.1146/annurev-arplant-042817-040314

Kong, X., Pan, J., Zhang, D., Jiang, S., Cai, G., Wang, L., et al. (2013). Identification of mitogen-activated protein kinase kinase gene family and MKK-MAPK interaction network in maize. *Biochem. Biophys. Res. Commun.* 441, 964–969. doi: 10.1016/j.bbrc.2013.11.008

Köpp, M., Passos, L., Verneue, R., Lédo, F. J., Coimbra, J. L., de Oliveira, A. (2011). Effects of nutrient solution pH on growth parameters of alfalfa (*Medicago sativa* L.) genotypes. *Comun. Sci.e* 2, 135–141. doi: 10.14295/cs.v2i3.39

Kosetsu, K., Matsunaga, S., Nakagami, H., Colcombet, J., Sasabe, M., Soyano, T., et al. (2010). The MAP kinase MPK4 is required for cytokinesis in *Arabidopsis thaliana*. *Plant Cell* 22, 3778–3790. doi: 10.1105/tpc.110.077164

Kosturkova, G. (1993). Expression of foreign genes following electroporation of Medicago protoplasts. *Biotechnol. Biotech. Equip.* 7, 43–46. doi: 10.1080/13102818.1993.10818692

Křenek, P., Šamajová, O., Luptovčiak, I., Doskočilová, A., Komis, G., Šamaj, J. (2015). Transient plant transformation mediated by *Agrobacterium tumefaciens*: Principles, methods and applications. *Biotechnol. Adv.* 33, 1024–1042. doi: 10.1016/j.biotechadv.2015.03.012

Kumar, K. R., Kirti, P. B. (2010). A mitogen-activated protein kinase, AhMPK6 from peanut localizes to the nucleus and also induces defense responses upon transient expression in tobacco. *Plant Physiol. Biochem.* 48, 481–486. doi: 10.1016/j.plaphy.2010.03.010

Kumar, T., Bao, A. K., Bao, Z., Wang, F., Gao, L., Wang, S. M. (2018). The progress of genetic improvement in alfalfa (*Medicago sativa* L.). *Czech J. Genet. Plant Breed.* 54, 41–51. doi: 10.17221/46/2017-CJGPB

Laberge, S., Castonguay, Y., Vézina, L. P. (1993). New cold-and drought-regulated gene from *Medicago sativa*. *Plant Physiol.-Rockville Pike Bethesda*, 101, 1411–1411. doi: 10.1104/pp.101.4.1411

Laffont, C., Ivanovici, A., Gautrat, P., Brault, M., Djordjevic, M. A., Frugier, F. (2020). The NIN transcription factor coordinates CEP and CLE signaling peptides that regulate nodulation antagonistically. *Nature Com.* 11, 1–13. doi: 10.1038/s41467-020-16968-1

Lai, F. M., McKersie, B. D. (1994). Regulation of starch and protein accumulation in alfalfa (*Medicago sativa* L.) somatic embryos. *Plant Sci.* 100, 211–219. doi: 10.1016/0168-9452(94)90078-7

Landy, A. (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. *Ann. Rev. Biochem.* 58, 913–949. doi: 10.1146/annurev.bi.58.070189.004405

Lardi, M., Pessi, G. (2018). Functional genomics approaches to studying symbioses between legumes and nitrogen-fixing rhizobia. *High Throughput.* 7, 15. doi: 10.3390/ht7020015

Lawson, C. G. R., Rolfe, B. G., Djordjevic, M. A. (1996). *Rhizobium* inoculation induces conditiondependent changes in the flavonoid composition of root exudates from *Trifolium subterraneum*. *Funct. Plant Biol.* 23, 93–101. doi: 10.1071/PP9960093

Lee, H., Kim, J., Im, J. H., Kim, H. B., Oh, C. J., An, C. S. (2008). Mitogen-activated protein kinase is involved in the symbiotic interaction between *Bradyrhizobium japonicum* USDA110 and soybean. *J. Plant Biol.* 51, 291–296. doi: 10.1007/BF03036129

Le Nguyen, K., Grondin, A., Courtois, B., Gantet, P. (2018). Next-generation sequencing accelerates crop gene discovery. *Trends Plant Sci.* 24. doi: 10.1016/j.tplants.2018.11.008

Le, D. T., Nishiyama, R., Watanabe, Y., Tanaka, M., Seki, M., Ham, L. H., et al. (2012). Differential gene expression in soybean leaf tissues at late developmental stages under drought stress revealed by genome-wide transcriptome analysis. *PLoS One* 7, e49522. doi: 10.1371/journal.pone.0049522

Lei, Y., Hannoufa, A., Yu, P. (2017). The use of gene modification and advanced molecular structure analyses towards improving alfalfa forage. *Int. J. Mol. Sci.* 18, 298. doi: 10.3390/ijms18020298

Lei, Y., Xu, Y., Hettenhausen, C., Lu, C., Shen, G., Zhang, C., et al. (2018). Comparative analysis of alfalfa (*Medicago sativa* L.) leaf transcriptomes reveals genotype-specific salt tolerance mechanisms. *BMC Plant Biol.* 18, 35. doi: 10.1186/s12870-018-1250-4

Lewis, T. S., Shapiro, P. S., Ahn, N. G. (1998). Signal transduction through MAP kinase cascades. *Adv. Can. Res.* 74, 49–139. doi: 10.1016/s0065-230x(08)60765-4

Li, B., Li, Y. Y., Wu, H. M., Zhang, F. F., Li, C. J., Li, X. X., et al. (2016). Root exudates drive interspecific facilitation by enhancing nodulation and N_2 fixation. *Proc Natl. Acad. Sci.* 113, 6496–6501. doi: 10.1073/pnas.1523580113

Li, H., Wang, Z., Ke, Q., Ji, C. Y., Jeong, J. C., Lee, H. S., et al. (2014). Overexpression of *codA* gene confers enhanced tolerance to abiotic stresses in alfalfa. *Plant Physiol. Biochem.* 85, 31–40. doi: 10.1016/j.plaphy.2014.10.010

Li, S., Jia, S., Hou, L., Nguyen, H., Sato, S., Holding, D., Yu, B. (2019). Mapping of transgenic alleles in soybean using a nanopore-based sequencing strategy. *J. Exp. Bot.* 70, 3825–3833. doi: 10.1093/jxb/erz202

Li, X., Brummer, E. C. (2012). Applied genetics and genomics in alfalfa breeding. *Agronomy* 2, 40–61. doi: 10.3390/agronomy2010040

Li, X., Wei, Y., Acharya, A., Jiang, Q., Kang, J., and Brummer, E. C. (2014). A saturated genetic linkage map of autotetraploid alfalfa (*Medicago sativa* L.) developed using genotyping-by-sequencing is highly syntenous with the *Medicago truncatula* genome. *G3* 4, 1971–1979. doi: 10.1534/g3.114.012245

Limpens, E., Franken, C., Smit, P., Willemse, J., Bisseling, T., Geurts, R. (2003). LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science* 302, 630–633. doi: 10.1126/science.1090074

Lin, J., Frank, M., Reid, D. (2020). No home without hormones: how plant hormones control legume nodule organogenesis. *Plant Commun.* 1, 100104. doi: 10.1016/j.xplc.2020.100104

Liu, C. W., Murray, J. (2016). The role of flavonoids in nodulation host-range specificity: an update. *Plants* 5, 33. doi: 10.3390/plants5030033

Liu, C. Z., Yan, L., Wei, L. X., Zhang, F., Qian, X. J. (2008). Effects of cutting on the population dynamics of main insect pests on alfalfa. *Ying Yong Sheng Tai Xue Bao* 193, 691–694.

Liu, H., Zhang, C., Yang, J., Yu, N., Wang, E. (2018). Hormone modulation of legume-*rhizobial* symbiosis. *J. Int. Plant Biol.* 60, 632–648. doi: 10.1111/jipb.12653

Liu, Y., Jiang, X., Guan, D., Zhou, W., Ma, M., Zhao, B., et al. (2017). Transcriptional analysis of genes involved in competitive nodulation in *Bradyrhizobium diazoefficiens* at the presence of soybean root exudates. *Sci. Rep.* 7, 1–11. doi: 10.1038/s41598-017- 11372-0

Liu, Z., Chen, T., Ma, L., Zhao, Z., Zhao, P. X., Nan, Z., et al. (2013). Global transcriptome sequencing using the Illumina platform and the development of EST-SSR markers in autotetraploid alfalfa. *PLoS One* 8, e83549. doi: 10.1371/journal.pone.0083549

Loh, J., Pierson, E. A., Pierson III, L. S., Stacey, G., Chatterjee, A. (2002). Quorum sensing in plant-associated bacteria. *Curr. Opin. Plant Biol.*, 5, 285–290. doi: 10.1016/S1369-5266(02)00274-1

Long, R., Zhang, F., Zhang, Z., Li, M., Chen, L., Wang, X., Yang, Q. (2021). Assembly of chromosome-scale and allele-aware autotetraploid genome of the Chinese alfalfa cultivar Zhongmu-4 and identification of SNP loci associated with 27 agronomic traits. *bioRxiv*. doi: 10.1101/2021.02.21.428692

Long, S. R. (1996). Rhizobium symbiosis: nod factors in perspective. *Plant Cell* 8, 1885–1898. doi: 10.1105/tpc.8.10.1885

Long, S. R. (2001). Genes and signals in the rhizobium-legume symbiosis. *Plant Physiol.* 125, 69–72. doi: 10.1104/pp.125.1.69

Lopez-Gomez, M., Sandal, N., Stougaard, J., Boller, T. (2012). Interplay of flg22-induced defence responses and nodulation in *Lotus japonicus*. *J. Exp. Bot.* 63, 393–401. doi: 10.1093/jxb/err291

Lopez-Solanilla, E., García-Olmedo, F., Rodríguez-Palenzuela, P. (1998). Inactivation of the *sapA* to *sapF* locus of *Erwinia chrysanthemi* reveals common features in plant and animal bacterial pathogenesis. *Plant Cell* 10, 917–924. doi: 10.1105/tpc.10.6.917

LPWG – The Legume Phylogeny Working Group (2017). A new subfamily classification of the *Leguminosae* based on a taxonomically comprehensive phylogeny. *Taxon* 66, 44–77. doi: 10.12705/661.3

Lu, D. Y., Davey, M. R., Cocking, E. C. (1983). A comparison of the cultural behaviour of protoplasts from leaves, cotyledons and roots of *Medicago sativa*. *Plant Sci. Lett.* 31, 87–99. doi: 10.1016/0304-4211(83)90132-3

Lu, H., Giordano, F., Ning, Z. (2016). Oxford Nanopore MinION sequencing and genome assembly. *Genom. Proteom. Bioinf.* 14, 265–279. doi: 10.1016/j.gpb.2016.05.004

Luo, D., Wu, Y., Liu, J., Zhou, Q., Liu, W., Wang, Y., et al. (2019a). Comparative transcriptomic and physiological analyses of *Medicago sativa* L. indicates that multiple regulatory networks are activated during continuous aba treatment. *Int. J. Mol. Sci.* 20, E47. doi: 10.3390/ijms20010047

Luo, D., Zhou, Q., Wu, Y., Chai, X., Liu, W., Wang, Y., et al. (2019b). Full-length transcript sequencing and comparative transcriptomic analysis to evaluate the contribution of osmotic and ionic stress components towards salinity tolerance in the roots of cultivated alfalfa (*Medicago sativa* L.). *BMC Plant Biol.* 19, 32. doi: 10.1186/s12870-019-1630-4

Luo, M., Lin, L., Hill, R. D., Mohapatra, S. S. (1991). Primary structure of an environmental stress and abscisic acid-inducible alfalfa protein. *Plant Mol. Biol.* 17, 1267–1269. doi: 10.1007%2FBF00028745

Luo, M., Liu, J. H., Mohapatra, S., Hill, R. D., Mohapatra, S. S. (1992). Characterization of a gene family encoding abscisic acid-and environmental stress-inducible proteins of alfalfa. *J. Biol. Chem.* 267, 15367–15374. doi: 10.1016/S0021-9258(19)49543-4

Mackie, J. M., Musial, J. M., Armour, D. J., Phan, H. T. T., Ellwood, S. E., et al. (2007). Identification of QTL for reaction to three races of *Colletotrichum trifolii* and further analysis of inheritance of resistance in autotetraploid lucerne. *Theor. Appl. Genet.* 114, 1417–1426. doi: 10.1007/s00122-007-0527-z

Macovei, A., Gill, S. S., Tuteja, N. (2012). microRNAs as promising tools for improving stress tolerance in rice. *Plant Sig. Beh.* 7, 1296–1301. doi: 10.4161/psb.21586

Madhani, H. D., Fink, G. R. (1998). The riddle of MAP kinase signaling specificity. *Trends Gen.* 14, 151–155. doi: 10.1016/s0168-9525(98)01425-5

Madsen, L. H., Tirichine, L., Jurkiewicz, A., Sullivan, J. T., Heckmann, A. B., Bek, A. S., et al. (2010). The molecular network governing nodule organogenesis and infection in the model legume *Lotus japonicus*. *Nat. Commun.* 1, 10. doi: 10.1038/ncomms1009

Maekawa, M. Maekawa-Yoshikawa, N. Takeda, H. Imaizumi-Anraku, Y. Murooka, M. Hayashi, M. (2009). Gibberellin controls the nodulation signaling pathway in *Lotus japonicus. Plant J.*, 58, 183–-194. doi: 10.1111/j.1365-313X.2008.03774.x

Maldonado-Bonilla, L. D., Eschen-Lippold, L., Gago-Zachert, S., Tabassum, N., Bauer, N., Scheel, D., et al. (2013). The Arabidopsis tandem zinc finger 9 protein binds RNA and mediates pathogen-associated molecular pattern-triggered immune responses. *Plant Cell Physiol.* 55, 412–425. doi: 10.1093/pcp/pct175

Mannetje, L. T. (1967). A re-examination of the taxonomy of the genus *Rhizobium* and related genera using numerical analysis. *Antonie van Leeuwenhoek* 33, 477–491. doi: 10.1007/BF02045600

MAPK-Group. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends Plant Sci.* 7, 301–308. doi: 10.1016/S1360-1385(02)02302-6

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437, 376–380. doi: 10.1038/nature03959

Masonbrink, R. E., Severin, A. J., Seetharam, A. S. (2017). "Comparative genomics of soybean and other legumes," in: The Soybean Genome, eds. H. Nguyen, M. Bhattacharyya (Cham: Springer). 83–93. doi: 10.1007/978-3-319-64198-0_6

Masoud, S. A., Zhu, Q., Lamb, C., Dixon, R. A. (1996). Constitutive expression of an inducible β-1,3-glucanase in alfalfa reduces disease severity caused by the oomycete pathogen *Phytophthora megasperma* f. sp. *medicaginis*, but does not reduce disease severity of chitin-containing fungi. *Transgenic Res.* 5, 313–323. doi: 10.1007/BF01968941

Mathesius, U., Schlaman, H. R. M., Spaink, H. P., Sautter, C., Rolfe, B. G., Djordjevic, M. A. (1998). Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. *Plant J.* 14, 23–34. doi: 10.1046/j.1365-313X.1998.00090.x

Matiru, V. N., Dakora, F. D. (2005a). The rhizosphere signal molecule lumichrome alters seedling development in both legumes and cereals. *New Phytol.* 166, 439–444. doi: 10.1111/j.1469-8137.2005.01344.x

Matiru, V. N., Dakora, F. D. (2005b). Xylem transport and shoot accumulation of lumichrome, a newly recognized rhizobial signal, alters root respiration, stomatal conductance, leaf transpiration and photosynthetic rates in legumes and cereals. *New Phytol.* 165, 847–855. doi: 10.1111/j.1469-8137.2004.01254.x

Matthews, C., Arshad, M., Hannoufa, A. (2019). Alfalfa response to heat stress is modulated by microRNA156. *Physiol. Plant.* 165, 830–842. doi: 10.1111/ppl.12787

McCaslin, M., Temple, S. J., Tofte, J. E. (2002). Methods for maximizing expression of transgenic traits in autopolyploid plants. US Patent Appl US-2002-0042928-A1.

Meilhoc, E., Boscari, A., Bruand, C., Puppo, A., Brouquisse, R. (2011). Nitric oxide in legume–rhizobium symbiosis. *Plant Sci.* 181, 573–581. doi: 10.1016/j.plantsci.2011.04.007

Mendis M. H., Power J. B., Davey M. R. (1991). Somatic hybrids of the forage legumes *Medicago sativa* L. and *M. falcata* L. *J. Exp. Bot.* 42245, 1565–1573.

Menke, F. L., Kang, H. G., Chen, Z., Park, J. M., Kumar, D., and Klessig, D. F. (2005). Tobacco transcription factor WRKY1 is phosphorylated by the MAP kinase SIPK and mediates HR-like cell death in tobacco. *Mol. Plant Microbe Interact.* 18, 1027–1034. doi: 10.1094/MPMI-18-1027

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., et al. (2006). Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium–legume symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5230–5235. doi: 10.1073/pnas.0600912103

Merkle, S. A., Parrot, W. A., Flim, B. S. (1995). “Morphogenic aspects of somatic embryogenesis,” in: In Vitro Embryogenesis in Plants, eds. T. A. Thorpe (Kluwer Academic Publishers: Dordrecht, The Netherlands). 155–204.

Meskiene, I., Baudouin, E., Schweighofer, A., Liwosz, A., Jonak, C., Rodriguez, P. L., et al. (2003). Stress-induced protein phosphatase 2C is a negative regulator of a mitogen-activated protein kinase. *J. Biol. Chem.* 278, 18945–18952. doi: 10.1074/jbc.M300878200

Mishra, N. S., Tuteja, R., Tuteja, N. (2006). Signaling through MAP kinase networks in plants. *Arch. Biochem. Biophys.* 452, 55–68. doi: 10.1016/j.abb.2006.05.001

Mitten, D. H., Sato, S. J., Skokut, T. A. (1984). In vitro regeneration potential of alfalfa germplasm sources. *Crop Sci.* 24, 943–945. doi: 10.2135/cropsci1984.0011183X002400050028x

Mittler, R., Blumwald, E. (2015). The roles of ROS and ABA in systemic acquired acclimation. *Plant Cell* 27, 64–70. doi: 10.1105/tpc.114.133090

Miyahara, A., Richens, J., Starker, C., Morieri, G., Smith, L., Long, S., et al. (2010). Conservation in function of a SCAR/WAVE component during infection thread and root hair growth in *Medicago truncatula*. *Mol. Plant-Microbe Interact.* 23, 1553–1562. doi: 10.1094/MPMI-06-10-0144

Moffett, M. L., Colwell, R. R. (1968). Adansonian analysis of the *Rhizobiaceae. J. Gen. Microbial.* 51, 245–266. doi: 10.1099/00221287-51-2-245

Mohd-Radzman, N. A., Laffont, C., Ivanovici, A., Patel, N., Reid, D., Stougaard, J., Djordjevic, M. A. (2016). Different pathways act downstream of the CEP peptide receptor CRA2 to regulate lateral root and nodule development. *Plant Physiol.* 171, 2536–2548. doi: 10.1104/pp.16.00113

Mortier, V., Den Herder, G., Whitford, R., Van de Velde, W., Rombauts, S., D’Haeseleer, K., et al. (2010). CLE peptides control *Medicago truncatula* nodulation locally and systemically. *Plant Physiol.* 153, 222–237. doi: 10.1104/pp.110.153718

Mortier, V., Holsters, M., Goormachtig, S. (2012). Never too many? How legumes control nodule numbers. *Plant Cell Environ.* 35, 245–258. doi: 10.1111/j.1365-3040.2011.02406.x

Müller, J., Beck, M., Mettbach, U., Komis, G., Hause, G., Menzel, D., et al. (2010). Arabidopsis MPK6 is involved in cell division plane control during early root development, and localizes to the pre-prophase band, phragmoplast, trans-Golgi network and plasma membrane. *Plant J.* 61, 234–248. doi: 10.1111/j.1365-313X.2009.04046.x

Munnik, T., Ligterink, W., Meskiene, I., Calderini, O., Beyerly, J., Musgrave, A. (1999). Distinct osmo-sensing protein kinase pathways are involved in signalling moderate and severe hyper-osmotic stress. *Plant J.* 20, 381–388. doi: 10.1046/j.1365-313x.1999.00610.x

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Murray, J. D., Karas, B. J., Sato, S., Tabata, S., Amyot, L., Szczyglowski, K. (2007). A cytokinin perception mutant colonized by *Rhizobium* in the absence of nodule organogenesis. *Science* 315, 101–4. doi: 10.1126/science.1132514

Mus, F., Crook, M. B., Garcia, K., Costas, A. G., Geddes, B. A., Kouri, E. D., et al. (2016). Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. *Appl. Environ. Microbiol.* 82, 3698–3710. doi: 10.1128/AEM.01055-16

Musial, J. M., Mackie, J. M., Armour, D. J., Phan, H. T. T., Ellwood, S. E., Aitken, K. S., et al. (2007). Identification of QTL for resistance and susceptibility to *Stagonospora meliloti* in autotetraploid lucerne. *Theor. Appl. Gen.* 114, 1427–1435. doi: 10.1007/s00122-007-0528-y

Nakagami, H., Kiegerl, S., Hirt, H. (2004). OMTK1, a novel MAPKKK, channels oxidative stress signaling through direct MAPK interaction. *J. Biol. Chem.* 279, 26959–26966. doi: 10.1074/jbc.M312662200

Nakagami, H., Soukupová, H., Schikora, A., Zárský, V., Hirt, H. (2006). A mitogen-activated protein kinase kinase kinase mediates reactive oxygen species homeostasis in Arabidopsis. *J. Biol. Chem.* 281, 38697–38704. doi: 10.1074/jbc.M605293200

Nakano, K., Shiroma, A., Shimoji, M., Tamotsu, H., Ashimine, N., Ohki, S., et al. (2017). Advantages of genome sequencing by long-read sequencer using SMRT technology in medical area. Hum. *Cell* 30, 149–161. doi: 10.1007/s13577-017-0168-8

Neupane, A., Nepal, M. P., Piya, S., Subramanian, S., Rohila, J. S., Reese, R. N. and Benson, B. V. (2013). Identification, nomenclature, and evolutionary relationships of mitogen-activated protein kinase (MAPK) genes in soybean. *Evol. Bioinf.* 9, EBO–S12526. doi: 10.4137/EBO.S12526

Newton, W. E. (2007). "Physiology, biochemistry and molecular biology of nitrogen fixation," in: Biology of nitrogen cycle, eds. H. Bothe, S. Ferguson, W. E. Newton (Elsevier, Amsterdam), 109–130. doi: 10.1016/B978-044452857-5.50009-6

O'Rourke, J. A., Fu, F., Bucciarelli, B., Yang, S. S., Samac, D. A., Lamb, J. F. S., et al. (2015). The *Medicago sativa* gene index 1.2: a web-accessible gene expression atlas for investigating expression differences between *Medicago sativa* subspecies. *BMC Genom.* 16, 502. doi: 10.1186/s12864-015-1718-7

Oldroyd, G. E. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Rev. Microbiol.* 11, 252–264. doi: 10.1038/nrmicro2990

Oldroyd, G. E., Downie, J. A. (2004). Calcium, kinases and nodulation signalling in legumes. *Nat. Rev. Mol. Cell Biol.* 5, 566–576. doi: 10.1038/nrm1424

Oldroyd, G. E., Downie, J. A. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annu. Rev. Plant Biol.* 59, 519–546. doi: 10.1146/annurev.arplant.59.032607.092839

Oldroyd, G. E., Murray, J. D., Poole, P. S., Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Ann. Rev. Ann.* 45, 119–144. doi: 10.1146/annurev-genet-110410-132549

Olukolu, B. A., Tracy, W. F., Wisser, R., De Vries, B., Balint-Kurti, P. J. (2016). A genome-wide association study for partial resistance to maize common rust. *Phytopath.* 106, 745–751. doi: 10.1094/PHYTO-11-15-0305-R

Otegui, M., Staehelin, L. A. (2000). Cytokinesis in flowering plants: more than one way to divide a cell. *Curr. Opin. Plant Biol.* 3, 493–502. doi: 10.1016/S1369-5266(00)00119-9

Ovečka, M., Lichtscheidl, I. K., Baluška, F., Šamaj, J., Volkmann, D., Hirt, H. (2008). "Regulation Of Root Hair Tip Growth: Can Mitogen-Activated Protein Kinases Be Taken Into Account?," in: The Plant Cytoskeleton: a Key Tool for Agro-Biotechnology, eds. Y. B. Blume, W. V. Baird, A. I. Yemets, D. Breviario (Springer, Dordrecht), 91–128. doi: 10.1007/978-1-4020-8843-8

Ovečka, M., Takáč, T., Komis, G., Vadovič, P., Bekešová, S., Doskočilová, A., et al. (2014). Salt-induced subcellular kinase relocation and seedling susceptibility caused by overexpression of *Medicago* SIMKK in *Arabidopsis*. *J. Exp. Bot.* 65, 2335–2350. doi: 10.1093/jxb/eru115

Palmer, E., Freeman, T. (2004). Investigation into the use of C-and N-terminal GFP fusion proteins for subcellular localization studies using reverse transfection microarrays. *Comp. Funct. Genomics* 5, 342–353. doi: 10.1002/cfg.405

Pan, J., Zhang, M., Kong, X., Xing, X., Liu, Y., Zhou, Y., et al. (2012). *ZmMPK17*, a novel maize group D MAP kinase gene, is involved in multiple stress responses. *Planta* 235, 661–676. doi: 10.1007/s00425-011-1510-0

Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., et al. (1991). Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J.* 10, 885–892. doi: 10.1002/j.1460-2075.1991.tb08021.x

Peck, M. C., Fisher, R. F., Long, S. R. (2006). Diverse flavonoids stimulate NodD1 binding to nod gene promoters in *Sinorhizobium meliloti*. *J. Bacteriol.* 188, 5417–5427. doi: 10.1128/JB.00376-06

Pei, W., Du, F., Zhang, Y., He, T., Ren, H. (2012). Control of the actin cytoskeleton in root hair development. *Plant Sci.* 187, 10–18.

Pellock, B. J., Cheng, H. P., Walker, G. C. (2000). Alfalfa Root Nodule Invasion Efficiency Is Dependent on *Sinorhizobium meliloti*Polysaccharides. *J. Bacteriol.* 182, 4310–4318. doi: 10.1128/JB.182.15.4310-4318.2000

Pereira, L. F., Erickson, L. (1995). Stable transformation of alfalfa (*Medicago sativa* L.) by particle bombardment. *Plant Cell Rep.* 14, 290–293. doi: 10.1007/BF00232030

Perret, X., Staehelin, C., Broughton, W. J. (2000). Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* 64, 180–201. doi: 10.1128/mmbr.64.1.180-201.2000

Persak, H., Pitzschke, A. (2013). Tight interconnection and multi-level control of Arabidopsis MYB44 in MAPK cascade signalling. *PLoS ONE* 8:e57547. doi: 10.1371/journal.pone.0057547

Peters, N. K., Frost, J. W., Long, S. R. (1986). A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science* 233, 977–980. doi: 10.1126/science.3738520

Phillips, D. A., Joseph, C. M., Yang, G. P., Martínez-Romero, E., Sanborn, J. R., Volpin, H., (1999). Identification of lumichrome as a *Sinorhizobium* enhancer of alfalfa root respiration and shoot growth. *Proc. Natl. Acad. Sci. U.S.A.*, 96, 12275–12280. doi: 10.1073/pnas.96.22.12275

Piano, E., Pecetti, L. (2010). "Minor legume species," in: Fodder Crops and Amenity Grassesm Handbook of Plant Breeding, Vol.5, eds B. Boller, U.K. Posselt, and F. Veronesi (NewYork, NY: Springer), 477–500. doi: 10.1007/978-1-4419-0760-8_20

Pierce, M., Bauer, W. D. (1983). A rapid regulatory response governing nodulation in soybean. *Plant Physiol.* 73, 286–290. doi: 10.1104/pp.73.2.286

Pitzschke, A., Datta, S., Persak, H. (2014). Salt stress in Arabidopsis: lipid transfer protein AZI1 and its control by mitogen-activated protein kinase MPK3. *Mol. Plant* 7, 722–738. doi: 10.1093/mp/sst15

Pitzschke, A., Schikora, A., Hirt, H. (2009). MAPK cascade signaling networks in plant defence. *Curr. Opin. Plant Biol.* 12, 421–426. doi: 10.1016/j.pbi.2009.06.008

Playne, M. J., McDonald, P. (1966). The buffering constituents of herbage and silage. *J. Sci. Food Agric.* 17, 264–268. doi: 10.1002/jsfa.2740170609

Plet, J., Wasson, A., Ariel, F., Le Signor, C., Baker, D., et al. (2011). MtCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in *Medicago truncatula*. *Plant J.* 65, 622–33. doi: 10.1111/j.1365-313X.2010.04447.x

Postnikova, O. A., Hult, M., Shao, J., Skantar, A., Nemchinov, L. G. (2015). Transcriptome analysis of resistant and susceptible alfalfa cultivars infected with root-knot nematode *Meloidogyne incognita*. *PLoS One* 10, e0123157. doi: 10.1371/journal.pone.0118269

Postnikova, O. A., Shao, J., Nemchinov, L. G. (2013). Analysis of the alfalfa root transcriptome in response to salinity stress. *Plant Cell Physiol.* 54, 1041–1055. doi: 10.1093/pcp/pct056

Pratt, R. G., Rowe D. E. (2002). Enhanced resistance to *Sclerotium rolfsii* in populations of alfalfa selected for quantitative resistance to *Sclerotinia trifoliorum. J. Phytopathol.* 92, 204–209. doi: 10.1094/PHYTO.2002.92.2.204

Prinsen, E., Chauvaux, N., Schmidt, J., John, M., Wieneke, U., De Greef, J., Van Onckelen, H. (1991). Stimulation of indole-3-acetic acid production in *Rhizobium* by flavonoids. *FEBS L.* 282, 53–55. doi: 10.1016/0014-5793(91)80442-6

Puppo, A., Pauly, N., Boscari, A., Mandon, K., Brouquisse, R. (2013). Hydrogen peroxide and nitric oxide: key regulators of the legume—rhizobium and mycorrhizal symbioses. *Ant. Red. Sig.* 18, 2202–2219. doi: 10.1089/ars.2012.5136

Préstamo, G., Testillano, P. S., Vicente, O., González-Melendi, P., Coronado, M. J., Wilson, C., et al. (1999). Ultrastructural distribution of a MAP kinase and transcripts in quiescent and cycling plant cells and pollen grains. *J. Cell Sci.* 112, 1065–1076.

Putnam, D., Russelle, M., Orloff, S., Kuhn, J., Fitzhugh, L., Godfrey, L., et al. (2001) "Alfalfa, wildlife and the environment," in: The importance and benefits of alfalfa in the 21st century (California Alfalfa and Forage Association), 1–24.

Radović, J., Sokolović, D., Marković, J. (2009). Alfalfa-most important perennial forage legume in animal husbandry. *Biotechnol. Anim. Husb.* 25, 465–475. doi: 10.2298/BAH0906465R

Rai, M. K., Asthana, P., Jaiswal, V. S., Jaiswal, U. (2010). Biotechnological advances in guava (*Psidium guajava* L.): recent developments and prospects for further research. *Trees Struct. Funct.* 24, 1–12. doi: 10.1007/s00468-009-0384-2

Raja, V., Majeed, U., Kang, H., Andrabi, K. I., John, R. (2017). Abiotic stress: interplay between ROS, hormones and MAPKs. *Environ. Exp. Bot.* 137, 142–157. doi: 10.1016/j.envexpbot.2017.02.010

Rajwar, A., Sahgal, M., Johri, B. N. (2013). "Legume–rhizobia symbiosis and interactions in agroecosystems," in: Plant microbe symbiosis: fundamentals and advances, eds. N. K. Arora (Springer, New Delhi), 233–265. doi: 10.1007/978-81-322-1287-4

Ramaiah, S. M., Skinner, D. Z. (1997). Particle bombardment: A simple and efficient method of alfalfa (*Medicago sativa* L.) pollen transformation. *Curr. Science*, 674–682.

Rashid, M. H. O., Young, J. P. W., Everall, I., Clercx, P., Willems, A., Braun, M. S., et al. (2015). Average nucleotide identity of genome sequences supports the description of *Rhizobium lentis* sp. nov., *Rhizobium bangladeshense* sp. nov. and *Rhizobium binae* sp. nov. from lentil (*Lens culinaris*) nodules. *Int. J. Syst. Evol. Microbiol.* 65, 3037–3045. doi: 10.1099/ijs.0.000373

Rashmi, R., Sarkar, M., Vikramaditya, T. (1997). Cultivaton of alfalfa (*Medicago sativa L.). Anc. Sci. Life* 17, 117–119.

Rasmussen, M. W., Roux, M., Petersen, M., Mundy, J. (2012). MAP Kinase Cascades in Arabidopsis Innate Immunity. *Front. Plant Sci.* 3, 169. doi: 10.3389/fpls.2012.00169

Redmond, J. W., Batley, M., Djordjevic, M. A., Innes, R. W., Kuempel, P. L., Rolfe, B. G. (1986). Flavones induce expression of nodulation genes in *Rhizobium. Nature* 323, 632-635.

Reid, D. E., Ferguson, B. J., Hayashi, S., Lin, Y. H., Gresshoff, P. M. (2011). Molecular mechanisms controlling legume autoregulation of nodulation. *Ann. Bot.* 108, 789–795. doi: 10.1093/aob/mcr205

Reinert, J. (1958). Morphogenese und ihre Kontrolle an Gewebekulturen aux Carotten. *Naturwissenenschaften* 45, 344–345.

Rhodes, D., Hanson, A. D. (1993). Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Ann. Rev. Plant Biol.* 44, 357–384. doi: 10.1146/annurev.pp.44.060193.002041

Ribeiro, C. W., Alloing, G., Mandon, K., Frendo, P. (2015). Redox regulation of differentiation in symbiotic nitrogen fixation. *Biochim. Biophys. Acta, Gen. Subj*. 1850, 1469–1478. doi: 10.1016/j.bbagen.2014.11.018

Riday, H., Brummer, E. C. (2002). Forage yield heterosis in alfalfa. *Crop Sci.* 42, 716–723. doi: 10.2135/cropsci2002.7160

Rinaudi-Marron, L. V., González, J. E. (2013). Role of quorum sensing in the *Sinorhizobium Meliloti*–Alfalfa Symbiosis. *Mol. Mic. Ecol. Rhizos.* 1, 535-540. doi: 10.1002/9781118297674.ch50

Ringli, C., Baumberger, N., Diet, A., Frey, B., Keller, B. (2002). ACTIN2 is essential for bulge site selection and tip growth during root hair development of *Arabidopsis. Plant Physiol.* 129, 1464–72. doi: 10.1104/pp.005777

Rivera, A. L., Gómez-Lim, M., Fernández, F., Loske, A. M. (2012). Physical methods for genetic plant transformation. *Phys. Life Rev.* 9, 308–345. doi: 10.1016/j.plrev.2012.06.002

Robins, J. G., Luth, D., Campbell, T. A., Bauchan, G. R., He, C., Viands, D. R., Brummer, E. C. (2007). Genetic mapping of biomass production in tetraploid alfalfa. *Crop Sci.* 47, 1–10. doi: 10.2135/cropsci2005.11.0401

Robinson, M. J., Cobb, M. H. (1997). Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* 9**,** 180–186. doi: 10.1016/S0955-0674(97)80061-0

Rodriguez, M. C., Petersen, M., Mundy, J. (2010). Mitogen-activated protein kinase signaling in plants. *Annu. Rev. Plant Biol.* 61, 621–649. doi: 10.1146/annurev-arplant-042809-112252

Rostas, K., Kondorosi, E., Horvath, B., Simoncsits, A., Kondorosi, A. (1986). Conservation of extended promoter regions of nodulation genes in *Rhizobium*. *Proc. Natl. Acad. Sci. U.S.A.* 83, 1757–1761. doi: 10.1073/pnas.83.6.1757

Rothberg, J. M., Hinz, W., Rearick, T. M., Schultz, J., Mileski, W., Davey, M., et al. (2011). An integrated semiconductor device enabling non-optical genome sequencing. *Nature* 475, 348–352. doi: 10.1038/nature10242

Roumen, E. C. (1994). "A strategy for accumulating genes for partial resistance to blast disease in rice within a conventional breeding program," in: Rice Blast Disease, eds R. S. Zeigler, S. A. Leong, P. S. Teng (Cambridge: CAB International), 245–265.

Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., et al. (2020). Celebrating 20 years of genetic discoveries in legume nodulation and symbiotic nitrogen fixation. *Plant Cell* 32, 15–41. doi: 10.1105/tpc.19.00279

Rubiales D., Fondevilla S., Chen W., Gentzbittel L., Higgins T.J.V., Castillejo M.A., et al. (2015). Achievements and challenges in legume breeding for pest and disease resistance. *Crit. Rev. Plant Sci.* 34, 195–236. doi: 10.1080/07352689.2014.898445

Ryu, H., Laffont, C., Frugier, F., Hwang, I. (2017). MAP kinase-mediated negative regulation of symbiotic nodule formation in *Medicago truncatula*. *Mol. Cells* 40, 17. doi: 10.14348/molcells.2017.2211

Sakiroglu, M., Brummer, E. C. (2017). Identification of loci controlling forage yield and nutritive value in diploid alfalfa using GBS-GWAS. *Theor. Appl. Gen.* 130, 261–268. doi: 10.1007/s00122-016-2782-3

Samac D. A., Temple S. J. (2004). "Development and utilization of transformation in Medicago species," in: Genetically Modified Crops, Their Development, Uses and Risks, eds. G. H. Liang, D. Z. Skinner (New York, NY: The Haworth Press), 165–202.

Samac, D. A., Austin-Phillips, S. (2006). Alfalfa (*Medicago sativa* L.). Agrobacterium protocols, 301-312. doi: 10.1385/1-59745-130-4:301

Šamaj, J., Baluška, F., Menzel, D. (2004). New signalling molecules regulating root hair tip growth. *Trends Plant Sci.* 9, 217–220. doi: 10.1016/j.tplants.2004.03.008

Šamaj, J., Ovečka, M., Hlavacka, A., Lecourieux, F., Meskiene, I., Lichtscheidl, I., Hirt, H. (2002). Involvement of the mitogen-activated protein kinase SIMK in regulation of root hair tip growth. *EMBO J.* 21, 3296–3306. doi: 10.1093/emboj/cdf349

Šamaj, J., Ovečka, M., Hlavačka, A., Lecourieux, F., Meskiene, I., Lichtscheidl, I., et al. (2003). Involvement of MAP kinase SIMK and actin cytoskeleton in the regulation of root hair tip growth. *Cell Biol. Int.* 27, 257–259. doi.org/10.1016/S1065-6995(02)00344-X

Šamajová, O., Komis, G., Šamaj, J. (2013b). Emerging topics in the cell biology of mitogen-1190 activated protein kinases. *Trends Plant Sci.* 18, 140–148. doi.org/10.1016/j.tplants.2012.11.004. 1191

Šamajová, O., Plíhal, O., Al-Yousif, M., Hirt, H., Šamaj, J. (2013a). Improvement of stress tolerance in plants by genetic manipulation of mitogen-activated protein kinases. *Biotech A.* 31, 118–128. 1193 doi.org/10.1016/j.biotechadv.2011.12.002

Šamajová, O., Takáč, T., von Wangenheim, D., Stelzer, E., Šamaj, J. (2012). "Update on methods and techniques to study endocytosis in plants," in: Endocytosis in Plants, (Springer, Berlin, Heidelberg), 1–36. doi: 10.1007/978-3-642-32463-5_1

Sangra, A., Shahin, L., Dhir, S. K. (2019). Long-term maintainable somatic embryogenesis system in alfalfa (*Medicago sativa*) using leaf explants: embryogenic sustainability approach. *Plants 8*, 278. doi: 10.3390/plants8080278

Sato, S., Nakamura, Y., Kaneko, T., Asamizu, E., Kato, T., Nakao, M., et al. (2008). Genome structure of the legume, *Lotus japonicus*. *DNA Res.* 15, 227–239. doi: 10.1093/dnares/dsn008

Saulis, G., Venslauskas, M. S., Naktinis, J. (1991). Kinetics of pore resealing in cell membranes after electroporation. *J. Electroanal. Chem. Interfacial Electrochem.* 321, 1–13. doi: 10.1016/0022-0728(91)85564-6

Saunders, J.W., Bingham, E.T. (1972). Production of alfalfa plants from callus tissue. *Crop Sci.* 12, 804–808. doi: 10.2135/cropsci1972.0011183X001200060026x

Saur, I., Oakes, M., Djordjevic, M. A., Imin, N. (2011). Crosstalk between the nodulation signaling pathway and the autoregulation of nodulation in *Medicago truncatula*. *New Phytolog.* 190, 865–874. doi: 10.1111/j.1469-8137.2011.03738.x

Schaeffer, H. J., Weber, M. J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell Biol.* 19, 2435–2444. doi: 10.1128/mcb.19.4.2435

Scheben, A., Verpaalen, B., Lawley, C. T., Chan, C. K. K., Bayer, P. E., Batley, J., et al. (2019). CropSNPdb: a database of SNP array data for Brassica crops and hexaploid bread wheat. *Plant J.* 98, 142–152. doi: 10.1111/tpj.14194

Schena, M., Shalon, D., Davis, R. W., Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467–470. doi: 10.1126/science.270.5235.467

Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., et al. (2010). Genome sequence of the palaeopolyploid soybean. *Nature* 463, 178–183. doi: 10.1038/nature08670

Schnabel, E., Journet, E. P., de Carvalho-Niebel, F., Duc, G., Frugoli, J. (2005). The *Medicago truncatula SUNN* gene encodes a CLV1-like leucine-rich repeat receptor kinase that regulates nodule number and root length. *Plant Mol. Biol.* 58, 809–822. doi: 10.1007/s11103-005-8102-y

Schreiber, M., Stein, N., Mascher, M. (2018). Genomic approaches for studying crop evolution. *Genom. Biol.* 19, 140. doi: 10.1186/s13059-018-1528-8

Schultze, M., Kondorosi, A. (1998). Regulation of symbiotic root nodule development. *Annu. Rev. Genet.* 32, 33–57. doi: 10.1146/annurev.genet.32.1.33

Schweighofer, A., Kazanaviciute, V., Scheikl, E., Teige, M., Doczi, R., Hirt, H., et al. (2007). The PP2C-type phosphatase AP2C1, which negatively regulates MPK4 and MPK6, modulates innate immunity, jasmonic acid, and ethylene levels in Arabidopsis. *Plant Cell* 19, 2213–2224. doi: 10.1105/tpc.106.049585

Seguí-Simarro, J. M., Testillano, P. S., Jouannic, S., Henry, Y., Risueño, M. C. (2005). Mitogen-activated protein kinases are developmentally regulated during stress-induced microspore embryogenesis in *Brassica napus* L. *Histochem. Cell Biol.* 123, 541–551. doi: 10.1007/s00418-004-0749-y

Segura, A., Moreno, M., Madueno, F., Molina, A., Garcia-Olmedo, F. (1999). Snakin-1, a peptide from potato that is active against plant pathogens. *Mol. Plant Microbe Interact.* 12, 16–23. doi: 10.1094/MPMI.1999.12.1.16

Severin, A. J., Cannon, S. B., Graham, M. M., Grant, D., Shoemaker, R. C. (2011). Changes in twelve homoeologous genomic regions in soybean following three rounds of polyploidy. *Plant Cell* 23, 3129–3136. doi: 10.1105/tpc.111.089573

Shao, C. Y., Russinova, E., Iantcheva, A., Atanassov, A., McCormac, A., Chen, D. F., et al. (2000). Rapid transformation and regeneration of alfalfa (*Medicago falcata* L.) via direct somatic embryogenesis. *Plant Growth Reg.* 31, 155–166. doi: 10.1023/A:1006306909722

Sheikh, A. H., Raghuram, B., Jalmi, S. K., Wankhede, D. P., Singh, P., Sinha, A. K. (2013). Interaction between two rice mitogen activated protein kinases and its possible role in plant defense. *BMC Plant Biol.* 13, 121. doi: 10.1186/1471-2229-13-121

Shen, C., Du, H., Chen, Z., Lu, H., Zhu, F., Chen, H., et al. (2020). The Chromosome-level genome sequence of the autotetraploid alfalfa and resequencing of core germplasms provide genomic resources for alfalfa research. *Mol. Plant* 13, 1250–1261. doi: 10.1016/j.molp.2020.07.003

Shetty, K., McKersie, B. D. (1993). Proline, thioproline and potassium mediated stimulation of somatic embryogenesis in alfalfa (*Medicago sativa* L.). *Plant Sci*. 88, 185–193. doi: 10.1016/0168-9452(93)90090-M

Shi, J., Zhang, L., An, H., Wu, C., Guo, X. (2011). GhMPK16, a novel stress-responsive group D MAPK gene from cotton, is involved in disease resistance and drought sensitivity. *BMC Mol. Biol.* 12, 1–15. doi: 10.1186/1471-2199-12-22

Sieberer, B. J., Timmers, A. C., Emons, A. M. C. (2005). Nod factors alter the microtubule cytoskeleton in *Medicago truncatula* root hairs to allow root hair reorientation. *Mol. Plant-Micr. Interact.* 18, 1195–1204. doi: 10.1094/MPMI-18-1195

Singer, S. D., Hannoufa, A., Acharya, S. (2018). Molecular improvement of alfalfa for enhanced productivity and adaptability in a changing environment. *Plant Cell Environ.* 41, 1955–1971. doi: 10.1111/pce.13090

Singh, R., Lee, M. O., Lee, J. E., Choi, J., Park, J. H., Kim, E. H., et al. (2012). Rice mitogen-activated protein kinase interactome analysis using the yeast two-hybrid system. *Plant Physiol.* 160, 477–487. doi: 10.1104/pp.112.200071

Sinha, A. K., Jaggi, M., Raghuram, B., Tuteja, N. (2011). Mitogen-activated protein kinase signaling in plants under abiotic stress. *Plant Sig. Behav.* 6, 196–203.

Skorupska, A., Kidaj, D., Wielbo, J. (2017). "Flavonoids and Nod Factors: Importance in Legume-Microbe Interactions and Legume Improvement," in: Microbes for Legume Improvement, eds. A. Zaidi, M. S. Khan and J. Musarrat (Springer, Cham.). doi: 10.1007/978-3-319-59174-2

Smékalová, V., Doskočilová, A., Komis, G., Šamaj, J. (2014). Crosstalk between secondary messengers, hormones and MAPK modules during abiotic stress signalling in plants. *Biotechnol. Adv.* 32, 2–11. doi: 10.1016/j.biotechadv.2013.07.009

Smith, L. G., Oppenheimer, D. G. (2005). Spatial control of cell expansion by the plant cytoskeleton. *Annu. Rev. Cell Dev. Biol.* 21, 271–95. doi: 10.1146/annurev.cellbio.21.122303.114901

Somasegaran, P., Hoben, H. J. (2012). "Handbook for rhizobia: methods in legume-Rhizobium technology," (Springer Verlag New York). 450. doi: 10.1007/978-1-4613-8375-8

Song, L., Jiang, L., Chen, Y., Shu, Y., Bai, Y., Guo, C. (2016). Deep-sequencing transcriptome analysis of field-grown *Medicago sativa* L. crown buds acclimated to freezing stress. *Func. Integr. Genomics* 16, 495–511. doi: 10.1007/s10142-016-0500-5

Sorkin, A., Von Zastrow, M. (2002). Signal transduction and endocytosis: close encounters of many kinds. *Nat. Rev. Mol. Cell Biol.* 3, 600–614. doi: 10.1038/nrm883

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kämpfer, P., Maiden, M. C., et al. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int. J. Syst. Evol. Microbiol.* 52, 1043–1047. doi: 10.1099/00207713-52-3-1043

Staehelin, C., Schultze, M., Tokuyasu, K., Poinsot, V., Promé, J. C., Kondorosi, E., et al. (2000). N-deacetylation of *Sinorhizobium meliloti* Nod factors increases their stability in the *Medicago sativa* rhizosphere and decreases their biological activity. *Mol. Plant Microbe Interact.* 13, 72–79. doi: 10.1094/MPMI.2000.13.1.72

Stambulska, U. Y., Bayliak, M. M. (2020). "Legume-rhizobium symbiosis: Secondary metabolites, free radical processes, and effects of heavy metals," in: Co-Evolution of Secondary Metabolites, eds. J.-M. Mérillon and K. G. Ramawat (Springer International Publishing). 291–322. doi: 10.1007/978-3-319-96397-6

Stefanova, G., Slavov, S., Gecheff, K., Vlahova, M., Atanassov A. (2013). Expression of recombinant human lactoferrin in transgenic alfalfa plants. *Biol. Plant.* 57, 457–464. doi: 10.1007/s10535-013-0305-5

Steward, F.C., Mapes, M.O., Mears, K. (1958). Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspend cells. *Am. J. Bot.* 45, 705–708. doi: 10.1002/j.1537-2197.1958.tb10599.x.

Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., et al. (2002). A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature* 417, 959–962. doi: 10.1038/nature00841

Strizhov, N., Keller, M., Mathur, J., Koncz-Kálmán, Z., Bosch, D., Prudovsky, E., Zilberstein, A. (1996). A synthetic cryIC gene, encoding a *Bacillus thuringiensis* δ-endotoxin,

confers *Spodoptera* resistance in alfalfa and tobacco. *Proc. Natl. Acad. Sci. U.S.A.* 93, 15012–-15017. doi: 10.1073/pnas.93.26.15012

Subramanian, S., Stacey, G., Yu, O. (2007). Distinct, crucial roles of flavonoids during legume nodulation. *Trends Plant Sci* 12, 282–285. doi: 10.1016/j.tplants.2007.06.006

Suneja, P., Duhan, J. S., Bhutani, N., Dudeja, S. S. (2017). "Recent biotechnological approaches to study taxonomy of legume nodule forming rhizobia," in: Plant Biotechnology: Recent Advancements and Developments, eds. S. K. Gahlawat, R. K. Salar, P. Siwach, J. S. Duhan, S. Kumar, P. Kaur (Springer, Singapore), 101–124. doi: 10.1007/978-981-10-4732-9

Suominen, L., Roos, C., Lortet, G., Paulin, L., Lindström, K. (2001). Identification and structure of the *Rhizobium galegae* common nodulation genes: evidence for horizontal gene transfer. *Mol. Biol. Evol.* 18, 907–916. doi: 10.1093/oxfordjournals.molbev.a003891

Suzaki,T., Yoro, E., Kawaguchi, M. (2015). Leguminous plants: inventors of root nodules to accommodate symbiotic bacteria. *Int. Rev. Cell Mol. Biol.* 316, 111–158.

Tabe, L. M., Wardley-Richardson, T., Ceriotti, A., Aryan, A., McNabb, W., Moore, A., et al. (1995). A biotechnological approach to improving the nutritive value of alfalfa. *J. Anim. Biol.* 73, 2752–2759. doi: 10.2527/1995.7392752x

Taji, A., Lakshmanan, P., Williams, R. (2004). "Emerging themes in in vitro culture and their applications to horticulture," in: V International Symposium on In Vitro Culture and Horticultural Breeding 725, 241–254. doi: 10.17660/ActaHortic.2006.725.31

Tang, H., Krishnakumar, V., Bidwell, S., Rosen, B., Chan, A., Zhou, S., et al. (2014). An improved genome release (version Mt4.0) for the model legume *Medicago truncatula. BMC genom.* 15, 312. doi: 10.1186/1471-2164-15-312

Teale, W. D., Paponov, I. A., Palme, K. (2006). Auxin in action: signalling, transport and the control of plant growth and development. *Nat. Rev. Mol. Cell Biol.* 7, 847–859. doi: 10.1038/nrm2020

Takáč, T., Šamajová, O., Luptovčiak, I., Pechan, T., Šamaj, J. (2017). Feedback microtubule control and microtubule-actin cross-talk in Arabidopsis revealed by integrative proteomic and cell biology analysis of KATANIN 1 mutants. *Mol. Cel. Prot.* 16, 1591–1609. doi: 10.1074/mcp.M117.068015

Tesfaye, M., Denton, M. D., Samac, D. A., Vance, C. P. (2005). Transgenic alfalfa secretes a fungal endochitinase protein to the rhizosphere. *Plant Soil* 2691–2692, 233–243. doi: 10.1007/s11104-004-0520-0

Tesfaye, M., Liu, J., Vance, C. P. (2007). Genomic and genetic control of phosphate stress in legumes. *Plant Physiol.* 144, 594–603. doi: 10.1104/pp.107.097386

Theunis, M., Kobayashi, H., Broughton, W. J., Prinsen, E. (2004). Flavonoids, NodD1, NodD2, and nod-box NB15 modulate expression of the y4wEFG locus that is required for indole-3-acetic acid synthesis in Rhizobium sp. strain NGR234. *Mol. Plant-Mic. Int.* 17, 1153–1161. doi: 10.1094/MPMI.2004.17.10.1153

Tichá, M., Hlaváčková, K., Hrbáčková, M., Ovečka, M., Šamajová, O., Šamaj, J. (2020a). Super-resolution imaging of microtubules in *Medicago sativa*. *Met. Cell Biol.* 160, 237–251. doi: 10.3390/ijms22041903

Tichá, M., Illésová, P., Hrbáčková, M., Basheer, J., Novák, D., Hlaváčková, K., et al. (2020b). Tissue culture, genetic transformation, interaction with beneficial microbes, and modern bio-imaging techniques in alfalfa research. *Crit. Rev. Biotechnol.* 40, 1265–1280. doi: 10.1080/07388551.2020.1814689

Timmers, A. C., Auriac, M. C., Truchet, G. (1999). Refined analysis of early symbiotic steps of the *Rhizobium–Medicago* interaction in relationship with microtubular cytoskeleton rearrangements. *Develop.* 126, 3617–3628.

Tirichine, L., Sandal, N., Madsen, L. H., Radutoiu, S., Albrektsen, A. S., et al. (2007). A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. *Science* 315, 104–7. doi: 10.1126/science.1132397

Tohidfar, M., Zare, N., Jouzani, G. S., Eftekhari, S. M. (2013). Agrobacterium-mediated transformation of alfalfa (*Medicago sativa*) using a synthetic *cry3a* gene to enhance resistance against alfalfa weevil. *Plant Cell T. O. Cult.* 113, 227–235. doi: 10.1007/s11240-012-0262-2?shared-article-renderer

Toth, E., Bakheit, B. R. (1983). Results of resistance breeding in alfalfa. II. Resistance to *Verticillium wilt*. *Acta Biol. Hung.* 32, 78–85.

Tripathi, P., Rabara, R. C., Reese, R. N., Miller, M. A., Rohila, J. S., Subramanian, S., et al. (2016). A toolbox of genes, proteins, metabolites and promoters for improving drought tolerance in soybean includes the metabolite coumestrol and stomatal development genes. *BMC Genom.* 17, 102. doi: 10.1186/s12864-016-2420-0

Tsuda, K., Mine, A., Bethke, G., Igarashi, D., Botanga, C. J., Tsuda, Y., et al. (2013). Dual regulation of gene expression mediated by extended MAPK activation and salicylic acid contributes to robust innate immunity in *Arabidopsis thaliana*. *PLoS Genet.* 9:e1004015. doi: 10.1371/journal.pgen.1004015

Tu, X., Zhao, H., Zhang, Z. (2018). Transcriptome approach to understand the potential mechanisms of resistant and susceptible alfalfa (*Medicago sativa* L.) cultivars in response to aphid feeding. *J. Integr. Agric*. 17, 2518–2527. doi: 10.1016/S2095-3119(17)61843-4

Ubersax, J. A., Ferrell, J. E. Jr. (2007). Mechanisms of specificity in protein phosphorylation. *Nat. Rev. Mol. Cell Biol.* 8, 530–541. doi: 10.1038/nrm2203

Umbrasaite, J., Schweighofer, A., Kazanaviciute, V., Magyar, Z., Ayatollahi, Z., Unterwurzacher, V., et al. (2010). MAPK phosphatase AP2C3 induces ectopic proliferation of epidermal cells leading to stomata development in Arabidopsis. *PLoS ONE* 5:e15357. doi: 10.1371/journal.pone.0015357

Vahdati, K., Bayat, S., Ebrahimzadeh, H., Jariteh, M., Mirmasoumi, M. (2008). Effect of exogenous ABA on somatic embryo maturation and germination in Persian walnut (*Juglans regia* L.). *Plant Cell T. O. Cult.* 93, 163–171. doi: 10.1007/s11240-008-9355-3

van Noorden, G. E., Ross, J. J., Reid, J. B., Rolfe, B. G., Mathesius, U. (2006). Defective long-distance auxin transport regulation in the *Medicago truncatula* super numeric nodules mutant. *Plant Physiol.* 140, 1494–1506. doi: 10.1104/pp.105.075879

Valliyodan, B., Ye, H., Song, L., Murphy, M., Shannon, J. G., Nguyen, H. T. (2017). Genetic diversity and genomic strategies for improving drought and waterlogging tolerance in soybeans. *J. Exp. Bot.* 68, 1835–1849. doi: 10.1093/jxb/erw433

van Zeijl, A., den Camp, R. H. O., Deinum, E. E., Charnikhova, T., Franssen, H., den Camp, H. J. O., et al. (2015). Rhizobium lipo-chitooligosaccharide signaling triggers accumulation of cytokinins in *Medicago truncatula* roots. *Mol. Plant* 8, 1213–1226. doi: 10.1016/j.molp.2015.03.010

Van, K., Rastogi, K., Kim, K. H., Lee, S. H. (2013). Next-generation sequencing technology for crop improvement. SABRAO *J. Breed. Genet.* 45, 84–99. doi: 10.3389/fpls.2014.00367

Vance, C. P. (2001). Symbiotic nitrogen fixation and phosphorous acquisition: plant nutrition in a world of declining renewable resources. *Plant Physiol.* 127, 390–397. doi: 10.1104/pp.010331

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev.* 60, 407–438.

Vasil, I. K. (1988). Progress in the regeneration and genetic manipulation of cereal crops. *Nat. Biotechnol.* 6, 397–402. doi: 10.1038/nbt0488-397.

Voinnet, O., Rivas, S., Mestre, P., Baulcombe, D. (2003). Retracted: An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J.* 33, 949–956. doi: 10.1046/j.1365-313X.2003.01676.x

Volenec, J. J., Cunningham, S. M., Haagenson, D. M., Berg, W. K., Joern, B. C., Wiersma, D. W. (2002). Physiological genetics of alfalfa improvement: past failures, future prospects. *Field Crops Res.* 75, 97–110. doi: 10.1016/S0378-4290(02)00020-5

Volkov, V., Wang, B., Dominy, P. J., Fricke, W., Amtmann, A. (2004). Thellungiella halophila, a salt-tolerant relative of *Arabidopsis thaliana*, possesses effective mechanisms to discriminate between potassium and sodium. *Plant Cell Environ.* 27, 1–14. doi: 10.1046/j.0016-8025.2003.01116.x

von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., Filonova, L. (2002). Developmental pathways of somatic embryogenesis. *Plant Cell Tiss. Org.* 69, 233–249. doi: 10.1023/A:1015673200621.

Wang, E. T. (2019). "Working on the Taxonomy, Biodiversity, Ecology and Evolution of Rhizobia," in: Ecology and Evolution of Rhizobia, (Springer, Singapore), 251–273). doi: 10.1007/978-981-32-9555-1_11

Wang, K., Wang, Z., Li, F., Ye, W., Wang, J., et al. (2012). The draft genome of a diploid cotton *Gossypium raimondii*. *Nature Gen.* 44, 1098–1103. doi: 10.1038/ng.2371

Wang, Q., Liu, J., Zhu, H. (2018). Genetic and molecular mechanisms underlying symbiotic specificity in legume-rhizobium interactions. *Front. Plant Sci.* 9, 1–8. doi: 10.3389/ fpls.2018.00313

Wang, Z., Gerstein, M., Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10, 57–63. doi: 10.1038/nrg2484

Wang, Z., Li, H., Ke, Q., Jeong, J. C., Lee, H. S., Xu, B., et al. (2014). Transgenic alfalfa plants expressing AtNDPK2 exhibit increased growth and tolerance to abiotic stresses. *Plant Physiol. Biochem.* 84, 67–77. doi: 10.1016/j.plaphy.2014.08.025

Wankhede, D. P., Misra, M., Singh, P., Sinha, A. K. (2013). Rice mitogen activated protein kinase kinase and mitogen activated protein kinase interaction network revealed by in-silico docking and yeast two-hybrid approaches. *PLoS One* 8:e65011. doi: 10.1371/journal.pone.0065011

Wasson, A. P., Pellerone, F. I., Mathesius, U. (2006). Silencing the flavonoid pathway in Medicago truncatula inhibits root nodule formation and prevents auxin transport regulation by rhizobia. *Plant Cell* 18, 1617–1629. doi: 10.1105/tpc.105.038232

Weaver, J. C., Chizmadzhev, Y. A. (1996). Theory of electroporation: a review. *Bioelectrochem. Bioenerg.* 41, 135–160. doi: 10.1016/S0302-4598(96)05062-3

Webb, D. H., Nutter Jr, F. W. (1997). Effects of leaf wetness duration and temperature on infection efficiency, latent period, and rate of pustule appearance of rust in alfalfa. *Phytopatol.* 87, 946-950. doi: 10.1094/PHYTO.1997.87.9.946

Weeks, J. T., Ye, J., Rommens, C. M. (2008). Development of an in planta method for transformation of alfalfa (*Medicago sativa*). *Trans. Res.* 17, 587–597. doi: 10.1007/s11248-007-9132-9

Wei, Z., Liu, Y., Lin, C., Wang, Y., Dong, Y., Xing, S. (2011). Transformation of alfalfa chloroplasts and expression of green fluorescent protein in a forage crop. *Biotechnol. Lett.* 33, 2487–2494. doi: 10.1007/s10529-011-0709-2

Whitmarsh, A. J., Davis, R. J. (1998). Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. *Trends Biochem. Sci.* 23, 481–485. doi: 10.1016/S0968-0004(98)01309-7

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.* 126, 485–493. doi: 10.1104/pp.126.2.485

Wong, C. E., Li, Y., Moffatt, B. A. (2006). Transcriptional profiling implicates novel interactions between abiotic stress and hormonal responses in *Thellungiella*, a close relative of Arabidopsis. *Plant Physiol.* 140, 1437–1450. doi: 10.1104/pp.105.070508

Wu, H. S., Shi, X., Li, J., Wu, T. Y., Ren, Q. Q., Zhang, Z. H., et al. (2016). Effects of root exudates of bivalent transgenic cotton (Bt+CpTI) plants on antioxidant proteins and growth of conventional cotton (Xinluhan 33). *J. Environ. Biol.* 37, 13–19.

Xia, T., Apse, M. P., Aharon, G. S., Blumwald, E. (2002). Identification and characterization of a NaCl-inducible vacuolar Na^+/H^+ antiporter in *Beta vulgaris*. *Physiol. Plant.* 116, 206–212. doi: 10.1034/j.1399-3054.2002.1160210.x

Xiao, T. T., Schilderink, S., Moling, S., Deinum, E. E., Kondorosi, E., Franssen, H., et al. (2014). Fate map of *Medicago truncatula* root nodules. *Develop.* 141, 3517–3528. doi: 10.1242/dev.110775

Xiong, L., Lee, H., Ishitani, M., Zhu, J. K. (2002). Regulation of osmotic stress-responsive gene expression by thelos6/aba1 locus in *Arabidopsis. J. Biol. Chem.* 277, 8588–8596. doi: 10.1074/jbc.M109275200

Xiong, L., Lee, M. W., Qi, M., Yang, Y. (2001). Identification of defense-related rice genes by suppression subtractive hybridization and differential screening. *Mol. Plant Microbe Interact.* 14, 685–692. doi: 10.1094/MPMI.2001.14.5.685

Xu, B., Wang, Y., Zhang, S., Guo, Q., Jin, Y., Chen, J., et al. (2017). Transcriptomic and physiological analyses of *Medicago sativa* L. roots in response to lead stress. *PLoS ONE* 12, e0175307. doi: 10.1371/journal.pone.0175307

Xu, C., Liu, R., Zhang, Q., Chen, X., Qian, Y., Fang, W. (2017). The diversification of evolutionarily conserved MAPK cascades correlates with the evolution of fungal species and development of lifestyles. *Genome Biol. Evol.* 9, 311–322. doi: 10.1093/gbe/evw051

Yan, Z., Cao, J., Fan, Q., Chao, H., Guan, X., Zhang, Z., et al. (2020). Dephosphorylation of LjMPK6 by Phosphatase LjPP2C is Involved in Regulating Nodule Organogenesis in *Lotus japonicus. Int. J. Mol. Sci.* 21, 5565. doi: 10.3390/ijms21155565

Yang, S. S., Tu, Z. J., Cheung, F., Xu, W. W., Lamb, J. F., Jung, H. J. G., et al. (2011). Using RNA-Seq for gene identification, polymorphism detection and transcript profiling in two alfalfa genotypes with divergent cell wall composition in stems. *BMC genom.* 12, 199. doi: 10.1186/1471-2164-12

Yang, S. S., Xu, W. W., Tesfaye, M., Lamb, J. F., Jung, H. J. G., VandenBosch, K. A., et al. (2010). Transcript profiling of two alfalfa genotypes with contrasting cell wall composition in stems using a cross-species platform: optimizing analysis by masking biased probes. *BMC genom.* 11, 323. doi: 10.1186/1471-2164-11-323

Yang, S., Gao, M., Deshpande, S., Lin, S., Roe, B. A., Zhu, H. (2007). Genetic and physical localization of an anthracnose resistance gene in *Medicago truncatula*. *Theor. Appl. Genet.* 116, 45–52. doi: 10.1007/s00122-007-0645-7

Yang, S., Gao, M., Xu, C., Gao, J., Deshpande, S., Lin, S., Zhu, H. (2008). Alfalfa benefits from *Medicago truncatula*: the RCT1 gene from *M. truncatula* confers broad-spectrum

resistance to anthracnose in alfalfa. *Proc. Natl. Acad. Sci. U.S.A.* 105, 12164–12169. doi: 10.1073/pnas.0802518105

Yang, X., Islam, M. S., Sood, S., Maya, S., Hanson, E. A., Comstock, J., et al. (2018). Identifying quantitative trait loci (QTLs) and developing diagnostic markers linked to orange rust resistance in sugarcane (*Saccharum* spp.). *Front. Plant Sci.* 9, 350. doi: 10.3389/fpls.2018.00350/full

Yang, Y., Li, R., Qi, M. (2000). In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. *Plant J.* 22, 543–551. doi: 10.1046/j.1365-313x.2000.00760.x

Yap, Y.-K., Kodama, Y., Waller, F., Chung, K. M., Ueda, H., Nakamura, K., et al. (2005). Activation of a novel transcription factor through phosphorylation by wipk, a wound-induced mitogen-activated protein kinase in tobacco plants. *Plant Physiol.* 139, 127–137. doi: 10.1104/pp.105.065656

Yin, J., Guan, X., Zhang, H., Wang, L., Li, H., Zhang, Q., et al. (2019). An MAP kinase interacts with LHK1 and regulates nodule organogenesis in *Lotus japonicus*. *Sci. Chin. Life Sci.* 62, 1203–1217. doi: 10.1007/s11427-018-9444-9

Yokota, K., Fukai, E., Madsen, L. H., Jurkiewicz, A., Rueda, P., Radutoiu, S., et al. (2009). Rearrangement of actin cytoskeleton mediates invasion of *Lotus japonicus* roots by *Mesorhizobium loti*. *Plant Cell* 21, 267–284. doi: 10.1105/tpc.108.063693

Yoo, S. D., Cho, Y. H., Tena, G., Xiong, Y., Sheen, J. (2008). Dual control of nuclear EIN3 by bifurcate MAPK cascades in C_2H_4 signalling. *Nature* 451, 789–795. doi: 10.1038/nature06543

Young, N. D., Debellé, F., Oldroyd, G. E., Geurts, R., Cannon, S. B., Udvardi, M. K., et al. (2011). The Medicago genome provides insight into the evolution of rhizobial symbioses. *Nature* 480, 520–524. doi: 10.1038/nature10625

Yu, L. X., Liu, X., Boge, W., Liu, X. P. (2016). Genome-wide association study identifies loci for salt tolerance during germination in autotetraploid alfalfa (*Medicago sativa* L.) using genotyping-by-sequencing. *Front. Plant Sci.* 7, 956.

Yu, L., Nie, J., Cao, C., Jin, Y., Yan, M., Wang, F., Zhang, W. (2010). Phosphatidic acid mediates salt stress response by regulation of MPK6 in *Arabidopsis thaliana*. *New Phytol.* 188, 762–773. doi: 10.1111/j.1469-8137.2010.03422.x

Zaïdi, I., Ebel, C., Touzri, M., Herzog, E., Evrard, J. L., Schmit, A. C., et al. (2010). TMKP1 is a novel wheat stress responsive MAP kinase phosphatase localized in the nucleus. *Plant Mol. Biol.* 73, 325–338. doi: 10.1007/s11103-010-9617-4

Zanke, B. W., Boudreau, K., Rubie, E., Winnett, E., Tibbles, L. A., Zon, L., et al. (1996). The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. *Curr. Biol.* 6, 606–613. doi: 10.1016/S0960-9822(02)00547-X

Zeke, A., Lukács, M., Lim, W. A., Reményi, A. (2009). Scaffolds: interaction platforms for cellular signalling circuits. *Trends Cell Biol.* 19, 364–374. doi: 10.1016/j.tcb.2009.05.007

Zemenchik, R. A., Albrecht, K. A. (2002). Nitrogen use efficiency and apparent nitrogen recovery of Kentucky bluegrass, smooth bromegrass, and orchardgrass. *Agron. J.* 94, 421–428. doi: 10.2134/agronj2002.4210

Zemenchik, R. A., Albrecht, K. A., Wollenhaupt, N. C. (1997). Soil erosion and forage production from established alfalfa and smooth bromegrass in rotation with corn. *In Proc.* 6, 251–255.

Zemenchik, R. A., Wollenhaupt, N. C., Albrecht, K. A., Bosworth, A. H. (1996). Runoff, erosion, and forage production from established alfalfa and smooth bromegrass. *Agronom. J.* 88, 461–466. doi: 10.2134/agronj1996.00021962008800030017x

Zeng, N., Yang, Z., Zhang, Z., Hu, L., Chen, L. (2019). Comparative transcriptome combined with proteome analyses revealed key factors involved in alfalfa (*Medicago sativa*) response to waterlogging stress. *Int. J. Mol. Sci.* 20, 1359. doi: 10.3390/ijms20061359

Zhang, J. Y., Broeckling, C. D., Blancaflor, E. B., Sledge, M. K., Sumner, L. W., Wang, Z.Y. (2005). Overexpression of WXP1, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). *Plant J.* 42, 689–707. doi: 10.1111/j.1365-313X.2005.02405.x

Zhang, J., Subramanian, S., Stacey, G., Yu, O. (2009). Flavones and flavonols play distinct critical roles during nodulation of *Medicago truncatula* by *Sinorhizobium meliloti*. *Plant J.* 57, 171–183. doi: 10.1111/j.1365-313X.2008.03676.x

Zhang, L. Q., Niu, Y. D., Huridu, H., Hao, J. F., Qi, Z., Hasi, A. (2014). *Salicornia europaea* L. Na^+/H^+ antiporter gene improves salt tolerance in transgenic alfalfa (*Medicago sativa* L.). *Genet. Mol. Res.* 13, 5350–5360. doi: 10.4238/2014.July.24.14

Zhang, L., Xi, D., Li, S., Gao, Z., Zhao, S., Shi, J., et al. (2011). A cotton group C MAP kinase gene, *GhMPK2*, positively regulates salt and drought tolerance in tobacco. *Plant Mol. Biol.* 77, 17–31. doi: 10.1007/s11103-011-9788-7

Zhang, S., Shi, Y., Cheng, N., Du, H., Fan, W., Wang, C. (2015). De novo characterization of fall dormant and nondormant alfalfa (*Medicago sativa* L.) leaf transcriptome and identification of candidate genes related to fall dormancy. *PloS One* 10:e0122170. doi: 10.1371/journal.pone.0122170

Zhang, Y. M., Liu, Z. H., Wen, Z. Y., Zhang, H. M., Yang, F., Guo, X. L. (2012). The vacuolar Na^+- H^+ antiport gene *TaNHX2* confers salt tolerance on transgenic alfalfa (*Medicago sativa*). *Funct. Plant Biol.* 39, 708–716. doi: 10.1071/FP12095

Zhang, T., Yu, L. X., Zheng, P., Li, Y., Rivera, M., Main, D., et al. (2015). Identification of loci associated with drought resistance traits in heterozygous autotetraploid alfalfa (*Medicago*

sativa L.) using genome-wide association studies with genotyping by sequencing. *PLoS One* 10:e0138931. doi: 10.1371/journal.pone.0138931

Zhao, B., Liang, R., Ge, L., Li, W., Xiao, H., Lin, H., et al. (2007). Identification of drought-induced microRNAs in rice. *Biochem. Biophys. Res. Comm.* 354, 585–590. doi: 10.1016/j.bbrc.2007.01.022

Zhao, C., Nie, H., Shen, Q., Zhang, S., Lukowitz, W., Tang, D. (2014). EDR1 physically interacts with MKK4/MKK5 and negatively regulates a MAP kinase cascade to modulate plant innate immunity. *PLoS Genet.* 10:e1004389. doi: 10.1371/journal.pgen.1004389

Zhou, C., Han, L., Pislariu, C., Nakashima, J., Fu, C., Jiang, Q., et al. (2011). From model to crop: Functional analysis of a STAY-GREEN gene in the model legume *Medicago truncatula* and effective use of the gene for alfalfa improvement. *Plant Physiol.* 157, 1483–1496. doi: 10.1104/pp.111.185140

Zhou, M., Luo, H. (2013). MicroRNA-mediated gene regulation: potential applications for plant genetic engineering. *Plant Mol. Biol.* 83, 59–75. doi: 10.1007/s11103-013-0089-1

Zhu, J. K. (2001). Plant salt tolerance. *Trends Plant Sci.* 6, 66–71. doi: 10.1016/S1360-1385(00)01838-0

Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. *Ann. Rev. Plant Biol.* 53, 247–273. doi: 10.1146/annurev.arplant.53.091401.143329

Ziegelhoffer, T. J., Will, J., Austin-Phillips, S. (1999) Expression of bacterial cellulase genes in transgenic alfalfa (*Medicago sativa* L.), potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabacum* L.). *Mol. Breed*. 5**,** 309–318. doi: 10.1023%2FA%3A1009646830403

Zipfel, C. (2014). Plant pattern-recognition receptors. *Trends Immunol.* 35, 345–351. doi: 10.1016/j.it.2014.05.004

Zong, X., Li, D. P., Gu, L., Li, D. Q., Liu, L., Hu, X. L. (2009). Abscisic acid and hydrogen peroxide induce a novel maize group C MAP kinase gene, *ZmMPK7*, which is responsible for the removal of reactive oxygen species. *Planta* 229, 485–495. doi: 10.1007/s00425-008-0848-4

Zuanazzi, J. A. S., Clergeot, P. H., Quirion, J. C., Husson, H. P., Kondorosi, A., Ratet, P. (1998). Production of *Sinorhizobium meliloti* nod gene activator and repressor flavonoids from *Medicago sativa* roots. *Mol. Plant-Microbe Interact.* 11, 784–794. doi: 10.1094/MPMI.1998.11.8.784

7 Abbreviations

1D	1 dimensional
2,4-D	2,4-dichlorophenoxyacetid acid
5NG4	AUXIN-INDUCED PROTEIN
A	Average number
AADA	AMINOGLYCOSIDE 3-ADENYLYLTRANSFERASE
ABA	Abscisic acid
ACT	Actin
AHL	*N*-ACETYL-HOMOSERINE-LACTONES
AON	Autoregulation of nodulation
ARP2/3	ACTIN RELATED PROTEIN
ATP	Adenosine triphosphate
BADH	BETAINE ALDEHYDE DEHYDROGENASE
BAR	PHOSPHINOTHRICIN ACETYL TRANSFERASE
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BP	Recombination attB and attP recombination reaction in Gateway
BSA	Bovine serum albumin
C	Carbon
Ca^{2+}	Calcium ion
CAT	CHLORAMPHENICOL ACETYLTRANSFERASE
CBL	CALCINEURIN B-LIKE
CCaMK	Ca^{2+} CALMODULIN-DEPENDENT PROTEIN KINASE
cDNA	Complementary DNA
CEP	C-terminally ENCODED PEPTIDE
CHS	CHALCON SYNTHASE
CIPK	CBL INTERACTING PROTEIN KINASE

CLE	CLAVATA (CLV)/EMBRYO SURROUNDING REGION (ESR)-RELATED PROTEIN
CLSM	Confocal laser scanning microscopy
CODA	CHOLINE OXIDASE
COG	Clusters of Orthologous Group
CRA2	COMPACT ROOT ARCHITECTURE 2
DAPI	4′,6-diamidino-2-phenylindole
DEI	DIFFERENTIALLY EXPRESSED ISOFORMS
DFR	DIHYDROFLAVONOL REDUCTASE
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	Days-post-inoculation
DPK2	NUCLEOSIDE DIPHOSPHATE KINASE 2
DREB1	DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 1
DRI	Drought resistance index
DSP	DUAL-SPECIFITY PHOSPHATASE
ECH4	ENDOCHITINASE GENE 4
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ENOD	EARLY NODULIN
EPSI	Exopolysaccharide 1
ERD	EARLY RESPONSE TO DEHYDRATION
ERF	ETHYLENE RESPONSE FACTOR
EST	Expressed sequence tag
EST-SSR	Expressed sequence tags simple sequence repeat marker
F-actin	Filamentous actin
FAH	Fåhreus medium

Fw	Forward primer
G-actin	Globular actin
GB	Glycibebetaine
GBS	Genotyping by sequencing
GFP	GREEN FLUORESCENT PROTEIN
GGDP	GERANYLGERANYL DISPHOSPHATE
GPX	GLUTATHIONE PEROXIDASE
GST	GLUTATHIONE S-TRANSFERASE
GUS	β-GLUCURONIDASE
GWAS	Genome-wide associated studies
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hi-C	High-throughput sequencing
HRP	Horseradish peroxidase
IAA	Indole-2-acetic acid
INDEL	Insertions/deletions
IOMT	ISOFLAVONE-O-METHYLTRANFERASE
IP5P2	INOSITOL-145-TRISPHOSPHATE 5-PHOSPHATASE
Ipr	InterPro database
IT	Infection thread
K^+	Potassium ion
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	DNA ladder/protein ladder
LB	Latrunculin B
LD	Linkage disequilibrium
LF	LACTOFERRIN
LR reaction	attL and attR recombination reaction in Gateway
M	Median value

MAPK	MITOGEN-ACTIVATED PROTEIN KINASE
MAPKK	MITOGEN-ACTIVATED PROTEIN KINASE KINASE
mapkk4/+	MITOGEN ACTIVATED PROTEIN KINASE 4 heterozygous mutant
MAPKKK	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE
MEKK1	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 1
$MgCl_2$	Magnessium chloride
miRNA	Micro ribonucleic acid
MKK4	MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4
MKK9	MITOGEN-ACTIVATED PROTEIN KINASE KINASE 9
MLSA	Multilocus sequence analysis
MMK2	MITOGEN-ACTIVATED PROTEIN KINASE 2
MMK3	MITOGEN-ACTIVATED PROTEIN KINASE 3
Mn-P	MANGANESE-DEPENDENT LIGNIN PEROXIDASE
MPK1	MITOGEN-ACTIVATED PROTEIN KINASE 1
MPK10	MITOGEN-ACTIVATED PROTEIN KINASE 10
MPK11	MITOGEN-ACTIVATED PROTEIN KINASE 11
MPK12	MITOGEN-ACTIVATED PROTEIN KINASE 12
MPK13	MITOGEN-ACTIVATED PROTEIN KINASE 13
MPK14	MITOGEN-ACTIVATED PROTEIN KINASE 14
MPK15	MITOGEN-ACTIVATED PROTEIN KINASE 15
MPK17	MITOGEN-ACTIVATED PROTEIN KINASE 17
MPK19	MITOGEN-ACTIVATED PROTEIN KINASE 19
MPK2	MITOGEN-ACTIVATED PROTEIN KINASE 2
MPK4	MITOGEN-ACTIVATED PROTEIN KINASE 4
MPK5	MITOGEN-ACTIVATED PROTEIN KINASE 5
MPK6	MITOGEN-ACTIVATED PROTEIN KINASE 6
MPK7	MITOGEN-ACTIVATED PROTEIN KINASE 7
MPK8	MITOGEN-ACTIVATED PROTEIN KINASE 8

MS	Murashige & Skoog
N	Number of samples
N	Nitrogen
N_2	Dinitrogen
NA	Numeric aperture
Na^+	Sodium ion
NaCl	Sodium chloride
NaF	Sodium fluoride
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NH_3	Ammonium
NHX1	SODIUM/HYDROGEN EXCHANGER 1
NHX2	SODIUM/HYDROGEN EXCHANGER 2
NO	Nitric oxide
Nod	Nodulation
NPTII	NEOMYCIN PHOSPHOTRANSFERASE II
Nr	Nonredundant protein
NSP1	NODULATION SIGNALING PATHWAY 1
NTF3	MITOGEN-ACTIVATED PROTEIN KINASE HOMOLOG NTF3
NTF6	MITOGEN-ACTIVATED PROTEIN KINASE HOMOLOG NTF6
OMTK1	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE
OMTK1	OXIDATIVE STRESS ACTIVATED MAPKKK
ORF	Open reading frame
P	Phosphor
P5CS	Δ1-PYRROLINE-5-CARBOXYLATE SYNTHASE
PacBio SMRT	Pacific Biosciences Single-molecule real-time sequencing
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
POG	PEROXYGENASE
PP2C	PROTEIN PHOSPHATASE OF TYPE 2C
PRKK	MITOGEN-ACTIVATED PROTEIN KINASE KINASE
PSTP	SERINE/THREONINE PHOSPHATASE
PTP	PROTEIN TYROSINE PHOSPHATASE
PVDF	Polyvinylidene difluoride
QTL	Quantitative trait loci
RCT1	RESISTANCE TO *C. trifolli* RACE 1
RD	RESPONSIVE TO DESICCATION
Rev	Reverse primer
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RS	RESVERATROL SYNTHASE
RSY	*Medicago sativa* cv. Regen-SY
RT-qPCR	Quantitative real-time PCR
RUBISCO	RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE-OXYGENASE
RWC	Relative leaf water content
S	Serine
SAMK	STRESS-ACTIVATED MAPK
SDA	Single dose allele
SDS	Sodium dodecyl
Ser	Serine
SIMK	STRESS-INDUCED MITOGEN ACTIVATED PROTEIN KINASE

SIMKK	STRESS-INDUCED MITOGEN ACTIVATED PROTEIN KINASE KINASE
SIMKKi	SIMKK RNAi line
SIP2	MITOGEN-ACTIVATED PROTEIN KINASE KINASE
SIPK	MITOGEN-ACTIVATED PROTEIN KINASE
SN1	SNAKIN-1 PEPTIDE
SNP	Single nucleotide polymorphism
SOS1	SALT OVERLAY SENSITIVE 1
SPL13	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 13
SUNN	SUPER NUMERIC NODULES
SWPA2	SWEETPOTATO PEROXIDASE ANIONIC 2
T	Threonine
tagRFP	RED FLUORESCENT PROTEIN
TBS	Tris-buffered-saline
TBS-T	TBS, 0.1% Tween 20
T-DNA	Transfer DNA
TDY1	MITOGEN-ACTIVATED PROTEIN KINASE
TEY	Phosphorylation motif in MPKs (Thr-Glu-Tyr)
TFs	Transcription factors
Thr	Threonine
TrEMBL	Translated EMBL
WIPK	MITOGEN-ACTIVATED PROTEIN KINASE
Y	Tyrosine
YFP	Yellow fluorescent protein

8 *Curriculum vitae*

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"Developmental and subcellular localization of annexin 1 in Arabidopsis root using advanced microscopy methods" (IGA_PřF_2018_031), position: Ph.D. student.

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List of Attended Conferences

Poster

Hrbáčková, M., Ovečka, M., Luptovčiak, I., Šamaj, J. (2019). Subcellular localization of fluorescently tagged MAPKs in alfalfa. Plant Biotechnology: Green for Good V, Olomouc, Czech Republic, 10. – 13. June 2019, abstract 14.

Fields of Interest

- Molecular biology methods (DNA and RNA isolation, cDNA preparation, PCR based techniques, Multisite Gateway cloning, bimolecular fluorescence complementation).
- Biochemical methods (protein extraction, SDS-PAGE, Western blotting, Immunoprecipitation of GFP-tagged proteins with magnetic anti-GFP beads).
- Cytological and histological techniques (whole mount immunolabelling).
- Transformation methods (*Nicotiana benthamiana*, *Arabidopsis thaliana*, *Medicago sativa*).
- Genetics (phenotype studies).
- Microscopy (light microscopy, epifluorescence microscopy, zoom fluorescence microscopy, confocal laser scanning microscopy, confocal laser scanning microscopy with Airyscan module, spinning disk, light-sheet fluorescence microscopy).

List of Publications

- **Hrbáčková, M.**, Dvořák, P., Takáč, T., Tichá, M., Luptovčiak, I., Šamajová, O., Ovečka, M., Šamaj, J. (2020). Biotechnological Perspectives of Omics and Genetic Engineering Methods in Alfalfa. *Frontiers in Plant Science 11, 592,* doi: 10.3389/fpls.2020.00592.
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- Tichá, M., Richter, H., Ovečka, M., Maghelli, N., **Hrbáčková, M.,** Dvořák, P., Šamaj, J., Šamajová, O. (2020). Advanced microscopy reveals complex developmental and subcellular localization patterns of ANNEXIN 1 in Arabidopsis. *Frontiers in Plant Science 11, 1153,* doi: 1153.10.3389/fpls.2020.01153.
- Tichá, M., Illésová, P., **Hrbáčková, M.,** Basheer, J., Novák, D., Hlaváčková, K., Šamajová, O., Niehaus, K., Ovečka, M., Šamaj, J. (2020). Tissue culture, genetic transformation and modern bio-imaging techniques in alfalfa research. *Critical Reviews in Biotechnology 40, 1265-1280,* doi: 10.1080/07388551.2020.1814689.

- Tichá, M., Hlaváčková, K., **Hrbáčková, M.**, Ovečka, M., Šamajová, O., Šamaj, J. (2020). Super-resolution imaging of microtubules in Medicago sativa. *Methods in Cell Biology 160, 237-251,* doi: 10.1016/bs.mcb.2020.03.004.

9 Supplements

Supplement I

Review

Biotechnological perspectives of omics and genetic engineering methods in alfalfa

Hrbáčková, M., Dvořák, P., Takáč, T., Tichá, M., Luptovčiak, I., Šamajová, O., Ovečka, M., Šamaj, J.

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REVIEW
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Biotechnological Perspectives of Omics and Genetic Engineering Methods in Alfalfa

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For several decades, researchers are working to develop improved major crops with better adaptability and tolerance to environmental stresses. Forage legumes have been widely spread in the world due to their great ecological and economic values. Abiotic and biotic stresses are main factors limiting legume production, however, alfalfa (*Medicago sativa* L.) shows relatively high level of tolerance to drought and salt stress. Efforts focused on alfalfa improvements have led to the release of cultivars with new traits of agronomic importance such as high yield, better stress tolerance or forage quality. Alfalfa has very high nutritional value due to its efficient symbiotic association with nitrogen-fixing bacteria, while deep root system can help to prevent soil water loss in dry lands. The use of modern biotechnology tools is challenging in alfalfa since full genome, unlike to its close relative barrel medic (*Medicago truncatula* Gaertn.), was not released yet. Identification, isolation, and improvement of genes involved in abiotic or biotic stress response significantly contributed to the progress of our understanding how crop plants cope with these environmental challenges. In this review, we provide an overview of the progress that has been made in high-throughput sequencing, characterization of genes for abiotic or biotic stress tolerance, gene editing, as well as proteomic and metabolomics techniques bearing biotechnological potential for alfalfa improvement.

Keywords: alfalfa, *Medicago sativa*, genomics, metabolomics, proteomics, stress resistance genes

INTRODUCTION

Legumes are important food crops for the exponentially growing population, owing to their micronutrient, macronutrient, and secondary metabolite content (Le et al., 2007). Some of these organic compounds (e.g., phytoalexins and chitinases) play roles in plant defense against pathogens and pests (He and Dixon, 2000). Moreover, Fabaceae is one of the most studied plant families, and it has gained high agricultural importance, especially owing to its ability to fix nitrogen in symbiosis with rhizobia (Doyle and Luckow, 2003).

Medicago sativa L., commonly known as alfalfa or "lucerne," belongs to Fabaceae, and its first cultivated form most likely originates from western Persia. It then spread to many regions in Asia, Europe, and America. In addition, Rashmi et al. (1997) and Samac and Temple (2004) reported that alfalfa ranks fourth in terms of acreage and economic value, following corn, soybean, and wheat.

The genus *Medicago* includes both perennial and annual species. Alfalfa is a highly valuable perennial deep-rooted forage legume, especially because of its widespread production, soil protection, and ability to improve nitrogen-limited soils (Radović et al., 2009). It is also widely cultivated for livestock feed (Flajoulot et al., 2005), and is used as a biofuel feedstock for ethanol production, either as hay or silage (McCoy and Bingham, 1988). The biological and agronomical potential of alfalfa, like all other members of the whole legume family, is extraordinary because it requires little to no nitrogen fertilizer for optimal growth (Ebert, 2007). In addition, alfalfa plays an important role as a free fertilizer providing nitrogen to subsequent crops (Triboi and Triboi-Blondel, 2014).

Alfalfa shows a high content of proteins, enzymes (amylase, coagulase, peroxidase, erepsin, lipase, invertase, and pectinase), antioxidants, minerals, and vitamins A, C, K, and E, as well as valuable phytopharmaceutical components (Bora and Sharma, 2011 and references therein). Moreover, alfalfa and some other species of Fabaceae family possess two different thiol redox compounds, namely glutathione (GSH) and the homoglutathione (hGSH), with higher content of hGSH (Klapheck, 1988; Baldacci-Cresp et al., 2012). More specifically, alfalfa shows different ratios of hGSH/GSH in diverse organs such as leaves, stems, and roots (Pasternak et al., 2014). Thus, alfalfa represents one of the most valuable and important forage crops, and can also be used in grasslands as a cover crop for improved weed control. Finally, alfalfa is also suitable for use in the production of recombinant pharmaceutical proteins (Fu et al., 2015) and in phytoremediation (Nirola et al., 2016).

The tetraploid genome of alfalfa and outbreeding mating systems have made selective breeding harder (Zhou et al., 2011; Annicchiarico et al., 2015). Advanced methods such as genomic, proteomic, and metabolomic approaches, as well as gene editing, could lead to the practical applications of genes that have biotechnological value for alfalfa improvement, especially if applied in an integrated and targeted manner. As a result, single or multiple genes might show desirable effects on several agronomically important alfalfa traits, which can significantly accelerate research in comparison to conventional breeding (Singer et al., 2018). Alfalfa is a major source of proteins in the livestock and dairy industries. In the last years, alfalfa production has been displaced to saline environments by major cereals. Therefore, the incorporation of transgenic traits into alfalfa with varying degrees of tolerance to salinity has been developed and this robust approach can improve the productivity and quality of nitrogen-fixing crops (Kang et al., 2016; Stritzler et al., 2018). Genetically engineered glyphosate-resistant alfalfa was commercialized in the United States in 2010. Another alfalfa variety with reduced lignin content stacked to glyphosate resistance trait has been available since 2015. Reduced lignin content in forage legumes can improve their digestibility by animals, thus it is an important forage quality trait (Li et al., 2016; Barros et al., 2019).

The purpose of this review is to provide a perspective on the current state of alfalfa biotechnology research. It focuses mainly on the biotechnological potential of genomic and transcriptomic approaches, biotechnologically valuable genes, gene editing, proteomics, and metabolomics. When appropriate it is compared to barrel medic.

GENOMIC APPROACHES

The identification of genes that affect legume crop production represents an important aim of current genomic studies (Bevan et al., 2017), and this requires knowledge of their full genomic sequences. Technologies for sequencing DNA and RNA have undergone revolutionary improvements (Ari and Arikan, 2016). It is known that after the evolutionary split between monocots and eudicots, several whole genome duplications and triplications had occurred in legumes (Severin et al., 2011; Masonbrink et al., 2017), which might delay whole genome sequencing efforts. The major strength of next-generation sequencing (NGS) is its ability to detect abnormalities across the entire genome. NGS is less costly and has a faster turnaround time compared to classical sequencing methods. New NGS platforms, such as the Roche/454 system (Margulies et al., 2005), Illumina platform (Wang et al., 2012), real-time DNA sequencing by Pacific Biosciences (Eid et al., 2009), Oxford Nanopore system (Lu et al., 2016), and Ion Torrent system (Rothberg et al., 2011), were used for sequencing crop and legume genomes. They have had a major impact on plant research, since they enable the understanding of genomic complexity as well as the identification of genomic variations, such as single nucleotide polymorphisms (SNPs) or insertions/deletions (INDELs; Valliyodan et al., 2017; Abdelrahman et al., 2018). NGS and bioinformatics approaches for high-throughput data analysis are major tools in modern plant breeding programs (Abdelrahman et al., 2015, 2017a,b; Pavlovich, 2017). These modern technologies are also used in legume research, and several recent studies have been devoted to alfalfa genomics using high-throughput genome sequencing (reviewed by Hawkins and Yu, 2018).

High-Throughput NGS in Genomics and Transcriptomics

Genome sequencing and assembly have been applied to many plant species, including crops. Such genome assemblies serve as common references for alignment with re-sequenced plants (Huang et al., 2012; Schreiber et al., 2018). Large-scale systematic genome sequencing has been carried out in leguminous plants such as *Lotus japonicus* (Sato et al., 2008), *M. truncatula* (release 3.0)[1], and *Glycine max* (Schmutz et al., 2010). The genome sequence of alfalfa has not yet been published, and current transcriptomic studies and SNP discoveries rely on the barrel medic genome sequence alignment (genome version[2] *Mt4*.0v1; Young et al., 2011; Tang et al., 2014). Currently, the most advanced genome sequencing method is NGS. It has become the major tool for the development of new molecular markers and for gene identification (Edwards and Batley, 2010). Together with the rapid development of NGS, the number of plants with completely sequenced genomes has dramatically increased (Van et al., 2013;

[1]https://www.jcvi.org/research/medicago-truncatula-genome-database
[2]phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Mtruncatula

Le Nguyen et al., 2018; Kersey, 2019). Advantages of NGS include lower costs and shorter time requirements. The development of NGS technology contributed to the identification of new genes that had evolved by whole-genome duplication and structural variations in chromosomes (Barabaschi et al., 2012; Van et al., 2013). Reference genome sequences of several legume and crop species are now available, and candidate genes of important SNPs can be rapidly and easily identified (Gao et al., 2012; Van et al., 2013; Le Nguyen et al., 2018; Scheben et al., 2019). Alfalfa is an outbred, tetrasomic tetraploid ($2n = 4x = 32$) with eight basic chromosomes and a genome size of 800–1000 Mbp (Blondon et al., 1994). Genetic and genomic resources have been widely explored and developed, but in the absence of a fully sequenced and assembled reference genome for alfalfa, genome of closely related barrel medic is used as a model organism (Zhou et al., 2011). Barrel medic is a diploid species ($2n = 2x = 16$) with smaller genome (about 550 Mbp; Piano and Pecetti, 2010). NGS technologies could speed up the discovery of quantitative trait loci (QTLs) and candidate SNPs, which represent common sequence variations among plants and are functionally important. Numerous molecular markers are used in high-throughput genotyping by sequencing (GBS) platforms associated with alfalfa mapping (Hawkins and Yu, 2018), population diversity studies (Herrmann et al., 2018), and genomic selection (Annicchiarico et al., 2016). In the past years, low density linkage maps were constructed on diploid alfalfa (Brummer et al., 1993; Kiss et al., 1993; Echt et al., 1994; Julier et al., 2003). Although several genetic linkage maps have been constructed for tetraploid alfalfa, most of them were framework maps with only few markers (Brouwer and Osborn, 1999; Julier et al., 2003; Musial et al., 2007; Robins et al., 2007; Khu et al., 2013). Li X. et al. (2014) have constructed a saturated genetic linkage map of autotetraploid alfalfa by using GBS. They have shown high synteny between linkage groups of alfalfa and barrel medic, and clearly identified translocations between chromosomes 4 and 8, and small inversion on chromosome 1. The high-density linkage maps contained 3,591 SNP markers on 64 linkage groups across both maternal and paternal genomes of an autotetraploid alfalfa F_1 population (Li X. et al., 2014).

Genome-wide associated studies (GWAS) are a modern and powerful strategy that can be used to overcome the limitations of conventional QTL mapping. GWAS map genetic loci in a breeding population, relying on linkage disequilibrium (LD; Liu X. P. et al., 2019). Recently, GWAS have been used in the identification of genetic loci in crop species such as soybean (Hwang et al., 2014), maize (Olukolu et al., 2016), barrel medic (Kang et al., 2015), and alfalfa. Zhang T. et al. (2015) evaluated two important features associated with drought resistance, namely drought resistance index (DRI) and relative leaf water content (RWC) under greenhouse conditions in 198 alfalfa cultivars and landraces. These results were then correlated with genomic data obtained through GBS. Subsequent to the QTL mapping approach, GWAS provided identification of 15 loci associated with DRI and RWC. Markers associated with DRI are located at all chromosomes, whereas markers associated with RWC are located at chromosomes 1, 2, 3, 4, 5, 6, and 7. Co-localization of markers for DRI and WRC were found on chromosomes 3, 5, and 7 (Zhang T. et al., 2015). A GWAS approach using more than 15,000 genome-wide SNPs obtained through GBS was applied to examine forage yield and nutritive value-related traits. Five genes, containing known SNPs aligned to the barrel medic genome, were found as candidates in determining fall dry matter yield (*TUBBY-LIKE PROTEIN*), summer dry matter yield (*E3 SUMO-PROTEIN LIGASE SIZ1, RNA-DEPENDENT RNA POLYMERASE FAMILY PROTEIN*), fall stem weight (*UBIQUITIN-LIKE-SPECIFIC PROTEASE ESD4-LIKE PROTEIN*), and cell wall biogenesis (*NUCLEOTIDE-DIPHOSPHO-SUGAR TRANSFERASE FAMILY PROTEIN*; Sakiroglu and Brummer, 2017). Aiming to find markers for alfalfa forage quality, 154 plants originating from the second generation prepared by the outcrossing of three alfalfa cultivars were subjected to GBS, while their half-sib progenies were phenotyped for forage quality parameters under three different growing conditions. Subsequently, GWAS of SNPs was carried out using barrel medic as a reference genome, confirming a polygenic control of quality traits and indicating a substantially different genetic control of a given trait in stems and leaves (Biazzi et al., 2017). Important alfalfa loci for salt tolerance during germination were identified by similar marker-trait association using a GWAS approach (Yu et al., 2016). Remarkably, they used 198 different accessions with potential drought tolerance, whereas DNA libraries were sequenced in two lanes of an Illumina Hi-Seq2000 instrument. Identified SNP markers were located on all chromosomes, with the exception of chromosome 3. Several alfalfa loci showed similar genetic locations to the reported QTLs associated with salt tolerance in barrel medic. The results suggest the similarity of mechanisms controlling salt stress responses in these two species. This study resulted in the identification of 14 genes connected to 23 markers associated with salt tolerance during germination. These include *PEROXYGENASE, B3 DNA-BINDING PROTEIN,* and *CPR5 PROTEIN*, which are linked to cuticle wax biosynthesis and ABA signaling (Yu et al., 2016).

Over the last two decades, several methods have been developed that allowed the examination of global transcriptional changes. The most used ones are the hybridization of cDNAs (DNA microarrays) and the deep sequencing of cDNA (RNA-Seq; Schena et al., 1995; Wang et al., 2009; Lardi and Pessi, 2018). RNA-Seq, a massive parallel sequencing method for transcriptome analysis, was developed 10 years ago (Wang et al., 2009). Transcriptomic studies analyze only the transcribed portion of the genome and provides in-depth sequencing coverage and additional qualitative information such as isoform-specific expression (Abdelrahman et al., 2018). In contrast to microarrays, ribosomal RNA (rRNA) does not hybridize to the chip, as homologous probes are not present. In RNA-Seq, the abundant rRNA is removed (Lardi and Pessi, 2018). Originally, transcriptomic studies were based on Sanger sequencing of expressed sequence tags (ESTs) or microarrays, which was used in alfalfa and barrel medic (Aziz et al., 2005; Cheung et al., 2006; Yang et al., 2010). It has also been applied for other legumes such as *G. max* (Le et al., 2012; Ha et al., 2015; Tripathi et al., 2016), *L. japonicus* (Asamizu et al., 2004), and *Cicer arietinum* (Deokar et al., 2011).

Several studies contributed to the transcriptome sequencing of alfalfa with various coverage. These studies relied on NGS technologies such as 454 technology (Han et al., 2011) or RNA-Seq (Yang et al., 2011; Li and Brummer, 2012; Liu et al., 2013; O'Rourke et al., 2015). Liu et al. (2013) performed *de novo* transcriptome sequencing of *M. sativa* L. subsp. *sativa* using Illumina paired-end sequencing. Plant material included 15 tissue types, and the transcriptome coverage was 5.64 Gbp of clean nucleotides. About 40,433 unigenes were obtained, and 1649 potential expressed sequence tags simple sequence repeat markers (EST-SSRs) were annotated by alignment with the following databases: the National Center for Biotechnology Information (NCBI) nonredundant protein (Nr) database, the NCBI non-redundant nucleotide sequence (Nt) database, Swiss-Prot, The Kyoto Encyclopedia of Genes and Genomes (KEGG), the Clusters of Orthologous Group (COG), Translated EMBL (TrEMBL), and the InterPro (Ipr) database (Liu et al., 2013). RNA-Seq analysis of two alfalfa subspecies, namely *M. sativa* ssp. *sativa* (B47) and *M. sativa* ssp. *falcata* (F56) using roots, nitrogen-fixing root nodules, leaves, flowers, elongating stem internodes, and post-elongation stem internodes resulted in 112,626 unique transcript sequences, which were assembled into the alfalfa Gene Index 1.2 (MSGI 1.2; O'Rourke et al., 2015). Chao et al. (2019) used PacBio SMRT technology and identified 72,606 open reading frames (ORFs) including 46,616 full-length ORFs, 1670 transcription factors and 44,040 SSRs. A total of 7568 alternative splicing events and 17,740 long non-coding RNAs supported the feasibility of deep sequencing full length RNA from alfalfa transcriptome on a single-molecule level (Chao et al., 2019). Another approach developed to provide long-read sequencing of transcripts is Oxford Nanopore Technologies®. The MinION device, which was developed by Oxford Nanopore, is a portable apparatus compatible with a PC or laptop (Jain et al., 2016; Lu et al., 2016). Fleming et al. (2018) evaluated changes in mRNA in dry soybean seeds with use of MinION-based pipeline technology. Li et al. (2019) used MinION-based technology for high-throughput mapping of transgenic alleles in soybean. They rapidly mapped the transgene insertion positions in 51 transgenic soybean plants in a single 1D sequencing run. This method was optimized using a population of soybean lines, but it can be adapted to map the transgenes in any other crops.

TRANSCRIPTOMIC APPROACHES AND GENE EXPRESSION MODIFICATIONS

Resistance to Abiotic Stress

Salinity stress interferes with plant growth because it causes two main stresses on plants: hyperosmotic pressure and ion toxicity, especially due to Na^+ (Volkov et al., 2004). High salinity often triggers an increase in cytosolic Ca^{2+}, reactive oxygen species (ROS), abscisic acid (ABA), and mitogen activated protein kinase (MAPK) signaling (Ovečka et al., 2014; Mittler and Blumwald, 2015). These activated signal molecules affect plant transcriptomes by regulating transcription factors (Xiong et al., 2002; Zhu, 2002). One of the basic strategies in plant stress responses is the accumulation of water-soluble compounds of low molecular weight, such as betaines, polyols, sugars, and amino acids (Chen and Murata, 2002). These compounds accumulate to high concentrations under water or salt stress and protect plants via ROS detoxification and membrane integrity maintenance (Bohnert and Jensen, 1996). For example, glycinebetaine (GB) is a particularly effective protectant against abiotic stress (Chen and Murata, 2008), and accumulates rapidly in plants exposed to salt, drought, and low temperature stresses (Rhodes and Hanson, 1993).

Previous studies have shown that overexpression of stress-related genes caused enhanced tolerance of alfalfa to the salinity stress (Luo et al., 2019b). Li H. et al. (2014) successfully targeted *CHOLINE OXIDASE A* (*CODA)* cDNA derived from *Agrobacterium globiformis* to alfalfa chloroplasts under the control of the strong stress inducible *SWEETPOTATO PEROXIDASE ANIONIC 2* (*SWPA2*) promoter (Kim et al., 2003). Such transgenic alfalfa plants exhibited increased tolerance to oxidative, drought, and salt stress. Because salinity also causes cellular ionic imbalances, the Na^+/H^+ antiporter in the plasma membrane (SOS1 – SALT OVERLAY SENSITIVE 1) and tonoplast (NHX2 – SODIUM/HYDROGEN EXCHANGER 2) can maintain higher K^+/Na^+ ratios in the cytoplasm as a protection against sodium toxicity (Fukuda et al., 1999; Xia et al., 2002; Zhang L. Q. et al., 2014). Moreover, the expression of foreign genes, such as *TaNHX2* (*Triticum aestivum NHX2*), *AhBADH* (*Atriplex hortensis BETAINE ALDEHYDE DEHYDROGENASE*), *SsNHX1 (Suaeda salsa NHX1),* and *GmDREB1* (*G. max DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 1*), can increase salt tolerance in transgenic alfalfa plants (Zhang et al., 2012). As such, Zhang L. Q. et al. (2014) transformed the exogenous gene *SeNHX1* (*Salicornia europaea NHX1*) into alfalfa using *Agrobacterium*-mediated transformation; this enhanced tolerance to salt stress was manifested by improved photosynthesis and membrane stability. Another attempt to improve salt tolerance in alfalfa was reported by Jin et al. (2010) using transformation with the soybean *DREB* ortholog, *GmDREB1*, under the control of *Arabidopsis* stress-inducible *RD29A* (*RD – RESPONSIVE TO DESICCATION)* promoter. Ion leakage, chlorophyll fluorescence, total soluble sugars, transcript level of *Δ1-PYRROLINE-5-CARBOXYLATE SYNTHASE* (*P5CS*), and free proline contents were correlated with the higher salt tolerance of transgenic lines (Jin et al., 2010). Wang et al. (2014) generated and characterized transgenic alfalfa plants with heterologous expression of *AtNDPK2* (*NUCLEOSIDE DIPHOSPHATE KINASE 2*) under the control of oxidative stress inducible *SWPA2* promoter. These transgenic plants showed increased tolerance to oxidative, high temperature, salt and drought stresses. Such enhanced tolerance was mediated by activation of ROS scavenging, enhanced activity of NDPK2 enzyme, improved protection of membrane integrity, and increased proline accumulation (Wang et al., 2014).

First studies on drought responses of alfalfa started in the 1990s (Luo et al., 1991, 1992; Laberge et al., 1993). Metabolite profiling and proteomic approaches identified soluble sugars, amino acids, and proteins that respond to drought in leaves and nodules of alfalfa variety Magali (Aranjuelo et al., 2011). Simultaneously, Kang et al. (2011) have shown systematic analysis of two alfalfa varieties, Wisfal and Chilean, with different tolerance/sensitivity to the drought stress. They have

identified many genes involved in adaptation to the drought stress, including genes encoding transcription and regulatory factors, or genes involved in the biosynthesis of osmolytes and antioxidants. Knowledge of such genes can help in breeding programs. A number of microRNAs have been used to improve various crop species via genetic engineering (Macovei et al., 2012; Zhou and Luo, 2013; Aung et al., 2015). Researchers also characterized microRNAs and their target genes that respond to hypoxia, wounding, heat or oxidative stress (Zhao et al., 2007; Budak et al., 2015). Recent study by Arshad et al. (2017) suggested that overexpression of microRNA156 (*miR156OE*) is an emerging tool to improve drought tolerance of alfalfa since it silenced *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 13 (SPL13i)* leading to reduced water loss and enhanced stomatal conductance and photosynthetic assimilation. Another study proposed a role of *miR156OE* and *SPL13i* in heat stress tolerance since plants carrying these constructs showed increased antioxidant levels (Matthews et al., 2019). As found by NGS, plants possessing *miR156OE* exhibited broad changes in gene expression, including genes involved in nodulation, root development and phytohormone biosynthesis (Aung et al., 2017). Taking together, miR156 can improve drought or heat stress tolerance in alfalfa, at least partially by silencing *SPL13* (Feyissa et al., 2019; Matthews et al., 2019).

RNA-Seq analysis was utilized in the transcriptome profiling of alfalfa in order to study the molecular mechanisms underlying frost (Song et al., 2016), salinity (Postnikova et al., 2013; An et al., 2016; Lei et al., 2018), drought (Arshad et al., 2018), resistance to aluminum (Liu W. et al., 2017), lead (Xu et al., 2017) and waterlogging (Zeng et al., 2019), or fall dormancy (Zhang S. et al., 2015). For example, genes encoding membrane proteins, and proteins of hormonal signal transduction, and ubiquitin-mediated proteolysis pathways contribute to the freezing adaptation mechanisms in alfalfa (Song et al., 2016). Using high-throughput sequencing technology, Postnikova et al. (2013) have demonstrated that salinity stress affects a variety of alfalfa genes. Among the most affected ones were genes of known function, such as *DIHYDROFLAVONOL REDUCTASE* (*DFR)*, transcription factor *MYB59*, *SUGAR TRANSPORTER ERD6-like 16* (*ERD – EARLY RESPONSE TO DEHYDRATION*), and *INOSITOL-145-TRISPHOSPHATE 5-PHOSPHATASE* (*IP5P2*). This study revealed that 86 transcription factors responded to salinity stress; among them are those belonging to GRAS, ARR, JUMONJI, and MYB families that were preferentially upregulated in the tolerant alfalfa cultivar (Postnikova et al., 2013). Alfalfa fall dormancy is determined by genes involved in auxin (e.g., *AUXIN-INDUCED PROTEIN 5NG4)* and ethylene signaling (ethylene responsive TF *RAP2-11)* and carbohydrate transport *(ERD6-LIKE PROTEIN*; Zhang S. et al., 2015). Genes encoding *BETA-AMYLASE*, *ETHYLENE RESPONSE FACTOR (ERF)*, *CALCINEURIN B-LIKE (CBL) INTERACTING PROTEIN KINASES (CIPKs)*, *GLUTATHIONE PEROXIDASE (GPX)*, and *GLUTATHIONE S-TRANSFERASE (GST)* are among those important for waterlogging stress resistance in alfalfa (Zeng et al., 2019).

Plant damage caused by saline stress is usually divided into three categories: high pH damage, osmotic shock, and toxic cation stress. Nutrient solution pH variation significantly affected growth of alfalfa seedlings with the optimal pH values in the range between 5.0 and 6.0, as estimated by length and fresh weight of roots, hypocotyls, epicotyls, first leaf petioles, and leaf blades (Köpp et al., 2011). Alfalfa is a saline-alkaline stress-tolerant species (Zhu, 2001; Wong et al., 2006; Gong et al., 2014; An et al., 2016). An et al. (2016) performed transcriptomic analysis of whole alfalfa seedlings treated with saline-alkaline solutions using ion torrent sequencing technology to study changes in the gene expression pattern. This method detects hydrogen ions that are released during DNA polymerization. DEG profiles were obtained and annotated using two methods. Firstly, generated reads were mapped to barrel medic, which has a sequence that is highly homologous to alfalfa. Secondly, functional annotations of assembled unigenes were performed using BLASTX search against the Swiss-Prot databases of barrel medic, thale cress, and soybean. Gene ontology analysis revealed 14 highly enriched pathways. Specific responses of peroxidases, the expression level of *RUBISCO*, and flavonoids indicated antioxidant capacity as one of the main mechanisms behind the saline-alkaline stress tolerance in alfalfa (An et al., 2016). Another study provided a comprehensive transcriptome analysis of alfalfa roots under prolonged ABA treatment (Luo et al., 2019a). Sequences were assembled for many isoforms and were analyzed for their potential role. Differentially expressed isoforms (DEIs) regulated by ABA were mainly involved in transcriptional regulation, plant immunity, plant hormone signal transduction, and anti-oxidative defense.

Nevertheless, these studies were mainly focused on genotype-specific stress mechanisms. Functional and structural genomics studies are fundamental for the understanding of plant biology. Access to high-quality genome and transcriptome sequences is important to perform studies of this kind. Recently, the third-generation sequencing technology PacBio RSII has emerged as a unique method for constructing full-length transcripts (Dong et al., 2015; Nakano et al., 2017). PacBio RSII is an ideal tool for whole genome sequencing, targeted sequencing, RNA-Seq, and epigenetic characterization. This technique allows the sequencing of single DNA molecules in real-time (SMRT) without amplification by PCR (Dong et al., 2015). Using PacBio RSII, Luo et al. (2019b) studied salt stress as a major environmental factor that impacts alfalfa development and production (Zhang S. et al., 2015). They have constructed the first full-length transcriptome database of alfalfa root tips treated with mannitol (a non-ionic osmotic stress) and NaCl (an ionic osmotic stress), which provided evidence that the response to salinity stress includes both osmotic and ionic components. They have found 8,016 mannitol-regulated DEGs and 8,861 NaCl-regulated DEGs. These DEGs are involved in signal transduction, transcriptional regulation, anti-oxidative defense, and signal perceptions (Luo et al., 2019b).

Resistance to Biotic Stress

Biotic stress also considerably affects alfalfa growth and yield. Current methods of plant protection focus mostly on the elimination of pathogenic organisms using pesticides (Shafique et al., 2014). However, the improvement of plant resistance against such pathogens seems like a more beneficial alternative, since it might be more effective and more environmentally

friendly (Kudapa et al., 2013; Varshney and Kudapa, 2013). It is expected that climatic changes are linked to the spread of diseases and emergence of new ones and can raise the threat of parasites and pests (Kudapa et al., 2013; Shafique et al., 2014). Therefore, disease-resilient plants could provide higher production and yield, reflecting the importance of genetically engineering specific genes (de Zélicourt et al., 2011).

Disease resistance mechanisms in plants after encountering a pathogen have been well-described (Roumen, 1994; Zipfel, 2014; Rubiales et al., 2015). Plant infection is facilitated by effector molecules produced by pathogens, which can overcome the first line of plant defense, which is the pathogen−associated molecular pattern (PAMP) triggered immunity; subsequently, plant resistance is suppressed. On the other hand, specific plant resistance (R) proteins have been evolutionarily developed and can provide protection against specific pathogen effectors (Jones and Dangl, 2006; Singer et al., 2018). Nowadays, genes encoding R proteins are widely manipulated for introducing plant resistance to a specific pathogen (Rubiales et al., 2015).

Generally, the most frequently occurring pathogens are bacteria and fungi belonging to Ascomycetes and Basidiomycetes; these obtain nutrients by attacking various parts of the plant body (Shafique et al., 2014). Considerable declines in alfalfa production have been observed mostly due to root infections leading to wilting caused by the bacterium *Clavibacter michiganensis*, fungi *Fusarium oxysporum* and *Verticillium alfalfae*, and microscopic fungus *Phytophthora medicaginis*, or due to leaves infected by *Colletotrichum trifolii* (Nutter et al., 2002; Singer et al., 2018). Alfalfa varieties resistant to these diseases have been obtained by common breeding methods over decades (Toth and Bakheit, 1983; Elgin et al., 1988; Pratt and Rowe, 2002). However, it may not be enough to cover the world demand for crop yields, considering the influence of a retrogressive living environment. Because of alfalfa autopolyploidy and its out-crossing nature (Zhang T. et al., 2015; Yu et al., 2017), the comprehension of molecular and genetic mechanisms during pathogenesis leading to the introduction of specific resistance can be a demanding task. For this reason, barrel medic is widely used for such purposes. Different transcriptomic methods (Gao et al., 2012; Van et al., 2013; Le Nguyen et al., 2018; Scheben et al., 2019) were used to identify barrel medic loci correlated with QTLs, providing resistance to diseases caused by fungi such as *Uromyces striatus* and *Erysiphe pisi* (Bustos-Sanmamed et al., 2013).

C. trifolii is an agent of a highly destructive and prevalent foliar disease, anthracnose (Annicchiarico et al., 2015), which can cause up to 30% decrease in alfalfa yield (Yang et al., 2008). Recognition of this pathogen and induction of response in alfalfa are understudied and need further characterization by cloning techniques. Nevertheless, Yang et al. (2008) found out that overexpression of the gene for intracellular R protein, *RCT1* encoding TIR-NBS-LRR (TOLL/INTERLEUKIN-1 RECEPTOR NUCLEOTIDE BINDING SITE LEUCINE-RICH REPEAT) from barrel medic, ensured anthracnose resistance in alfalfa. Mackie et al. (2007) and Tesfaye et al. (2007) identified tetrasomic dominant *ANTHOCYANIN* genes *AN1* and *AN2* regulating resistance against *C. trifolii* (Elgin and Ostazeski, 1985). Mackie et al. (2007) mapped locations of QTLs for *C. trifolii* traits 1, 2, and 4 in autotetraploid alfalfa clone W126, which is resistant to this pest. Interactions between particular QTLs and phenotypic variations for three *C. trifolii* traits have been described. Obtained markers may be usable in alfalfa breeding for introducing multiple sources of resistance. Although genes for a specific resistance have been identified, new pathotypes of *C. trifolii* are still being discovered; therefore, the generation of new, long-lasting resistant plants is more difficult (Shafique et al., 2014).

Using the suppression subtractive hybridization library, Bustos-Sanmamed et al. (2013) proved the importance of pathogenesis-related (PR) proteins of group 10, as well as proteins engaged in ABA signaling for resistance against harmful fungi, e.g., *Aphanomyces enteiches*. Bahramnejad et al. (2010) designated and isolated the *MsPR10.1A* gene in alfalfa based on its homology to *PR10* genes from other Fabaceae plants, e.g., *Lupinus luteus* (Zhang, 2004). Expression levels of *MsPR10.1A* under different conditions such as ABA treatment, heat shock, wounding, and pathogen attack, were compared with the expression levels of a previously described gene, *PPRG2* (termed as *MsPR10.1B;* Borsics and Lados, 2002). Bahramnejad et al. (2010) observed faster induction of *MsPR10.1A* gene expression than that of *MsPR10.1B* gene after ABA and ethylene treatment, and after application of the pathogenic bacterium *Xanthomonas campestris*. However, inoculation of alfalfa leaves with compatible *X. campestris* led to markedly higher expression of both genes. On the other hand, gene *AAB41557* from the alfalfa *PR10* group did not respond to *X. campestris* inoculation (Esnault et al., 1993). Generally, most examples regarding *PR10* induction due to bacterial inoculation involve incompatible bacteria, such as activation of alfalfa genes *AAB41557* (Esnault et al., 1993) and *MsPR10.1B* (Borsics and Lados, 2002) after *Pseudomonas syringae pv. pisi* inoculation. The promoter of *YPR-10* (of the *RIBONUCLEASE-LIKE PR PROTEIN-10* gene) from *G. max* fused with *GUS* showed activity in the vasculature of *Nicotiana benthamiana* leaves after transient transformation (Walter et al., 1996). Moreover, Bahramnejad et al. (2010) suggested the importance of *MsPR10.1A* promoter expression in the leaf vasculature, resulting in resistance against diseases. *MsPR10.1A* and *MsPR10.1B* promoters have many similar functions in stress responses, but notable differences were found in their reactions to wounding. Thus, promoters of *PR10* genes may be potentially used in biotechnological applications for directing transgene expression in proper tissues.

Plant defense peptides are composed of five main groups: proteases, α-amylase inhibitors, lectins, chitinases, and polyphenol oxidases (Fürstenberg-Hägg et al., 2013). Singer et al. (2018) summarized several genes for the biosynthesis of substances with anti-pathogen effect, such as *AGLUL* encoding β-1,3-glucanase (Masoud et al., 1996), *IOMT* – Isoflavone-*O*-methyltransferase (He and Dixon, 2000), *LF* – encoding lactoferrin (Stefanova et al., 2013) and *RS* – encoding resveratrol synthase (Hipskind and Paiva, 2000). Highly effective protectants, such as protease inhibitors, naturally occur in plants, and they can inhibit proteolytic enzymes in the digestive system of insects or nematodes. Consequently, plant material is not digestible, leading to pathogen starvation and removal from the plant. Inhibitors of cysteine proteases called phytocystatins

were identified in many plants, showing potential in conferring resistance against pathogens. Rice *ORYZACYSTATIN-I* (*OC-I*) and *ORYZACYSTATIN II* (*OC-II*) genes driven by a potato wound-inducible promoter (*Protease inhibitor II*, *PinII*) were transferred to alfalfa attacked by root lesion nematode and leaf beetle. Such transgenic plants revealed a reduction in the *Pratylenchus penetrans* population and enhanced mortality of *Phytodecta fornicata* larvae (Ninković et al., 1995; Samac and Smigocki, 2003).

Tesfaye et al. (2005) generated alfalfa plants that secreted a fungal endochitinase (ECH42). These transgenic plants showed up to 25.7 times increased chitinase activity in vegetative organs and root exudates. Such secreted endochitinases not only retained the lytic activity against glycol chitin, but also showed antifungal activity by the inhibition of spore germination of two fungal pathogens, namely, *Phoma medicaginis* and *C. trifolii* (Tesfaye et al., 2005).

Based on the expression distribution of *SNAKIN* gene *StSN1* in *Solanum tuberosum*, Segura et al. (1999) hypothesized *SN1* as a component of constitutive defense barriers in reproductive and storage plant organs. *StSN2* is induced locally after wounding and pathogen attack; accordingly, it could play an important role in constitutive and inducible defense barriers (Kovalskaya and Hammond, 2009; Guzman-Rodriguez et al., 2013). Next, García et al. (2014) proposed SNAKIN proteins as antimicrobial compounds in plant innate immunity. Indeed, alfalfa transgenic plants carrying *SNAKIN-1* (*MsSN1*) under the control of a constitutive promoter showed improved tolerance against pathogenic fungi. Three independent transgenic lines carrying the *CaMV35S:MsSN1* construct showed significantly lower amounts of infected leaves than wild type plants when treated by *C. trifolii* and with the oomycete *P. medicaginis* (García et al., 2014).

Finally, it is worth mentioning that the genetic transformation of alfalfa with *Bacillus thuringiensis* gene *Cry1C* coding for δ-endotoxin has also been shown to be an effective protective strategy. After transformation, alfalfa was more resistant to *Nemapogon granellus* and *Spodoptera exigua* (Strizhov et al., 1996).

Transcriptomic studies contributed to the knowledge of alfalfa resistance to aphids, strips, and nematodes. Aphids are major insect pests causing a significant decrease of alfalfa yield. Tu et al. (2018b) performed a transcriptomic analysis of two alfalfa cultivars differing in aphid resistance. Genes involved in salicylic acid biosynthesis represented an important defense mechanism in both cultivars. The alfalfa resistance against aphids was mainly determined by induction of genes involved in linoleic acid synthesis important for jasmonic acid and flavonoid biosynthesis (Tu et al., 2018b). Genes participating in jasmonic acid biosynthesis, such as *LIPOXYGENASE, SERINE PROTEINASE INHIBITOR*, and *SEED LINOLEATE 9S-LIPOXYGENASE* were also important for alfalfa resistance to strips infestation. Moreover, genes involved in fatty acid degradation, chloroalkane and chloroalkene degradation, beta-alanine and phenylalanine metabolism and flavonoid biosynthesis also contributed to this resistance (Tu et al., 2018a). Another comparative transcriptomic analysis aimed to screen for genes determining alfalfa resistance to root-knot nematode *Meloidogyne incognita* (Postnikova et al., 2015). *LRR AND NB-ARC DOMAIN DISEASE RESISTANCE PROTEIN* (Medtr3g023030.1), *RECEPTOR-LIKE PROTEIN* (Medtr5g087320.1) and *DISEASE RESISTANCE PROTEIN* (TIR-NBS-LRR class, Medtr0277s0020.3) were up-regulated in the resistant cultivar, while susceptible one showed their down-regulation (Postnikova et al., 2015).

From the biotechnological point of view, ideal alfalfa cultivars should have better nutritional quality, enhanced biomass production and yield, and better resistance to biotic and abiotic stress. All such traits mentioned should be sustainable over a long period of time. Several experimental studies have been conducted to improve alfalfa, but detailed characterization and relationships between desired traits need further genetic and molecular research.

PROTEOMICS AND METABOLOMICS

Owing to its beneficial agronomical traits, alfalfa has been attracting substantial interest in the fields of proteomics and metabolomics during the past two decades. A strong effort was invested in the discovery of new proteins and metabolites involved in alfalfa development and abiotic stress response. In this section, we attempt to summarize the recent achievements of current alfalfa proteomic and metabolomic research. We also aim to highlight the relevance of these investigations for putative biotechnological applications.

Nitrogen and Carbon Metabolism in Alfalfa From a Proteomic Perspective

Proteomics and metabolomics have a remarkable capability to examine the balance between carbon and nitrogen metabolism under stress conditions in alfalfa during interactions with nitrogen-fixing bacteria (Aranjuelo et al., 2011, 2013). Water stress limits nitrogen fixation in nodules by the reduction of nitrogenase activity (Carter and Sheaffer, 1983; Aranjuelo et al., 2011) and Rubisco availability in leaves (Aranjuelo et al., 2005, 2011). The latter likely occurs due to Rubisco-enhanced proteolysis and lower abundance of RUBISCO ACTIVASE. Water stress also affected ammonia assimilation into amino acids, as evidenced by the upregulation of glutamine synthetase and decreased levels of glutamic acid and asparagine in leaves. The effects of water stress were followed by elevated photorespiration (exemplified by increased abundances of photorespiratory enzymes), lower demand for carbohydrates, and accumulation of soluble sugars. In nodules, water deprivation caused the attenuation of respiration, leading to CO_2 recycling by PHOSPHOENOLPYRUVATE CARBOXYLASE. This likely occurred in order to support carbon skeletons for amino acid biosynthesis. The reduced respiration may also be a consequence of increased demand for compounds with osmoregulation capacity such as glycerol (Aranjuelo et al., 2013). The dynamic behavior of ammonia assimilation seems to be important for abiotic stress tolerance. It is likely that nitrogen is relocated from glutamic acid and asparagine, which are the main nitrogen sources in control conditions,

to proline under stress conditions. Thus, proline might be an alternative nitrogen source under osmotic stress, and it seems that alfalfa may easily switch between proline biosynthesis and degradation (Zhang and Shi, 2018). Abiotic stresses caused accumulation of enzymes of nitrogen assimilation, such as GLUTAMINE SYNTHETASE and FERREDOXIN-DEPENDENT GLUTAMATE SYNTHASE (Rahman et al., 2016) as well as GLUTAMATE DEHYDROGENASE (Dai et al., 2017). Remarkably, heat stress positively affected the abundance of ASPARTATE AMINOTRANSFERASE and GLUTAMINE SYNTHETASE, indicating an enhancement of nitrogen metabolism (Li W. et al., 2013).

Clearly, Rubisco availability and homeostasis between carbon and nitrogen metabolism is crucial for plant performance under unfavorable environmental conditions. For this reason, the proteins regulating C and N metabolism, as well as stress related proteins (**Table 1**), appear to be prospective candidates for the biotechnological improvement of alfalfa.

Proteins and Pathways Found by Proteomics as Promising Candidates for Alfalfa Abiotic Stress Resistance Improvement

Seed priming involves a complex array of physiological as well as molecular processes leading to an improved ability of plants to withstand adverse environment (Paparella et al., 2015). A gel-based proteomic approach was employed to investigate proteome remodeling during osmoprimed alfalfa seed germination. This process was accompanied by intense accumulation of storage proteins (such as vicilins), proteins involved in protein folding, UDP glucose and methionine biosynthesis, annexins, and antioxidant enzymes, compared to seeds that were not osmoprimed. Osmopriming was also followed by remarkable induction of stress-related proteins and proteasome components (**Table 1**) (Yacoubi et al., 2011). A follow-up article highlighted that osmopriming has remarkable consequences on the proteome of seeds germinating under saline conditions. An increased seed vigor associated with osmopriming was related to the accumulation of storage proteins, annexins and RNA-BINDING PROTEIN. The last one indicated the possible importance of posttranscriptional regulation in the seedlings exposed to salt stress. On the other hand, seeds without osmopriming accumulated HEAT SHOCK PROTEINS (HSP), LATE EMBRYOGENESIS ABUNDANT (LEA) PROTEINS, SEED MATURATION PROTEINS, GLUTATHIONE *S*-TRANSFERASE 9, and HEME OXIDASE (**Table 1**) (Yacoubi et al., 2013). These data indicate that the transient genetic modification of genes encoding the above-mentioned stress-related proteins (for instance, by expression under an inducible tissue-specific promoter), might be of biotechnological importance.

Tolerance of alfalfa to the polyethylene glycol (PEG)-induced osmotic stress was accompanied by enhanced carbohydrate metabolism and energy production. Stress-related proteins such as glutathione *S*-transferases and LEA proteins are also correlated with osmotic stress tolerance (**Table 1**) (Zhang and Shi, 2018), and both represent promising candidates for biotechnological applications. A similar study revealed that proteins involved in protein folding (DISULFIDE ISOMERASE), NAD production (NAD SYNTHASE), methylation (ADENOSINE KINASE, *S*-ADENOSYL-METHIONINE) and antioxidant defense (represented mainly by peroxidases), are candidates to determine alfalfa salt tolerance (Rahman et al., 2015). Overabundance of proteins involved in the enzymatic antioxidant defense was commonly associated with an increased tolerance of alfalfa not only to the salt, but also to the drought and osmotic stresses (**Table 1**) (Rahman et al., 2015; Long et al., 2018; Zhang and Shi, 2018). According to a proteomic study, water stress increased the abundance of AGAMOUS-LIKE 65 and bHLH TRANSCRIPTION FACTORS, while it reduced the abundance of JADE-1 and JADE-3, transcriptional regulators belonging to a PHD (plant homeodomain)-type zinc fingers family (**Table 1**) (Rahman et al., 2016). These intriguing findings of transcriptional factors involved in water stress deserve further biotechnological investigations. Genetic modifications of hormone biosynthesis belong also to promising biotechnological approaches, since water stress elevated the abundances of ABA (9-*CIS*-EPOXYCAROTENOID DIOXYGENASE) and auxin (AUXIN-INDEPENDENT GROWTH PROMOTER) biosynthetic proteins in alfalfa (Rahman et al., 2016). In this regard, local stress-induced changes in the turnover of auxin regulatory proteins could modify plant developmental processes, such as cell elongation, lateral roots emergence, transition from cell division to cell differentiation, enabling plants to rapidly adapt to adverse environmental conditions (Korver et al., 2018). On the other hand, drought stress caused some common but also distinct responses when compared to salt stress at the level of the alfalfa proteome. Interestingly, both stresses targeted proteasome complex and translation. Nevertheless, the proteasome complex exhibits different sensitivity to these stressors, since the abundance of 26S PROTEASOME REGULATORY SUBUNIT 6 was increased by drought but subsequently reduced by salt stress (Ma et al., 2017).

Comparative proteomic studies point out to obvious similarities between alfalfa and barrel medic in their response to environmental stimuli. Proteome-wide comparison of salt-tolerant alfalfa and salt-sensitive barrel medic indicated that both species are capable of keeping photosynthetic activity during salt stress. Only heat shock protein (gi357476131) was differentially regulated under salt stress in these two Medicago species. It was upregulated in alfalfa but downregulated in barrel medic (Long et al., 2016), indicating its potential biotechnological significance for salt tolerance. A proteomic analysis of these two species at the early post-germination stage showed an important role of antioxidant defense, cell wall metabolism, and jasmonic acid biosynthesis during response to salt (Long et al., 2018). Enhanced salt tolerance of alfalfa, compared to salt sensitive barrel medic, was reflected by higher numbers of differentially regulated proteins, also suggesting higher proteome plasticity (Long et al., 2016, 2018).

Differences in the composition of differentially abundant proteins between two alfalfa cultivars with contrasting freezing tolerance were reported after cold stress treatment (Chen et al.,

2015). Freezing-tolerant cultivar exhibited higher abundances of Rubisco subunits as compared to the freezing susceptible one, but showed downregulation of proteins involved in methionine, lignin and terpenoid biosynthesis, and energy metabolism under cold stress (Chen et al., 2015). Heat stress caused an upregulation of proteins involved in energy production, signaling, and intracellular transport and defense, including chaperones, antioxidant enzymes and PR proteins (Li W. et al., 2013). Interestingly, only prolonged heat stress caused downregulation of Rubisco and photosynthetic enzyme activities. Lower abundance of photosynthetic proteins was associated with altered abundance of proteins involved in plastid protein import.

It is known that the external application of bioactive molecules such as hydrogen (H_2; Jin et al., 2013) may remarkably increase plant survival rate under adverse environmental conditions. Proteomic elucidation of the beneficial effects of H_2 on the alfalfa response to cadmium revealed that this is mainly determined by the modification of proteins involved in the cellular redox homeostasis. Among these proteins, enzymes involved in cysteine biosynthesis and CYSTEINE DESULFURYLASE are elevated by external H_2. Cysteine is a precursor for GSH and hGSH, an important redox buffering compounds (Baldacci-Cresp et al., 2012; Diaz-Vivancos et al., 2015), hGSH is specifically produced in species of Fabaceae family including alfalfa, in higher rate compared to GSH, having important role in nodulation (Klapheck, 1988; Matamoros et al., 1999; Frendo et al., 2005; Baldacci-Cresp et al., 2012; Pasternak et al., 2014). Similarly, the abundance of CuZn SUPEROXIDE DISMUTASE (SOD) increased along with a positive effect of external H_2 treatment on alfalfa Cd tolerance. Gaseous H_2 also enhances the abundance of defense related proteins such as PATHOGENESIS-RELATED PROTEIN BET V I FAMILY PROTEIN and PATHOGENESIS-RELATED THAUMATIN FAMILY PROTEIN (Dai et al., 2017). Such induction of defense related proteins, including chitinases and enzymes involved in cell wall modification, was also observed in alfalfa stems and leaves exposed to long-term Cd stress (Gutsch et al., 2018a,b). Remarkably, chitinases are also employed in the alfalfa response to osmotic stress and waterlogging (**Table 1**) (Zhang and Shi, 2018; Zeng et al., 2019). This implies that genetic modification of cell walls might improve alfalfa tolerance to multiple stresses.

Proteins Implicated in Development-Associated Agronomical Traits

Proteomics has also been proven as valuable for the evaluation of metabolic activities during alfalfa stem development. The apical region characterized by fiber development showed an overabundance of proteins involved in chloroplast protein synthesis and carbon fixation. The mature stem part possessed a pool of proteins involved in redox homeostasis (Printz et al., 2015). Moreover, the stem is an organ highly sensitive to perturbations of mineral nutrition. This was highlighted by recent proteomic studies reporting that copper availability greatly influenced the abundance of proteins involved in cell wall biogenesis, and in pectin and lignin biosynthesis (Printz et al., 2016). Thus, mineral homeostasis seems to be a crucial factor affecting alfalfa stem growth and rigidity, and also eventually affecting drought tolerance and pathogen resistance.

Flowering represents a critical developmental stage in alfalfa, mainly in terms of seed yield and quality. Pollination and post-pollination processes in alfalfa are linked to altered homeostasis of stress-related proteins such as DUAL SPECIFICITY KINASE SPLA-LIKE PROTEIN, NADPH: QUINONE OXIDOREDUCTASE-LIKE PROTEIN, and CARBONIC ANHYDRASE (Chen et al., 2016). Moreover, PROTEIN DISULFIDE ISOMERASE-LIKE PROTEIN, ASCORBATE PEROXIDASE, GLUTAREDOXIN, and PEROXIREDOXINS also showed fluctuations in their abundances. In addition, metabolic activity was enhanced during pollination and declined afterward.

Fall dormancy is a crucial phenomenon influencing alfalfa performance in autumn, but also during the following season. Based on a comparative proteomic study of terminal buds isolated from two alfalfa cultivars with contrasting fall dormancy, several new proteins were discovered as important for this physiological process (Du et al., 2018). It was suggested that lower abundance of L-ASPARAGINASE and CINNAMYL ALCOHOL DEHYDROGENASES may contribute to fall dormancy. In addition, CHALCONE AND STILBENE SYNTHASE FAMILY PROTEIN (a protein involved in flavonoid biosynthesis) and GLUTAREDOXIN S17 seemed to be important for shoot apical meristem maintenance. Both proteins also have a role in polar auxin transport (**Table 1**) (Du et al., 2018).

Finally, the nutritional value of alfalfa depends on the developmental stage. Cutting of alfalfa in later developmental stages, such as in full flowering, leads to increased fiber and decreased protein content in the biomass (Fan et al., 2018). Combined proteomic and metabolomic analyses underpinned this finding and showed changes in amino acid composition. These unfeasible nutritional changes are accompanied by increased hemicellulose content, due to the accumulation of D-mannose and higher abundance of ALPHA GLUCOSIDASE, ALPHA AMYLASE, and UDP-GLUCURONIC ACID DECARBOXYLASE, as well as lignin, due to the higher levels of lignin precursors and proteins involved in lignin biosynthesis (**Table 1**) (Fan et al., 2018).

GENE EDITING USING TALEN AND CRISPR/Cas TECHNOLOGIES

The process of gene editing is based on sequence-specific nucleases (SSNs) creating *in vivo* loci-specific DNA double-stranded breaks (DSBs) that are subsequently repaired. There are two main DNA repair systems: homology-directed repair (HDR), and the more efficient but less precise non-homologous end joining (NHEJ). NHEJ can result in the insertion or deletion (indel) of nucleotides and a frameshift mutation, which can consequently create a premature stop codon, thus rendering the gene non-functional and creating a genetic knockout. Gene targeting technologies include meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases

TABLE 1 | Overview of proteins and metabolites important for biotechnological improvement of alfalfa as revealed by proteomic and metabolomic studies.

Treatment, stress, condition	Sample	Methodological approach	Proteins and metabolites of biotechnological importance	References
Seed germination and osmopriming	Seeds	2-D gel electrophoresis (nano-LC MS/MS)	**Carbohydrate metabolism:** UDP glucose pyrophosphorylase **Protein destination and storage:** HSP70 and HSP20, GroEL-like chaperone, ATPase, vicilin, protein disulfide-isomerase precursor **Stress response:** annexin, peroxiredoxins, manganese superoxide dismutase, glyoxalase, lipoxygenase, glutathione *S*-transferase, thioredoxin **Proteolysis:** peptidase T1A, proteasome beta subunit, peptidase A1 pepsin	Yacoubi et al. (2011)
Osmoprimed seeds germinating under salt stress	Seeds	2-D gel electrophoresis (nano-LC MS/MS)	**Small HSPs:** 18.2 kDa class I HSP **Methionine synthesis:** methionine synthase, cysteine synthase **Dehydration defense:** LEA proteins, PM22 **Others:** annexin, RNA-binding protein, heme oxygenase, glutathione *S*-transferase 9	Yacoubi et al. (2013)
PEG-induced osmotic stress	Roots of varieties contrasting in drought tolerance	iTRAQ (strong cation exchange fractionation and LC MS/MS)	**Stress and defense:** glutathione *S*-transferases, disease resistance response protein, epoxide hydrolase, chitinase, reticuline oxidase-like protein, low-temperature-induced 65 kDa protein, aldo/keto reductase, pirin-like plant protein, glucan endo-1,3-beta-glucosidase **Protein metabolism:** HSPs, lysine-ketoglutarate reductase/saccharopine dehydrogenase, phosphatidylethanolamine-binding protein, homoglutathione synthetase **Signal transduction:** monooxygenases, cysteine-rich RLK (receptor-like kinase) protein, 12-oxophytodienoate reductase **Cell wall:** beta xylosidase, xyloglucan-specific endoglucanase inhibitor protein, expansin-B1-like protein	Zhang and Shi (2018)
Salt stress	Roots of two cultivars contrasting in salt resistance	2-D gel electrophoresis (MALDI TOF/TOF)	**Oxidative stress:** peroxidase, peroxiredoxin **Protein folding:** protein disulfide isomerase **Metabolism:** NAD synthetase, UTP-glucose 1 phosphate uridylyltransferase **Fatty acid metabolism:** biotin carboxylase 3 **Membrane transport:** V-ATPase	Rahman et al. (2015)
Salt and drought stress	Seedlings	2-D gel electrophoresis (MALDI TOF-MS/MS)	**Salt stress:** caffeoyl-CoA 3-*O*-methyltransferase, peroxiredoxin, ubiquitin-conjugating enzyme, UV excision repair protein rad23, glutathione peroxidase **Drought stress:** ubiquitin-conjugating enzyme, putative alcohol dehydrogenase, chaperonin 10	Ma et al. (2017)
Drought stress	Leaves of plants inoculated by *S. meliloti*	Proteomics: 2-D gel electrophoresis (LCMS/MS analysis) Metabolomics: GC TOF-MS	**Rubisco availability and regeneration:** rubisco activase, sedoheptulose-1,7-bisphosphatase, ribulose-phosphate 3-epimerase and phosphoribulokinase **Nitrogen metabolism:** glutamine synthetase **Stress and defense response:** superoxide dismutase, dehydroascorbate reductase, 2-cys peroxiredoxin-like protein, 14-3-3-like protein **Osmoprotectant metabolites:** proline, pinitol	Aranjuelo et al. (2011)
Drought stress	Nodules, roots, leaves	Proteomics: 2-D gel electrophoresis (LCMS/MS analysis) Metabolomics: GC TOF-MS	**Nodule proteome:** alpha 1,4-glucan protein synthase, lipoxygenase, PEP-carboxylase **Nodule N containing metabolites:** glutamine, asparagine **Nodule osmoprotectant metabolites:** glycerol, galactinol, myo-inositol, proline, sucrose, raffinose, fumaric acid and malate **Nodule metabolites with antioxidant capacity:** ascorbate, threonate	Aranjuelo et al. (2013)
Water deficit stress	Roots	2-D gel electrophoresis (MALDI TOF)	**Nitrogen metabolism:** glutamine synthetase, ferredoxin-dependent glutamate synthase **ABA biosynthesis:** 9-*cis*-epoxycarotenoid dioxygenase **Stress response and oxidative stress:** ascorbate peroxidase, peroxiredoxin, calreticulin, stress-induced phosphoprotein, annexin **Transcription:** basic helix-loop-helix (bHLH) transcription factor, agamous-like 65 **Other functions:** inward-rectifying potassium channel, auxin-independent growth promoter	Rahman et al. (2016)
Heat stress	Leaves	2-D gel electrophoresis (MALDI TOF/TOF)	**Rubisco availability:** Rubisco activase isoforms **Nitrogen metabolism:** aspartate aminotransferase and glutamine synthetase **Protein synthesis and processing:** peptidyl-prolyl *cis–trans* isomerases, protein disulfide isomerase-like protein precursor, porin, proteasome subunit β type, eukaryotic translation initiation factor 3 subunit I, BiP isoform A/glycine max, cysteine proteinase, outer plastidial membrane protein porin **Intracellular traffic, cell structure:** protein TOC75, translocon Tic40, profilin	Li W. et al. (2013)

(Continued)

TABLE 1 | Continued

Treatment, stress, condition	Sample	Methodological approach	Proteins and metabolites of biotechnological importance	Reference
			Defense response: 17 kDa HSP, 18.2 kDa class I HSP, 20 kDa chaperonin, HSP23, HSP70, thaumatin-like protein, ubiquitin, ascorbate peroxidases, glucan endo-1,3-beta-glucosidase	
Cold acclimation	Leaves of cultivars tolerant or sensitive to freezing	2-D gel electrophoresis (MALDI TOF/TOF)	**Oxidative stress:** monodehydroascorbate reductase, glutathione peroxidase, peptide methionine sulfoxide reductases A3, thioredoxin-like protein CDSP32, 2-cys peroxiredoxin BAS1-like	Chen et al. (2015)
			Methionine biosynthesis: 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	
			Lignin and terpenoid biosynthesis: cinnamoyl-CoA reductase, 1-deoxy-D-xylulose 5-phosphate reductoisomerase	
			Photosynthesis and Rubisco availability: Rubisco large subunit-binding protein subunit beta, Rubisco activase B, chlorophyll A/B binding protein, oxygen-evolving enhancer protein 1, cytochrome b6-f complex iron-sulfur subunit	
			Protein folding and disassembling: chaperone protein ClpC, GTPase, peptidyl-prolyl *cis–trans* isomerase CYP20-3	
Cadmium stress	Cell walls and soluble proteins from stems	2-D DIGE (MALDI TOF/TOF)	**Cell wall modification:** sucrose synthase, pectinesterase/pectinesterase inhibitor, polygalacturonase non-catalytic protein, polygalacturonase-inhibiting protein 1, b-xylosidase/alpha-L-arabinofuranosidase, trichome birefringence-like protein, xyloglucan endotransglucosylase/hydrolase family protein, dirigent protein 21-like	Gutsch et al. (2018a)
			Defense: chitinase (Class Ib)/hevein, chitinase, class I chitinase, disease resistance response protein, pathogenesis-related protein 1, pathogenesis-related thaumatin family protein, plant basic secretory protein (BSP) family protein, pre-hevein-like protein, stromal 70 kDa heat shock-related protein, CAP, cysteine-rich secretory protein, antigen 5	
			Oxidation-reduction process: anionic peroxidase swpb3 protein, class III peroxidase, peroxidase family protein, peroxidase1b, peroxidase2	
Cadmium stress	Stems (soluble and cell wall enriched proteins)	2-D DIGE (MALDI TOF/TOF)	**Cell wall modification:** pectinesterase/pectinesterase inhibitor, polygalacturonase non-catalytic protein, polygalacturonase-inhibiting protein 1 **Chloroplast protein degradation:** chloroplastic aspartyl protease isoforms **Cell wall:** class III peroxidase, lignin biosynthetic peroxidase, chitinases	Gutsch et al. (2018b)
Stem growth	Different regions of stems (apical, intermediate, and basal)	2-D gel electrophoresis (MALDI TOF/TOF)	**Chloroplast protein synthesis:** CSP41-b, EF-Tu, EF-G, Cpn 60, HSP70 **Lignin biosynthesis:** transketolase, enolase **Cytoplasmic protein synthesis:** eIF-5a, endoplasmic protein disulfide isomerase, HSP90, ribosomal protein P3-like	Printz et al. (2015)
			Vesicular trafficking: clathrin light chain	
			Stress response: peroxisomal membrane protein, monodehydroascorbate reductase, flavoprotein wrbA-like, Pprg2	
			Sieve element development: sieve element occlusion by forisomes 3	
Cadmium stress and hydrogen-rich water	Roots	iTRAQ (nano-LC MS/MS)	**Defense response:** mitogen-activated protein kinase, pathogenesis-related thaumatin family protein, pathogenesis-related protein bet V I family protein, disease-resistance response protein **Nitrogen metabolism:** glutamate dehydrogenase **Sulfur compound metabolic process:** cysteine synthase, ATP sulfurylase	Dai et al. (2017)
			Secondary metabolism: chalcone-flavonone isomerase family protein	
Waterlogging	Leaves of two cultivars contrasting in tolerance to waterlogging	iTRAQ (reverse-phase HPLC fractionation and LC-MS/MS)	**Cell wall and defense response:** acidic endochitinase, expansin-like B1, early nodulin-like protein 2, thaumatin-like protein, 1,4 alpha-glucan-branching enzyme 1, pathogenesis-related protein **Stress response:** glutathione *S*-transferase, protein C2-DOMAIN ABA-RELATED 9, aldo-keto reductase family 4 member C9, Fe superoxide dismutase 2, 1 aminocyclopropane-1-carboxylate oxidase homolog 5,	Zeng et al. (2019)
			Proteolysis: vacuolar-processing enzyme	
Different developmental stages (budding and mid-flowering)	Leaves	TMT labeling (nano-LC MS/MS)	**Metabolites:** D-mannose hemicellulose precursor (upregulated in mid flowering), L-phenylalanine, L-tyrosine, L-phenylalanine **Metabolism:** alpha glucosidase, alpha amylase **Cell wall modification:** UDP-glucuronic acid decarboxylase (xylan production), cinnamyl alcohol dehydrogenase (lignin biosynthesis)	Fan et al. (2018)
Fall dormancy	Terminal buds of fall dormant and non-fall dormant cultivars	iTRAQ (SCX fractionation, LC MS/MS)	**Nitrogen metabolism:** L-asparaginase **Auxin polar transport:** stilbene synthase family protein, monothiol glutaredoxin-S17 protein **Lignin biosynthesis:** cinnamyl alcohol dehydrogenase **Pyruvate metabolism and transport:** pyruvate carrier protein	Du et al. (2018)
			Vitamin B1 metabolism: thiazole biosynthetic enzyme	

(TAL effector nucleases or TALENs), and clustered regularly interspaced short palindromic repeat/CRISPR–associated protein 9 (CRISPR/Cas9). Among these, TALENs and CRISPR/Cas9 are the preferred SSNs for research purposes (Kanaar et al., 1998; Pastwa and Blasiak, 2003; Smith et al., 2006; Pâques and Duchateau, 2007; Hartlerode and Scully, 2009; Sander et al., 2011; Qi, 2015; Steinert et al., 2016; Malzahn et al., 2017; Shan et al., 2020).

The history of gene targeting technologies started in 1988 when the first gene-targeting experiment was performed on tobacco (*Nicotiana tabacum*) protoplasts (Paszkowski et al., 1988). Later, Puchta et al. (1993) discovered that gene-targeting efficiency can be improved by DSBs in plant cells. More than a decade later, ZFNs were adapted in tobacco and were used in a few plant species for trait improvement (Wright et al., 2005). Subsequently, TALENs were introduced into the group of plant genome editing technologies (Christian et al., 2010). Finally, CRISPR/Cas9 technology has been used in plants such as *Arabidopsis thaliana*, *N. benthamiana*, *Oryza sativa*, and *T. aestivum* (Li J. F. et al., 2013; Nekrasov et al., 2013; Shan et al., 2013, 2020).

TALENs

TALENs are created by the fusion of DNA binding TALE repeats to the Fok1 nuclease domain. TALENs are less toxic and are easier to engineer than ZFNs. Each of these two platforms has unique limitations, and they are not routinely used in plants. The main advantages of TALENs over CRISPR are that they have less off-target effects due to their ∼30 bp target requirement, as well as their lack of PAM requirement, as unlike CRISPR, TALENs are able to target any sequence. On the other hand, TALENs have more disadvantages: an increased time and financial investment due to the difficulty in protein engineering, a highly variable efficiency for each construct, an inability to target methylated DNA, and the difficulties in engineering nickase (Christian et al., 2010; Li et al., 2011; Mahfouz et al., 2011; Miller et al., 2011; Malzahn et al., 2017; Chen et al., 2019). So far, a successful application of TALEN technology has not been published for either alfalfa or barrel medic. Nevertheless, TALENs have been used for the targeted mutagenesis of another legume, namely soybean (Haun et al., 2014; Demorest et al., 2016; Du et al., 2016; Curtin et al., 2018). The use of TALENs for the mutagenesis of higher plants was recently reviewed by Malzahn et al. (2017) and Khan et al. (2017).

CRISPR/Cas9

In bacteria and archaea, CRISPR and Cas9 function together against invading phages, plasmids, and viruses in adaptive immune system by cleaving the invader's nucleic acids. The first component is single guide RNA (sgRNA) that associates with a Cas9 protein a Cas9/sgRNA complex. The second component Cas9 belongs to the single-protein effectors of Class 2 CRISPR-Cas systems and is composed of two endonuclease domains, namely, the RuvC-like domain and the HNH, each cutting one strand of DNA. The CRISPR/Cas9 constituents can be transformed into plant cells by different strategies, including *Agrobacterium*-mediated delivery, gene gun (biolistic delivery), or using virus-based guide RNA (gRNA). Out of the primary SSN classes, CRISPR/Cas9 technology has been the most used and adopted in recent years (Barrangou et al., 2007; Marraffini and Sontheimer, 2008; Wiedenheft et al., 2012; Graham and Root, 2015; Schiml and Puchta, 2016; Makarova et al., 2017; Malzahn et al., 2017; Chen et al., 2019). The CRISPR/Cas system has the potential for numerous applications, such as fusing dCas9 (deactivated Cas9) with other proteins, which can be used for DNA imaging, epigenome editing, gene regulation, and genomic labeling (Chen et al., 2019). One of the main limitations of CRISPR/Cas9 technology might be the generation of undesired off-target effects. Nevertheless, whole-genome sequencing revealed very limited off-target effect mutations in *Arabidopsis* (Feng et al., 2013), rice (Zhang H. et al., 2014; Tang et al., 2018), and tomato (Nekrasov et al., 2017). Using software tools such as CRISPR-P (Liu H. et al., 2017) and CRISPRGE (Xie et al., 2017) can further decrease any potential off-target occurrence by designing highly specific guide RNAs. Finally, breeding processes may remove any off-target mutations that have negative effects and may keep positive or neutral off-target mutations (Mao et al., 2019).

CRISPR/Cas9 in Alfalfa

CRISPR/Cas9 technology was very recently used for targeted mutagenesis in alfalfa. Selected *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9* (*SPL9*) gene was successfully mutagenized and transgenic lines were pre-selected by using droplet digital PCR (ddPCR) for high-throughput screening of large populations. It was further confirmed by restriction enzyme digestion after PCR amplification and sequencing of sub-clones. Comparison of editing efficiency with available data on barrel medic showed lower efficiency in alfalfa, which might be related to its tetraploid genome possessing highly repeated clusters (Meng et al., 2017, 2019; Gao et al., 2018). Gao et al. (2018) concluded that CRISPR/Cas9-mediated modifications of tetraploid alfalfa genome have been successfully performed, but there is still a need to improve editing efficiency. Alfalfa plants with silenced *SPL9* had no visible phenotype so ddPCR-based estimation of concentration of the event per μl was a direct indicator of the genome editing rate. Sequencing analysis showed no off-target effects in the alfalfa genome and proved that the sgRNAs of *SPL9* were highly specific to the recognition site. In other legumes such as barrel medic, CRISPR/Cas9 technology has been used as well (Michno et al., 2015; Meng et al., 2017, 2019; Curtin et al., 2018; Wen et al., 2019; Yin et al., 2020). Recently, Meng et al. (2019) developed an optimized *Agrobacterium*-dependent CRISPR/Cas9 system and successfully edited an endogenous *PHYTOENE DESATURASE* (*MtPDS)* gene. CRISPR/Cas9 technology for the mutagenesis was also used in *L. japonicus* (Wang et al., 2016, 2019), and *G. max* (Cai et al., 2015; Jacobs et al., 2015; Li et al., 2015; Sun et al., 2015; Du et al., 2016; Tang et al., 2016; Curtin et al., 2018; Bao et al., 2019; Wang et al., 2020). Utilization of CRISPR/Cas9-based mutagenesis in several non-leguminous plant species, including data on delivery method, integration into the genome, and editing efficiency, has been reviewed recently (Belhaj et al., 2013; Jaganathan et al., 2018; Liu X. et al., 2019; Kuluev et al., 2019; Mao et al., 2019;

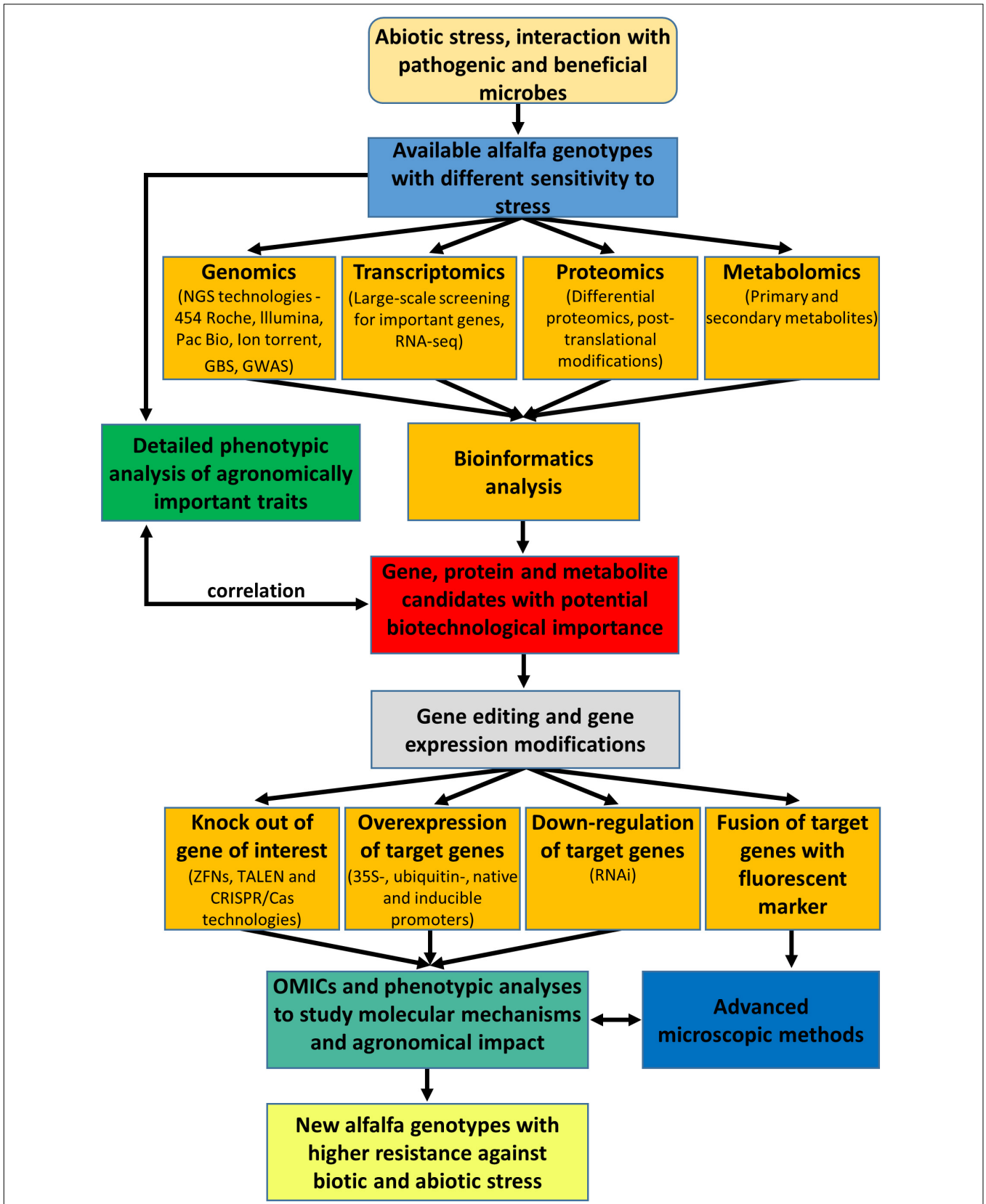

FIGURE 1 | Overview and integration of omics and molecular genetics approaches aiming to improve agronomic traits and performance of alfalfa.

Moradpour and Abdulah, 2020; Shan et al., 2020). Approaches such as transgene integration and gene stacking developed for diploid crop species (e.g., corn, cotton, soybean) might be less suitable for alfalfa due to its auto-tetraploid character (Kumar et al., 2018), but the CRISPR/Cas9 technology seems to work well.

PHOSPHORYLATION-DEPENDENT POST-TRANSLATIONAL MODIFICATION BY MAPKs

Multiple abiotic stress stimuli, such as wounding, cold, salinity, or drought, are perceived by plants through the activation of MAPKs (Šamajová et al., 2013b). Activated MAPKs phosphorylate, and thereby regulate, several intracellular targets including other protein kinases, cytoskeletal components, nuclear transcription factors, and proteins involved in vesicular trafficking (Komis et al., 2011; Šamajová et al., 2013a). In alfalfa, STRESS-INDUCED MAPK (SIMK), was identified as a salt- and elicitor- stress induced MAPK (Cardinale et al., 2002). SIMK in response to salt stress is specifically activated by upstream STRESS-INDUCED MAPKK (SIMKK; Kiegerl et al., 2000; Bekešová et al., 2015). SIMK is localized to nuclei and cytoplasm of root cells, while in developing root hairs it relocated from the nucleus to the growing tip (Šamaj et al., 2002). Moreover, stimulus-dependent activation and the subsequent subcellular relocation of both SIMK and its upstream SIMKK were induced by salt stress (Ovečka et al., 2014). Such activity-dependent and coordinated relocation of SIMK-SIMKK module from the nucleus to cytoplasm under salt stress were observed in alfalfa and thale cress. Transgenic thale cress plants stably producing SIMKK-YFP exhibited enhanced MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3) and MITOGEN-ACTIVATED PROTEIN KINASE 6 (MPK6) activation and conferred altered sensitivity to salt stress. These data suggested that SIMKK may serve as a negative regulator of the salt stress response in alfalfa (Ovečka et al., 2014).

CONCLUSION AND PERSPECTIVES

Alfalfa is a perennial, cross-pollinated, autotetraploid ($2n = 4x = 32$) plant with genome size of 800–900 Mbp. It is often mentioned as the "queen of forages" due to the very high production potential as hay, silage or as a biofuel feedstock for ethanol production (Blondon et al., 1994). However, tetraploid nature made understanding and improving of alfalfa by traditional breeding methods rather challenging. Therefore, the use of modern biotechnological, omics and genetic engineering approaches for alfalfa improvement is highly actual and desirable task for crop researchers.

This review provides an overview of the biotechnological potential of alfalfa based on the integration of various omics and molecular tools as depicted in the **Figure 1**. Recent advances in high-throughput sequencing technology have opened another scientific boundary, and many species, including economically important crops, have been subjected to whole-genome sequencing by *de novo* assembly and resequencing. Several novel genes have been identified owing to whole-genome duplications and structural variations in chromosomes (Van et al., 2013). Since plant responses to stresses are often very specific, proteomic and transcriptomic approaches should be targeted to individual cell types and tissues at different developmental stages. Such approach was already reported for root hairs and root border cells of barrel medic (Breakspear et al., 2014; Watson et al., 2015). In this respect, the integration of fast-developing omics methods and bioinformatics into systems biology at the single cell level might bring new opportunities to improve plant stress tolerance (Libault et al., 2017).

Biotechnological approaches provide a great potential to increase crop production for the constantly growing global population. Introducing tolerance to environmental abiotic and biotic stresses is crucial for improving the productivity of crop legumes (Farooq et al., 2017). Extensive research conducted on alfalfa stress tolerance suggests that it is able to cope with abiotic stresses using general mechanisms such as antioxidant defense, protein folding, and cell wall remodeling. Research in the field of alfalfa biotechnology also aimed to identify genes involved in the energy production pathway or in enhancing environmental tolerance (Pennycooke et al., 2008; Aranjuelo et al., 2011; Mo et al., 2011). Scientists grew alfalfa plants under different conditions in order to analyze gene expression profiles and to identify crucial genes and proteins, as well as to understand global correlations between genes, proteins, and metabolites using omics approaches.

The potentials of these methods have only partially been exploited in alfalfa research. Continued research toward the development of alfalfa proteome studies (Komatsu and Ahsan, 2009) should permit the rapid comparison of alfalfa cultivars, mutants, and transgenic lines.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. MH, PD, TT, MT, and IL drafted the review which was coordinated by OŠ, MO, and finally edited by JŠ.

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REFERENCES

Abdelrahman, M., El-Sayed, M., Sato, S., Hirakawa, H., Ito, S. I., Tanaka, K., et al. (2017a). RNA-sequencing-based transcriptome and biochemical analyses of steroidal saponin pathway in a complete set of *Allium fistulosum*-A. cepa monosomic addition lines. *PLoS One* 12:e0181784. doi: 10.1371/journal.pone.0181784

Abdelrahman, M., Suzumura, N., Mitoma, M., Matsuo, S., Ikeuchi, T., Mori, M., et al. (2017b). Comparative de novo transcriptome profiles in *Asparagus officinalis* and A. kiusianus during the early stage of *Phomopsis asparagi* infection. *Sci. Rep.* 7:2608. doi: 10.1038/s41598-017-02566-7

Abdelrahman, M., Jogaiah, S., Burritt, D. J., and Tran, L. S. P. (2018). Legume genetic resources and transcriptome dynamics under abiotic stress conditions. *Plant Cell Environ.* 41, 1972–1983. doi: 10.1111/pce.13123

Abdelrahman, M., Sawada, Y., Nakabayashi, R., Sato, S., Hirakawa, H., El-Sayed, M., et al. (2015). Integrating transcriptome and target metabolome variability in doubled haploids of *Allium cepa* for abiotic stress protection. *Mol. Breed.* 35:195. doi: 10.1007/s11032-015-0378-2

An, Y. M., Song, L. L., Liu, Y. R., Shu, Y. J., and Guo, C. H. (2016). De novo transcriptional analysis of alfalfa in response to saline-alkaline stress. *Front. Plant Sci.* 7:931. doi: 10.3389/fpls.2016.00931

Annicchiarico, P., Barrett, B., Brummer, E. C., Julier, B., and Marshall, A. H. (2015). Achievements and challenges in improving temperate perennial forage legumes. *Crit. Rev. Plant Sci.* 34, 327–380. doi: 10.1080/07352689.2014.898462

Annicchiarico, P., Nazzicari, N., and Brummer, E. C. (2016). ""Alfalfa genomic selection: challenges, strategies, transnational cooperation"," in *Breeding in a World of Scarcity*, eds I. Roldán-Ruiz, J. Baert, and D. Reheul (Cham: Springer), 145–149. doi: 10.1007/978-3-319-28932-8_22

Aranjuelo, I., Molero, G., Erice, G., Avice, J. C., and Nogués, S. (2011). Plant physiology and proteomics reveals the leaf response to drought in alfalfa (*Medicago sativa* L.). *J. Exp. Bot.* 62, 111–123. doi: 10.1093/jxb/erq249

Aranjuelo, I., Perez, P., Hernandez, L., Irigoyen, J. J., Zita, G., Martinez-Carrasco, R., et al. (2005). The response of nodulated alfalfa to water supply, temperature and elevated CO2: photosynthetic downregulation. *Physiol. Plant.* 123, 348–358. doi: 10.1111/j.1399-3054.2005.00459.x

Aranjuelo, I., Tcherkez, G., Molero, G., Gilard, F., Avice, J.-C., and Nogués, S. (2013). Concerted changes in N and C primary metabolism in alfalfa (*Medicago sativa*) under water restriction. *J. Exp. Bot.* 64, 885–897. doi: 10.1093/jxb/ers367

Ari, Ş, and Arikan, M. (2016). "Next-generation sequencing: advantages, disadvantages, and future," in *Plant Omics: Trends and Applications*, eds K. Hakeem, H. Tombuloglu, and G. Tombuloglu (Cham: Springer), 109–135. doi: 10.1007/978-3-319-31703-8_5

Arshad, M., Feyissa, B. A., Amyot, L., Aung, B., and Hannoufa, A. (2017). MicroRNA156 improves drought stress tolerance in alfalfa (*Medicago sativa*) by silencing SPL13. *Plant Sci.* 258, 122–136. doi: 10.1016/j.plantsci.2017.01.018

Arshad, M., Gruber, M. Y., and Hannoufa, A. (2018). Transcriptome analysis of microRNA156 overexpression alfalfa roots under drought stress. *Sci. Rep.* 8:9363. doi: 10.1038/s41598-018-27088-8

Asamizu, E., Nakamura, Y., Sato, S., and Tabata, S. (2004). Characteristics of the Lotus japonicus gene repertoire deduced from large-scale expressed sequence tag (EST) analysis. *Plant Mol. Biol.* 54, 405–414. doi: 10.1023/B:PLAN.0000036372.46942.b8

Aung, B., Gao, R., Gruber, M. Y., Yuan, Z. C., Sumarah, M., and Hannoufa, A. (2017). MsmiR156 affects global gene expression and promotes root regenerative capacity and nitrogen fixation activity in alfalfa. *Transgenic Res.* 26, 541–557. doi: 10.1007/s11248-017-0024-3

Aung, B., Gruber, M. Y., Amyot, L., Omari, K., Bertrand, A., and Hannoufa, A. (2015). Micro RNA 156 as a promising tool for alfalfa improvement. *Plant Biotechnol. J.* 13, 779–790. doi: 10.1111/pbi.12308

Aziz, N., Paiva, N. L., May, G. D., and Dixon, R. A. (2005). Transcriptome analysis of alfalfa glandular trichomes. *Planta* 221, 28–38. doi: 10.1007/s00425-004-1424-1

Bahramnejad, B., Goodwin, P. H., Zhang, J., Atnaseo, C., and Erickson, L. R. (2010). A comparison of two class 10 pathogenesis-related genes from alfalfa and their activation by multiple stresses and stress-related signaling molecules. *Plant Cell Rep.* 29, 1235–1250. doi: 10.1007/s00299-010-0909-6

Baldacci-Cresp, F., Chang, C., Maucourt, M., Deborde, C., Hopkins, J., Lecomte, P., et al. (2012). Homoglutathione deficiency impairs root-knot nematode development in *Medicago truncatula*. *PLoS Pathog.* 8:2471. doi: 10.1371/journal.ppat.1002471

Bao, A., Chen, H., Chen, L., Chen, S., Hao, Q., Guo, W., et al. (2019). CRISPR/Cas9-mediated targeted mutagenesis of *GmSPL9* genes alters plant architecture in soybean. *BMC Plant Biol.* 19:131. doi: 10.1186/s12870-019-1746-6

Barabaschi, D., Guerra, D., Lacrima, K., Laino, P., Michelotti, V., Urso, S., et al. (2012). Emerging knowledge from genome sequencing of crop species. *Mol. Biotech.* 50, 250–266. doi: 10.1007/s12033-011-9443-1

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., et al. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712. doi: 10.1126/science.1138140

Barros, J., Temple, S., and Dixon, R. A. (2019). Development and commercialization of reduced lignin alfalfa. *Curr. Opin. Biotech.* 56, 48–54. doi: 10.1016/j.copbio.2018.09.003

Bekešová, S., Komis, G., Křenek, P., Vyplelová, P., Ovečka, M., Luptovčiak, I., et al. (2015). Monitoring protein phosphorylation by acrylamide pendant Phos-TagTM in various plants. *Front. Plant Sci.* 6:336. doi: 10.3389/fpls.2015.00336

Belhaj, K., Chaparro-Garcia, A., Kamoun, S., and Nekrasov, V. (2013). Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. *Plant Methods* 9:39. doi: 10.1186/1746-4811-9-39

Bevan, M. W., Uauy, C., Wulff, B. B., Zhou, J., Krasileva, K., and Clark, M. D. (2017). Genomic innovation for crop improvement. *Nature* 543, 346–354. doi: 10.1038/nature22011

Biazzi, E., Nazzicari, N., Pecetti, L., Brummer, E. C., Palmonari, A., Tava, A., et al. (2017). Genome-wide association mapping and genomic selection for alfalfa (*Medicago sativa*) forage quality traits. *PLoS One* 12:e0169234. doi: 10.1371/journal.pone.0169234

Blondon, F., Marie, D., Brown, S., and Kondorosi, A. (1994). Genome size and base composition in *Medicago sativa* and M. *truncatula species*. *Genome* 37, 264–270. doi: 10.1139/g94-037

Bohnert, H. J., and Jensen, R. G. (1996). Strategies for engineering water-stress tolerance in plants. *Trends Biotechnol.* 14, 89–97. doi: 10.1016/0167-7799(96)80929-2

Bora, K. S., and Sharma, A. (2011). Phytochemical and pharmacological potential of *Medicago sativa*: a review. *Pharm. Biol.* 49, 211–220. doi: 10.3109/13880209.2010.504732

Borsics, T., and Lados, M. (2002). Dodder infection induces the expression of a pathogenesis-related gene of the family PR-10 in alfalfa. *J. Exp. Bot.* 53, 1831–1832. doi: 10.1093/jxb/erf039

Breakspear, A., Liu, C., Roy, S., Stacey, N., Rogers, C., Trick, M., et al. (2014). The root hair "infectome" of *Medicago truncatula* uncovers changes in cell cycle genes and reveals a requirement for auxin signaling in rhizobial infection. *Plant Cell* 26, 4680–4701. doi: 10.1105/tpc.114.133496

Brouwer, D. J., and Osborn, T. C. (1999). A molecular marker linkage map of tetraploid alfalfa (*Medicago sativa* L.). *Theor. Appl. Gen.* 99, 1194–1200. doi: 10.1007/s001220051324

Brummer, E. C., Bouton, J. H., and Kochert, G. (1993). Development of an RFLP map in diploid alfalfa. *Theor. Appl. Gen.* 86, 329–332. doi: 10.1007/BF00222097

Budak, H., Kantar, M., Bulut, R., and Akpinar, B. A. (2015). Stress responsive miRNAs and isomiRs in cereals. *Plant Sci.* 235, 1–13. doi: 10.1016/j.plantsci.2015.02.008

Bustos-Sanmamed, P., Mao, G., Deng, Y., Elouet, M., Khan, G. A., Bazin, J., et al. (2013). Overexpression of miR160 affects root growth and nitrogen-fixing nodule number in *Medicago truncatula*. *Funct. Plant Biol.* 40, 1208–1220. doi: 10.1071/FP13123

Cai, Y., Chen, L., Liu, X., Sun, S., Wu, C., Jiang, B., et al. (2015). CRISPR/Cas9-mediated genome editing in soybean hairy roots. *PLoS One* 10:e0136064. doi: 10.1371/journal.pone.0136064

Cardinale, F., Meskiene, I., Ouaked, F., and Hirt, H. (2002). Convergence and divergence of stress-induced mitogen-activated protein kinase signaling pathways at the level of two distinct mitogen-activated protein kinase kinases. *Plant Cell* 14, 703–711. doi: 10.1105/tpc.010256

Carter, P. R., and Sheaffer, C. C. (1983). Alfalfa response to soil water deficits. III. Nodulation and N2 fixation. *Crop Sci.* 23, 985–990. doi: 10.2135/cropsci1983.0011183X002300050041x

Chao, Y., Yuan, J., Guo, T., Xu, L., Mu, Z., and Han, L. (2019). Analysis of transcripts and splice isoforms in *Medicago sativa* L. by single-molecule

long-read sequencing. *Plant Mol. Biol.* 99, 219–235. doi: 10.1007/s11103-018-0813-y

Chen, J., Han, G., Shang, C., Li, J., Zhang, H., Liu, F., et al. (2015). Proteomic analyses reveal differences in cold acclimation mechanisms in freezing-tolerant and freezing-sensitive cultivars of alfalfa. *Front. Plant Sci.* 6:105. doi: 10.3389/fpls.2015.00105

Chen, K., Wang, Y., Zhang, R., Zhang, H., and Gao, C. (2019). CRISPR/Cas genome editing and precision plant breeding in agriculture. *Annu. Rev. Plant Biol.* 70, 667–697. doi: 10.1146/annurev-arplant-050718-100049

Chen, L., Chen, Q., Zhu, Y., Hou, L., and Mao, P. (2016). Proteomic identification of differentially expressed proteins during alfalfa (*Medicago sativa* L.) flower development. *Front. Plant Sci.* 7:1502. doi: 10.3389/fpls.2016.01502

Chen, T. H., and Murata, N. (2002). Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.* 5, 250–257. doi: 10.1016/s1369-5266(02)00255-8

Chen, T. H., and Murata, N. (2008). Glycinebetaine: an effective protectant against abiotic stress in plants. *Trends Plant Sci.* 13, 499–505. doi: 10.1016/j.tplants.2008.06.007

Cheung, F., Haas, B. J., Goldberg, S. M. D., May, G. D., Xiao, Y., and Town, C. D. (2006). Sequencing *Medicago truncatula* expressed sequenced tags using 454 Life Sciences technology. *BMC Genom.* 7:272. doi: 10.1186/1471-2164-7-272

Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., et al. (2010). Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186, 757–761. doi: 10.1534/genetics.110.120717

Curtin, S. J., Xiong, Y., Michno, J. M., Campbell, B. W., Stec, A. O., Čermák, T., et al. (2018). CRISPR/Cas9 and TALENs generate heritable mutations for genes involved in small RNA processing of Glycine max and *Medicago truncatula*. *Plant Biotech. J.* 16, 1125–1137. doi: 10.1111/pbi.12857

Dai, C., Cui, W., Pan, J., Xie, Y., Wang, J., and Shen, W. (2017). Proteomic analysis provides insights into the molecular bases of hydrogen gas-induced cadmium resistance in *Medicago sativa*. *J. Proteom.* 152, 109–120. doi: 10.1016/j.jprot.2016.10.013

de Zélicourt, A., Diet, A., Marion, J., Laffont, C., Ariel, F., Moison, M., et al. (2011). Dual involvement of a *Medicago truncatula* NAC transcription factor in root abiotic stress response and symbiotic nodule senescence. *Plant J.* 70, 220–230. doi: 10.1111/j.1365-313X.2011.04859.x

Demorest, Z. L., Coffman, A., Baltes, N. J., Stoddard, T. J., Clasen, B. M., Luo, S., et al. (2016). Direct stacking of sequence-specific nuclease-induced mutations to produce high oleic and low linolenic soybean oil. *BMC Plant Biol.* 16:225. doi: 10.1186/s12870-016-0906-1

Deokar, A. A., Kondawar, V., Jain, P. K., Karuppayil, S. M., Raju, N. L., Vadez, V., et al. (2011). Comparative analysis of expressed sequence tags (ESTs) between drought-tolerant and -susceptible genotypes of chickpea under terminal drought stress. *BMC Plant Biol.* 11:70. doi: 10.1186/1471-2229-11-70

Diaz-Vivancos, P., de Simone, A., Kiddle, G., and Foyer, C. H. (2015). Glutathione–linking cell proliferation to oxidative stress. *Free Radical Biol. Med.* 89, 1154–1164. doi: 10.1016/j.freeradbiomed.2015.09.023

Dong, L., Liu, H., Zhang, J., Yang, S., Kong, G., Chu, J. S., et al. (2015). Single-molecule real-time transcript sequencing facilitates common wheat genome annotation and grain transcriptome research. *BMC Genom.* 16:1039. doi: 10.1186/s12864-015-2257-y

Doyle, J. J., and Luckow, M. A. (2003). The rest of the iceberg. Legume diversity and evolution in a phylogenetic context. *Plant Physiol.* 131, 900–910. doi: 10.1104/pp.102.018150

Du, H., Shi, Y., Li, D., Fan, W., Wang, Y., Wang, G., et al. (2018). Proteomics reveals key proteins participating in growth difference between fall dormant and non-dormant alfalfa in terminal buds. *J. Proteom.* 173, 126–138. doi: 10.1016/j.jprot.2017.11.029

Du, H., Zeng, X., Zhao, M., Cui, X., Wang, Q., Yang, H., et al. (2016). Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. *J. Biotechnol.* 217, 90–97. doi: 10.1016/j.jbiotec.2015.11.005

Ebert, J. (2007). Alfalfa's bioenergy appeal. *Ethanol Prod. Mag.* 88–94.

Echt, C. S., Kidwell, K. K., Knapp, S. J., Osborn, T. C., and McCoy, T. J. (1994). Linkage mapping in diploid alfalfa (*Medicago sativa*). *Genome* 37, 61–71. doi: 10.1139/g94-008

Edwards, D., and Batley, J. (2010). Plant genome sequencing: applications for crop improvement. *Plant Biotechnol. J.* 8, 2–9. doi: 10.1111/j.1467-7652.2009.00459.x

Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., et al. (2009). Real-time DNA sequencing from single polymerase molecules. *Science* 323, 133–138. doi: 10.1126/science

Elgin, J. H. Jr., and Ostazeski, S. A. (1985). Inheritance of resistance to race 1 and race 2 anthracnose in Arc and Saranac AR alfalfa. *Crop Sci.* 25, 861–865. doi: 10.2135/cropsci1985.0011183X002500050032x

Elgin, J. H. Jr., Welty, R. E., and Gilchrist, D. B. (1988). Breeding for disease and nematode resistance. *Alfalfa Alfalfa Impr.* 29, 827–858. doi: 10.2134/agronmonogr29.c27

Esnault, R., Buffard, D., Breda, C., Sallaud, C., Turk, J., and Kondorosi, A. (1993). Pathological and molecular characterizations of alfalfa interactions with compatible and incompatible bacteria, Xanthomonas campestris pv. alfalfae and *Pseudomonas* syringae pv. pisi. *Mol. Plant Microbe Interact.* 6, 655–664. doi: 10.1094/MPMI-6-655

Fan, W., Ge, G., Liu, Y., Wang, W., Liu, L., and Jia, Y. (2018). Proteomics integrated with metabolomics: analysis of the internal causes of nutrient changes in alfalfa at different growth stages. *BMC Plant Biol.* 18:78. doi: 10.1186/s12870-018-1291-8

Farooq, M., Gogoi, N., Hussain, M., Barthakur, S., Paul, S., Bharadwaj, N., et al. (2017). Effects, tolerance mechanisms and management of salt stress in grain legumes. *Plant Physiol. Biochem.* 118, 199–217. doi: 10.1105/10.1016/j.plaphy.2017.06.020

Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D. L., Wei, P., et al. (2013). Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res.* 23, 1229–1232. doi: 10.1038/cr.2013.114

Feyissa, B. A., Arshad, M., Gruber, M. Y., Kohalmi, S. E., and Hannoufa, A. (2019). The interplay between miR156/SPL13 and DFR/WD40–1 regulate drought tolerance in alfalfa. *BMC plant Biol.* 19:2059. doi: 10.1186/s12870-019-2059-5

Flajoulot, S., Ronfort, J., Baudouin, P., Barre, P., Huguet, T., Huyghe, C., et al. (2005). Genetic diversity among alfalfa (*Medicago sativa*) cultivars coming from a breeding program, using SSR markers. *Theor. Appl. Genet.* 111, 1420–1429. doi: 10.1007/s00122-005-0074-4

Fleming, M. B., Patterson, E. L., Reeves, P. A., Richards, C. M., Gaines, T. A., and Walters, C. (2018). Exploring the fate of mRNA in aging seeds: protection, destruction, or slow decay? *J. Exp. Bot.* 69, 4309–4321. doi: 10.1093/jxb/ery215

Frendo, P., Harrison, J., Norman, C., and Jiménez, M. J. H. (2005). Glutathione and homoglutathione play a critical role in the nodulation process of *Medicago truncatula*. *Mol. Plant-Mic. Int.* 18, 254–259. doi: 10.1094/MPMI-18-0254

Fu, G., Grbic, V., Ma, S., and Tian, L. (2015). Evaluation of somatic embryos of alfalfa for recombinant protein expression. *Plant Cell Rep.* 34, 211–221. doi: 10.1007/s00299-014-1700-x

Fukuda, A., Nakamura, A., and Tanaka, Y. (1999). Molecular cloning and expression of the Na+/H+ exchanger gene in *Oryza sativa*. *Biochem. Biophys. Acta* 1446, 149–155. doi: 10.1016/s0167-4781(99)00065-2

Fürstenberg-Hägg, J., Zagrobelny, M., and Bak, S. (2013). Plant defense against insect herbivores. *Int. J. Mol. Sci.* 14, 10242–10297. doi: 10.3390/ijms140510242

Gao, R., Feyissa, B. A., Croft, M., and Hannoufa, A. (2018). Gene editing by CRISPR/Cas9 in the obligatory outcrossing *Medicago sativa*. *Planta* 247, 1043–1050. doi: 10.1007/s00425-018-2866-1

Gao, Z., Luo, W., Liu, H., Zeng, C., Liu, X., Yi, S., et al. (2012). Transcriptome analysis and SSR/SNP markers information of the blunt snout bream (*Megalobrama amblycephala*). *PLoS One* 7:42637. doi: 10.1371/journal.pone.0042637

García, A. N., Ayub, N. D., Fox, A. R., Gómez, M. C., Diéguez, M. J., Pagano, E. M., et al. (2014). Alfalfa snakin-1 prevents fungal colonization and probably coevolved with rhizobia. *BMC Plant Biol.* 14:248. doi: 10.1186/s12870-014-0248-9

Gong, B., Li, X., Bloszies, S., Wen, D., Sun, S., and Wei, M. (2014). Sodic alkaline stress mitigation by interaction of nitric oxide and polyamines involves antioxidants and physiological strategies in *Solanum lycopersicum*. *Free Radic. Biol. Med.* 71, 36–48. doi: 10.1016/j.freeradbiomed.2014.02.018

Graham, D. B., and Root, D. E. (2015). Resources for the design of CRISPR gene editing experiments. *Genome Biol.* 16:26. doi: 10.1186/s13059-015-0823-x

Gutsch, A., Keunen, E., Guerriero, G., Renaut, J., Cuypers, A., Hausman, J. F., et al. (2018b). Long-term cadmium exposure influences the abundance of proteins that impact the cell wall structure in *Medicago sativa* stems. *Plant Biol.* 20, 1023–1035. doi: 10.1111/plb.12865

Gutsch, A., Zouaghi, S., Renaut, J., Cuypers, A., Hausman, J. F., and Sergeant, K. (2018a). Changes in the proteome of *Medicago sativa* leaves in response to long-term cadmium exposure using a cell-wall targeted approach. *Int. J. Mol. Sci.* 19:2498. doi: 10.3390/ijms19092498

Guzman-Rodriguez, J. J., Ibarra-Laclette, E., Herrera-Estrella, L., Ochoa-Zarzosa, A., Suarez-Rodriguez, L. M., Rodriguez-Zapata, L. C., et al. (2013). Analysis of expressed sequence tags (ESTs) from avocado seed (*Persea americana* var. drymifolia) reveals abundant expression of the gene encoding the antimicrobial peptide snakin. *Plant Physiol. Biochem.* 70, 318–324. doi: 10.1016/j.plaphy.2013.05.045

Ha, C. V., Watanabe, Y., Tran, U. T., Le, D. T., Tanaka, M., Nguyen, K. H., et al. (2015). Comparative analysis of root transcriptomes from two contrasting drought-responsive Williams 82 and DT2008 soybean cultivars under normal and dehydration conditions. *Front. Plant Sci.* 6:551. doi: 10.3389/fpls.2015.00551

Han, Y., Kang, Y., Torres-Jerez, I., Cheung, F., Town, C. D., Zhao, P. X., et al. (2011). Genome-wide SNP discovery in tetraploid alfalfa using 454 sequencing and high resolution melting analysis. *BMC Genom.* 12:350. doi: 10.1186/1471-2164-12-350

Hartlerode, A. J., and Scully, R. (2009). Mechanisms of double-strand break repair in somatic mammalian cells. *Biochem. J.* 423, 157–168. doi: 10.1042/BJ20090942

Haun, W., Coffman, A., Clasen, B. M., Demorest, Z. L., Lowy, A., Ray, E., et al. (2014). Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. *Plant Biotechnol. J.* 12, 934–940. doi: 10.1111/pbi.12201

Hawkins, C., and Yu, L. X. (2018). Recent progress in alfalfa (*Medicago sativa* L.) *genomics and genomic selection*. *Crop J.* 6, 565–575. doi: 10.1016/j.cj.2018.01.006

He, X. Z., and Dixon, R. A. (2000). Genetic manipulation of isoflavone 7-O-methyltransferase enhances biosynthesis of 4'-O-methylated isoflavonoid phytoalexins and disease resistance in alfalfa. *Plant Cell* 12, 1689–1702. doi: 10.1105/tpc.12.9.1689

Herrmann, D., Flajoulot, S., Barre, P., Huyghe, C., Ronfort, J., and Julier, B. (2018). Comparison of morphological traits and molecular markers to analyse diversity and structure of alfalfa (*Medicago sativa* L.) *cultivars*. *Gen. Res. Crop Evol.* 65, 527–540. doi: 10.1007/s10722-017-0551-z

Hipskind, J. D., and Paiva, N. L. (2000). Constitutive accumulation of a resveratrol-glucoside in transgenic alfalfa increases resistance to Phoma medicaginis. *Mol. Plant Microbe Int.* 13, 551–556. doi: 10.1094/MPMI.2000.13.5.551

Huang, X., Kurata, N., Wang, Z. X., Wang, A., Zhao, Q., Zhao, Y., et al. (2012). A map of rice genome variation reveals the origin of cultivated rice. *Nature* 490, 497–501. doi: 10.1038/nature11532

Hwang, E. Y., Song, Q., Jia, G., Specht, J. E., Hyten, D. L., Costa, J., et al. (2014). A genome-wide association study of seed protein and oil content in soybean. *BMC Genom.* 15:2164. doi: 10.1186/1471-2164-15-1

Jacobs, T. B., LaFayette, P. R., Schmitz, R. J., and Parrott, W. A. (2015). Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnol.* 15:16. doi: 10.1186/s12896-015-0131-2

Jaganathan, D., Ramasamy, K., Sellamuthu, G., Jayabalan, S., and Venkataraman, G. (2018). CRISPR for crop improvement: an update review. *Front. Plant Sci.* 9:985. doi: 10.3389/fpls.2018.00985

Jain, M., Olsen, H. E., Paten, B., and Akeson, M. (2016). The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. *Gen. Biol.* 17:239. doi: 10.1186/s13059-016-1103-0

Jin, Q., Zhu, K., Cui, W., Xie, Y., Han, B., and Shen, W. (2013). Hydrogen gas acts as a novel bioactive molecule in enhancing plant tolerance to paraquat-induced oxidative stress via the modulation of heme oxygenase-1 signalling system. *Plant Cell Environ.* 36, 956–969. doi: 10.1111/pce.12029

Jin, T., Chang, Q., Li, W., Yin, D., Li, Z., Wang, D., et al. (2010). Stress-inducible expression of GmDREB1 conferred salt tolerance in transgenic alfalfa. *Plant Cell Trans. Organ Cult.* 100, 219–227. doi: 10.1007/s11240-009-9628-5

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Julier, B., Flajoulot, S., Barre, P., Cardinet, G., Santoni, S., Huguet, T., et al. (2003). Construction of two genetic linkage maps in cultivated tetraploid alfalfa (*Medicago sativa*) using microsatellite and AFLP markers. *BMC Plant B.* 3:9. doi: 10.1186/1471-2229-3-9

Kanaar, R., Hoeijmakers, J. H., and van Gent, D. C. (1998). Molecular mechanisms of DNA double strand break repair. *Trends Cell. Biol.* 8, 483–489. doi: 10.1016/S0962-8924(98)01383-X

Kang, P., Bao, A. K., Kumar, T., Pan, Y. Q., Bao, Z., Wang, F., et al. (2016). Assessment of stress tolerance, productivity, and forage quality in T1 transgenic alfalfa co-overexpressing ZxNHX and ZxVP1-1 from *Zygophyllum xanthoxylum*. *Front. Plant Sci.* 7:1598. doi: 10.3389/fpls.2016.01598

Kang, Y., Han, Y., Torres-Jerez, I., Wang, M., Tang, Y., Monteros, M., et al. (2011). System responses to long-term drought and re-watering of two contrasting alfalfa varieties. *Plant J.* 68, 871–889. doi: 10.1111/j.1365-313X.2011.04738.x

Kang, Y., Sakiroglu, M., Krom, N., Stanton-Geddes, J., Wang, M., Lee, Y. C., et al. (2015). Genome-wide association of drought-related and biomass traits with HapMap SNPs in *Medicago truncatula*. *Plant Cell Environ.* 38, 1997–2011. doi: 10.1111/pce.12520

Kersey, P. J. (2019). Plant genome sequences: past, present, future. *Curr. Opin. Plant Biol.* 48, 1–8. doi: 10.1016/j.pbi.2018.11.001

Khan, Z., Khan, S. H., Mubarik, M. S., Sadia, B., and Ahmad, A. (2017). Use of TALEs and TALEN technology for genetic improvement of plants. *Plant Mol. Biol. Rep.* 35, 1–19. doi: 10.1007/s11105-016-0997-8

Khu, D. M., Reyno, R., Han, Y., Zhao, P. X., Bouton, J. H., Brummer, E. C., et al. (2013). Identification of aluminum tolerance quantitative trait loci in tetraploid alfalfa. *Crop Sci.* 53, 148–163. doi: 10.2135/cropsci2012.03.0181

Kiegerl, S., Cardinale, F., Siligan, C., Gross, A., Baudouin, E., Liwosz, A., et al. (2000). SIMKK, a mitogen-activated protein kinase (MAPK) kinase, is a specific activator of the salt stress–induced MAPK, SIMK. *Plant Cell* 12, 2247–2258. doi: 10.1105/tpc.12.11.2247

Kim, K. Y., Kwon, S. Y., Lee, H. S., Hur, Y., Bang, J. W., and Kwak, S. S. (2003). A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. *Plant Mol. Biol.* 51, 831–838. doi: 10.1023/a:1023045218815

Kiss, G. B., Csanádi, G., Kálmán, K., Kaló, P., and Ökrész, L. (1993). Construction of a basic genetic map for alfalfa using RFLP, RAPD, isozyme and morphological markers. *Mol. Gen. Gen.* 238, 129–137. doi: 10.1007/BF00279539

Klapheck, S. (1988). Homoglutathione: isolation, quantification and occurrence in legumes. *Physiol. Plant.* 74, 727–732. doi: 10.1111/j.1399-3054.1988.tb02044.x

Komatsu, S., and Ahsan, N. (2009). Soybean proteomics and its application to functional analysis. *J. Proteomics* 72, 325–336. doi: 10.1016/j.jprot.2008.10.001

Komis, G., Illés, P., Beck, M., and Šamaj, J. (2011). Microtubules and mitogen-activated protein kinase signalling. *Curr. Oppin. Plant Biol.* 14, 650–657. doi: 10.1016/j.pbi.2011.07.008

Köpp, M., Passos, L., Verneue, R., Lédo, F. J., Coimbra, J. L., and de Oliveira, A. (2011). Effects of nutrient solution pH on growth parameters of alfalfa (*Medicago sativa* L.) *genotypes*. *Comun. Sci.e* 2, 135–141. doi: 10.14295/cs.v2i3.39

Korver, R. A., Koevoets, I. T., and Testerink, C. (2018). Out of shape during stress: a key role for auxin. *Trends Plant Sci.* 23, 783–793. doi: 10.1016/j.tplants.2018.05.011

Kovalskaya, N., and Hammond, R. W. (2009). Expression and functional characterization of the plant antimicrobial snakin-1 and defensin recombinant proteins. *Protein Expr. Purif.* 63, 12–17. doi: 10.1016/j.pep.2008.08.013

Kudapa, H., Ramalingam, A., Nayakoti, S., Chen, W., Zhuang, W., Liang, X., et al. (2013). Functional genomics to study stress responses in crop legumes: progress and prospects. *Funct. Plant Biol.* 40, 1221–1233. doi: 10.1071/FP13191

Kuluev, B. R., Gumerova, G. R., Mikhaylova, E. V., Gerashchenkov, G. A., Rozhnova, N. A., Vershinina, Z. R., et al. (2019). Delivery of CRISPR/Cas components into higher plant cells for genome editing. *Russ. J. Plant. Physl.* 66, 694–706. doi: 10.1134/S102144371905011X

Kumar, T., Bao, A. K., Bao, Z., Wang, F., Gao, L., and Wang, S. M. (2018). The progress of genetic improvement in alfalfa (*Medicago sativa* L.). *Czech. J. Genet. Plant Breed.* 54, 41–51. doi: 10.17221/46/2017-CJGPB

Laberge, S., Castonguay, Y., and Vézina, L. P. (1993). New cold-and drought-regulated gene from *Medicago sativa*. *Plant Physiol.* 101, 1411–1411. doi: 10.1104/pp.101.4.1411

Lardi, M., and Pessi, G. (2018). Functional genomics approaches to studying symbioses between legumes and nitrogen-fixing rhizobia. *High Throughput.* 7:15. doi: 10.3390/ht7020015

Le, B. H., Wagmaister, J. A., Kawashima, T., Bui, A. Q., Harada, J. J., and Goldberg, R. B. (2007). Using genomics to study legume seed development. *Plant Physiol.* 144, 562–574. doi: 10.1104/pp.107.100362

Le, D. T., Nishiyama, R., Watanabe, Y., Tanaka, M., Seki, M., Ham, L. H., et al. (2012). Differential gene expression in soybean leaf tissues at late developmental stages under drought stress revealed by genome-wide transcriptome analysis. *PLoS One* 7:e49522. doi: 10.1371/journal.pone.0049522

Le Nguyen, K., Grondin, A., Courtois, B., and Gantet, P. (2018). Next-generation sequencing accelerates crop gene discovery. *Trends Plant Sci.* 24, 8. doi: 10.1016/j.tplants.2018.11.008

Lei, Y., Xu, Y., Hettenhausen, C., Lu, C., Shen, G., Zhang, C., et al. (2018). Comparative analysis of alfalfa (*Medicago sativa* L.) leaf transcriptomes reveals genotype-specific salt tolerance mechanisms. *BMC Plant Biol.* 18:35. doi: 10.1186/s12870-018-1250-4

Li, H., Wang, Z., Ke, Q., Ji, C. Y., Jeong, J. C., Lee, H. S., et al. (2014). Overexpression of codA gene confers enhanced tolerance to abiotic stresses in alfalfa. *Plant Physiol. Biochem.* 85, 31–40. doi: 10.1016/j.plaphy.2014.10.010

Li, J. F., Norville, J. E., Aach, J., McCormack, M., Zhang, D., Bush, J., et al. (2013). Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nat. Biotechnol.* 31, 688–691. doi: 10.1038/nbt.2654

Li, S., Jia, S., Hou, L., Nguyen, H., Sato, S., Holding, D., et al. (2019). Mapping of transgenic alleles in soybean using a nanopore-based sequencing strategy. *J. Exp. Bot.* 70, 3825–3833. doi: 10.1093/jxb/erz202

Li, T., Huang, S., Jiang, W. Z., Wright, D., Spalding, M. H., Weeks, D. P., et al. (2011). TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. *Nucleic Acids Res.* 39, 359–372. doi: 10.1093/nar/gkq704

Li, W., Wei, Z., Qiao, Z., Wu, Z., Cheng, L., and Wang, Y. (2013). Proteomics analysis of alfalfa response to heat stress. *PLoS One* 8:e82725. doi: 10.1371/journal.pone.0082725

Li, X., and Brummer, E. C. (2012). Applied genetics and genomics in alfalfa breeding. *Agronomy* 2, 40–61. doi: 10.3390/agronomy2010040

Li, X., Hannoufa, A., Zhang, Y., and Yu, P. (2016). Gene-silencing-induced changes in carbohydrate conformation in relation to bioenergy value and carbohydrate subfractions in modeled plant (*Medicago sativa*) with down-regulation of HB12 and TT8 transcription factors. *Int. J. Mol. Sci.* 17:720. doi: 10.3390/ijms17050720

Li, X., Wei, Y., Acharya, A., Jiang, Q., Kang, J., and Brummer, E. C. (2014). A saturated genetic linkage map of autotetraploid alfalfa (*Medicago sativa* L.) developed using genotyping-by-sequencing is highly syntenous with the *Medicago truncatula* genome. *G3* 4, 1971–1979. doi: 10.1534/g3.114.012245

Li, Z., Liu, Z. B., Xing, A., Moon, B. P., Koellhoffer, J. P., Huang, L., et al. (2015). Cas9-guide RNA directed genome editing in soybean. *Plant Physiol.* 169, 960–970. doi: 10.1104/pp.15.00783

Libault, M., Pingault, L., Zogli, P., and Schiefelbein, J. (2017). Plant systems biology at the single-cell level. *T. Plant Sci.* 22, 949–960. doi: 10.1016/j.tplants.2017.08.006

Liu, H., Ding, Y., Zhou, Y., Jin, W., Xie, K., and Chen, L. L. (2017). CRISPR-P 2.0: *an improved CRISPR-Cas*9 tool for genome editing in plants. *Mol. Plant* 10, 530–532. doi: 10.1016/j.molp.2017.01.003

Liu, W., Xiong, C., Yan, L., Zhang, Z., Ma, L., Wang, Y., et al. (2017). Transcriptome analyses reveal candidate genes potentially involved in al stress response in alfalfa. *Front. Plant Sci.* 8:26. doi: 10.3389/fpls.2017.00026

Liu, X., Wu, S., Xu, J., Sui, C., and Wei, J. (2019). Application of CRISPR/Cas9 in plant biology. *Acta Pharm. Sin. B.* 7, 292–302. doi: 10.1016/j.apsb.2017.01.002

Liu, X. P., Hawkins, C., Peel, M. D., and Yu, L. X. (2019). Genetic loci associated with salt tolerance in advanced breeding populations of tetraploid alfalfa using genome-wide association studies. *Plant Genome* 12:26. doi: 10.3835/plantgenome2018.05.0026

Liu, Z., Chen, T., Ma, L., Zhao, Z., Zhao, P. X., Nan, Z., et al. (2013). Global transcriptome sequencing using the Illumina platform and the development of EST-SSR markers in autotetraploid alfalfa. *PLoS One* 8:e83549. doi: 10.1371/journal.pone.0083549

Long, R., Gao, Y., Sun, H., Zhang, T., Li, X., Li, M., et al. (2018). Quantitative proteomic analysis using iTRAQ to identify salt-responsive proteins during the germination stage of two Medicago species. *Sci. Rep.* 8:9553. doi: 10.1038/s41598-018-27935-8

Long, R., Li, M., Zhang, T., Kang, J., Sun, Y., Cong, L., et al. (2016). Comparative proteomic analysis reveals differential root proteins in *Medicago sativa* and *Medicago truncatula* in response to salt stress. *Front. Plant Sci.* 7:424. doi: 10.3389/fpls.2016.00424

Lu, H., Giordano, F., and Ning, Z. (2016). Oxford Nanopore MinION sequencing and genome assembly. *Genom. Proteom. Bioinf.* 14, 265–279. doi: 10.1016/j.gpb.2016.05.004

Luo, D., Wu, Y., Liu, J., Zhou, Q., Liu, W., Wang, Y., et al. (2019a). Comparative transcriptomic and physiological analyses of *Medicago sativa* L. indicates that multiple regulatory networks are activated during continuous aba treatment. *Int. J. Mol. Sci.* 20:E47. doi: 10.3390/ijms20010047

Luo, D., Zhou, Q., Wu, Y., Chai, X., Liu, W., Wang, Y., et al. (2019b). Full-length transcript sequencing and comparative transcriptomic analysis to evaluate the contribution of osmotic and ionic stress components towards salinity tolerance in the roots of cultivated alfalfa (*Medicago sativa* L.). *BMC Plant Biol.* 19:32. doi: 10.1186/s12870-019-1630-4

Luo, M., Lin, L., Hill, R. D., and Mohapatra, S. S. (1991). Primary structure of an environmental stress and abscisic acid-inducible alfalfa protein. *Plant Mol. Biol.* 17, 1267–1269. doi: 10.1007/bf00028745

Luo, M., Liu, J. H., Mohapatra, S., Hill, R. D., and Mohapatra, S. S. (1992). Characterization of a gene family encoding abscisic acid-and environmental stress-inducible proteins of alfalfa. *J. Biol. Chem.* 267, 15367–15374.

Ma, Q., Kang, J., Long, R., Zhang, T., Xiong, J., Zhang, K., et al. (2017). Comparative proteomic analysis of alfalfa revealed new salt and drought stress-related factors involved in seed germination. *Mol. Biol. Rep.* 44, 261–272. doi: 10.1007/s11033-017-4104-5

Mackie, J. M., Musial, J. M., Armour, D. J., Phan, H. T. T., and Ellwood, S. E. (2007). Identification of QTL for reaction to three races of *Colletotrichum trifolii* and further analysis of inheritance of resistance in autotetraploid lucerne. *Theor. Appl. Genet.* 114, 1417–1426. doi: 10.1007/s00122-007-0527-z

Macovei, A., Gill, S. S., and Tuteja, N. (2012). microRNAs as promising tools for improving stress tolerance in rice. *Plant Sig. Beh.* 7, 1296–1301. doi: 10.4161/psb.21586

Mahfouz, M. M., Li, L., Shamimuzzaman, M., Wibowo, A., Fang, X., and Zhu, J. K. (2011). De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. *Proc. Natl. Acad. Sci. U.S.A.* 108, 2623–2628. doi: 10.1073/pnas.1019533108

Makarova, K. S., Zhang, F., and Koonin, E. V. (2017). SnapShot: Class 2 CRISPR-Cas systems. *Cell* 168, 328–328. doi: 10.1016/j.cell.2016.12.038

Malzahn, A., Lowder, L., and Qil, Y. (2017). Plant genome editing with TALEN and CRISPR. *Cell Biosci.* 7:21. doi: 10.1186/s13578-017-0148-4

Mao, Y. F., Botella, J. R., Liu, Y. G., and Zhu, J. K. (2019). Gene editing in plants: progress and challenges. *Natl. Sci. Rev.* 6, 421–437. doi: 10.1093/nsr/nwz005

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437, 376–380. doi: 10.1038/nature03959

Marraffini, L. A., and Sontheimer, E. J. (2008). CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* 322, 1843–1845. doi: 10.1126/science.1165771

Masonbrink, R. E., Severin, A. J., and Seetharam, A. S. (2017). "Comparative genomics of soybean and other legumes," in *The Soybean Genome*, eds H. Nguyen and M. Bhattacharyya (Cham: Springer), 83–93. doi: 10.1007/978-3-319-64198-0_6

Masoud, S. A., Zhu, Q., Lamb, C., and Dixon, R. A. (1996). Constitutive expression of an inducible β-1,3-glucanase in alfalfa reduces disease severity caused by the oomycete pathogen *Phytophthora megasperma* f. sp. medicaginis, but does not reduce disease severity of chitin-containing fungi. *Transgenic Res.* 5, 313–323. doi: 10.1007/BF01968941

Matamoros, M. A., Moran, J. F., Iturbe-Ormaetxe, I., Rubio, M. C., and Becana, M. (1999). Glutathione and homoglutathione synthesis in legume root nodules. *Plant Physiol.* 121, 879–888. doi: 10.1104/pp.121.3.879

Matthews, C., Arshad, M., and Hannoufa, A. (2019). Alfalfa response to heat stress is modulated by microRNA156. *Physiol. Plant.* 165, 830–842. doi: 10.1111/ppl.12787

McCoy, T. J., and Bingham, E. T. (1988). "Cytology and cytogenetics of alfalfa," in *Alfalfa and Alfalfa Improvement*, ed. A. A. Hanson (Madison, WI: ASA), 737–776.

Meng, Y., Wang, C., Yin, P., Zhu, B., Zhang, P., Niu, L., et al. (2019). "Targeted mutagenesis by an optimized agrobacterium-delivered CRISPR/Cas 9 system in the model legume *Medicago truncatula*," in *The Model Legume Medicago truncatula*, ed. F. D. Bruijn (Hoboken, NJ: Wiley), 1015–1018. doi: 10.1002/9781119409144.ch130

Meng, Y. Y., Hou, Y. L., Wang, H., Ji, R. H., Liu, B., Wen, J. Q., et al. (2017). Targeted mutagenesis by CRISPR/Cas9 system in the model legume *Medicago truncatula*. *Plant Cell Rep.* 36, 371–374. doi: 10.1007/s00299-016-2069-9

Michno, J. M., Wang, X., Liu, J., Curtin, S. J., Kono, T. J., and Stupar, R. M. (2015). CRISPR/Cas mutagenesis of soybean and *Medicago truncatula* using a new web-tool and a modified Cas9 enzyme. *GM Crops Food* 6, 243–252. doi: 10.1080/21645698.2015.1106063

Miller, J. C., Tan, S., Qiao, G., Barlow, K. A., Wang, J., Xia, D. F., et al. (2011). A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.* 29, 143–148. doi: 10.1038/nbt.1755

Mittler, R., and Blumwald, E. (2015). The roles of ROS and ABA in systemic acquired acclimation. *Plant Cell* 27, 64–70. doi: 10.1105/tpc.114.133090

Mo, Y., Liang, G., Shi, W., and Xie, J. (2011). Metabolic responses of alfalfa (*Medicago Sativa* L.) leaves to low and high temperature induced stresses. *Afr. J. Biotechnol.* 10, 1117–1124. doi: 10.5897/AJB10.1433

Moradpour, M., and Abdulah, S. N. A. (2020). CRISPR/dCas9 platforms in plants: strategies and applications beyond genome editing. *Plant Biotechnol. J.* 18, 32–44. doi: 10.1111/pbi.13232

Musial, J. M., Mackie, J. M., Armour, D. J., Phan, H. T. T., Ellwood, S. E., Aitken, K. S., et al. (2007). Identification of QTL for resistance and susceptibility to *Stagonospora meliloti* in autotetraploid lucerne. *Theor. Appl. Gen.* 114, 1427–1435. doi: 10.1007/s00122-007-0528-y

Nakano, K., Shiroma, A., Shimoji, M., Tamotsu, H., Ashimine, N., Ohki, S., et al. (2017). Advantages of genome sequencing by long-read sequencer using SMRT technology in medical area. *Hum. Cell* 30, 149–161. doi: 10.1007/s13577-017-0168-8

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D., and Kamoun, S. (2013). Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31, 691–693. doi: 10.1038/nbt.2655

Nekrasov, V., Wang, C. M., and Win, J. (2017). Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Sci. Rep.* 7:482. doi: 10.1038/s41598-017-00578-x

Ninković, S., Miljuš-Đukić, J., and Nešković, M. (1995). Genetic transformation of alfalfa somatic embryos and their clonal propagation through repetitive somatic embryogenesis. *Plant Cell T. Organ Cult.* 42, 255–260. doi: 10.1007/BF00029996

Nirola, R., Megharaj, M., Beecham, S., Aryal, R., Thavamani, P., Vankateswarlu, K., et al. (2016). Remediation of metalliferous mines, revegetation challenges and emerging prospects in semi-arid and arid conditions. *Env. Sci. Poll. Res.* 23, 20131–20150. doi: 10.1007/s11356-016-7372-z

Nutter, F. W., Guan, J., Gotlieb, A. R., Rhodes, L. H., Grau, C. R., and Sulc, R. M. (2002). Quantifying alfalfa yield losses caused by foliar diseases in Iowa, Ohio, Wisconsin, and Vermont. *Plant Dis.* 863, 269–277. doi: 10.1094/PDIS.2002.86.3.269

Olukolu, B. A., Tracy, W. F., Wisser, R., De Vries, B., and Balint-Kurti, P. J. (2016). A genome-wide association study for partial resistance to maize common rust. *Phytopath.* 106, 745–751. doi: 10.1094/PHYTO-11-15-0305-R

O'Rourke, J. A., Fu, F., Bucciarelli, B., Yang, S. S., Samac, D. A., Lamb, J. F. S., et al. (2015). The *Medicago sativa* gene index 1.2: a web-accessible gene expression atlas for investigating expression differences between *Medicago sativa* subspecies. *BMC Genom.* 16:502. doi: 10.1186/s12864-015-1718-7

Ovečka, M., Takáč, T., Komis, G., Vadovič, P., Bekešová, S., Doskočilová, A., et al. (2014). Salt-induced subcellular kinase relocation and seedling susceptibility caused by overexpression of Medicago SIMKK in *Arabidopsis*. *J. Exp. Bot.* 65, 2335–2350. doi: 10.1093/jxb/eru115

Paparella, S., Araújo, S. S., Rossi, G., Wijayasinghe, M., Carbonera, D., and Balestrazzi, A. (2015). Seed priming: state of the art and new perspectives. *Plant Cell Rep.* 34, 1281–1293. doi: 10.1007/s00299-015-1784-y

Pâques, F., and Duchateau, P. (2007). Meganucleases and DNA double-strand break-induced recombination: perspectives for gene therapy. *Curr. Gene Ther.* 7, 49–66. doi: 10.2174/156652307779940216

Pasternak, T., Asard, H., Potters, G., and Jansen, M. A. (2014). The thiol compounds glutathione and homoglutathione differentially affect cell development in alfalfa (*Medicago sativa* L.). *Plant Phys. Biochem.* 74, 16–23. doi: 10.1016/j.plaphy.2013.10.028

Pastwa, E., and Blasiak, J. (2003). Non-homologous DNA end joining. *Acta. Biochim. Pol.* 50, 891–908. doi: 10.18388/abp.2003_3622

Paszkowski, J., Baur, M., Bogucki, A., and Potrykus, I. (1988). Gene targeting in plants. *EMBO J.* 7, 4021–4026. doi: 10.1002/j.1460-2075.1988.tb03295.x

Pavlovich, M. (2017). Computing in biotechnology: omics and beyond. *Trends Biotechnol.* 35, 450–497. doi: 10.1016/j.tibtech.2017.03.011

Pennycooke, J. C., Cheng, H., and Stockinger, E. J. (2008). Comparative genomic sequence and expression analyses of *Medicago truncatula* and alfalfa subspecies falcata COLD-ACCLIMATION-SPECIFIC genes. *Plant Physiol.* 146, 1242–1254.

Piano, E., and Pecetti, L. (2010). "Minor legume species," in *Fodder Crops and Amenity Grassesm Handbook of Plant Breeding*, Vol. 5, eds B. Boller, U. K. Posselt, and F. Veronesi (NewYork, NY: Springer), 477–500. doi: 10.1007/978-1-4419-0760-8_20

Postnikova, O. A., Hult, M., Shao, J., Skantar, A., and Nemchinov, L. G. (2015). Transcriptome analysis of resistant and susceptible alfalfa cultivars infected with root-knot nematode Meloidogyne incognita. *PLoS One* 10:e0123157. doi: 10.1371/journal.pone.0118269

Postnikova, O. A., Shao, J., and Nemchinov, L. G. (2013). Analysis of the alfalfa root transcriptome in response to salinity stress. *Plant Cell Physiol.* 54, 1041–1055. doi: 10.1093/pcp/pct056

Pratt, R. G., and Rowe, D. E. (2002). Enhanced resistance to *Sclerotium rolfsii* in populations of alfalfa selected for quantitative resistance to *Sclerotinia trifoliorum*. *Phytopathology* 92, 204–209. doi: 10.1094/PHYTO.2002.92.2.204

Printz, B., Guerriero, G., Sergeant, K., Audinot, J. N., Guignard, C., Renaut, J., et al. (2016). Combining-omics to unravel the impact of copper nutrition on alfalfa (*Medicago sativa*) stem metabolism. *Plant Cell Physiol.* 57, 407–422. doi: 10.1093/pcp/pcw001

Printz, B., Guerriero, G., Sergeant, K., Renaut, J., Lutts, S., and Hausman, J. F. (2015). Ups and downs in alfalfa: proteomic and metabolic changes occurring in the growing stem. *Plant Sci.* 238, 13–25. doi: 10.1016/j.plantsci.2015.05.014

Puchta, H., Dujon, B., and Hohn, B. (1993). Homologous recombination in plant cells is enhanced by in vivo induction of double strand breaks into DNA by a site-specific endonuclease. *Nucleic Acids Res.* 21, 5034–5040. doi: 10.1093/nar/21.22.5034

Qi, Y. (2015). "High efficient genome modification by designed zinc finger nuclease," in *Advances in New Technology for Targeted Modification of Plant Genomes*, eds F. Zhang, H. Puchta, and J. G. Thomson (New York, NY: Springer), 39–53. doi: 10.1007/978-1-4939-2556-8_3

Radović, J., Sokolović, D., and Marković, J. (2009). Alfalfa-most important perennial forage legume in animal husbandry. *Biotechnol. Anim. Husb.* 25, 465–475. doi: 10.2298/BAH0906465R

Rahman, M. A., Alam, I., Kim, Y. G., Ahn, N. Y., Heo, S. H., Lee, D. G., et al. (2015). Screening for salt-responsive proteins in two contrasting alfalfa cultivars using a comparative proteome approach. *Plant Physiol. Biochem.* 89, 112–122. doi: 10.1016/j.plaphy.2015.02.015

Rahman, M. A., Yong-Goo, K., Iftekhar, A., Liu, G., Hyoshin, L., Joo, L. J., et al. (2016). Proteome analysis of alfalfa roots in response to water deficit stress. *J. Integr. Agric.* 15, 1275–1285. doi: 10.1016/S2095-3119(15)61255-2

Rashmi, R., Sarkar, M., and Vikramaditya, T. (1997). Cultivaton of alfalfa (*Medicago sativa* L.). *Anc. Sci. Life* 17, 117–119.

Rhodes, D., and Hanson, A. D. (1993). Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Ann. Rev. Plant Biol.* 44, 357–384. doi: 10.1146/annurev.pp.44.060193.002041

Robins, J. G., Luth, D., Campbell, T. A., Bauchan, G. R., He, C., Viands, D. R., et al. (2007). Genetic mapping of biomass production in tetraploid alfalfa. *Crop Sci.* 47, 1–10. doi: 10.2135/cropsci2005.11.0401

Rothberg, J. M., Hinz, W., Rearick, T. M., Schultz, J., Mileski, W., Davey, M., et al. (2011). An integrated semiconductor device enabling non-optical genome sequencing. *Nature* 475, 348–352. doi: 10.1038/nature10242

Roumen, E. C. (1994). "A strategy for accumulating genes for partial resistance to blast disease in rice within a conventional breeding program," in *Rice Blast Disease*, eds R. S. Zeigler, S. A. Leong, and P. S. Teng (Cambridge: CAB International), 245–265.

Rubiales, D., Fondevilla, S., Chen, W., Gentzbittel, L., Higgins, T. J. V., Castillejo, M. A., et al. (2015). Achievements and challenges in legume breeding for pest and disease resistance. *CRC Crit. Rev. Plant Sci.* 34, 195–236. doi: 10.1080/07352689.2014.898445

Sakiroglu, M., and Brummer, E. C. (2017). Identification of loci controlling forage yield and nutritive value in diploid alfalfa using GBS-GWAS. *Theor. Appl. Gen.* 130, 261–268. doi: 10.1007/s00122-016-2782-3

Samac, D., and Smigocki, A. (2003). Expression of oryzacystatin I and II in alfalfa increases resistance to the root-lesion nematode. *Phytopathology* 93, 799–804. doi: 10.1094/PHYTO.2003.93.7.799

Samac, D. A., and Temple, S. J. (2004). "Development and utilization of transformation in Medicago species," in *Genetically Modified Crops, Their Development, Uses and Risks*, eds G. H. Liang and D. Z. Skinner (New York, NY: The Haworth Press), 165–202.

Šamaj, J., Ovečka, M., Hlavačka, A., Lecourieux, F., Meskiene, I., Lichtscheidl, I., et al. (2002). Involvement of the mitogen-activated protein kinase SIMK in regulation of root hair tip growth. *EMBO J.* 21, 3296–3306. doi: 10.1093/emboj/cdf349

Šamajová, O., Komis, G., and Šamaj, J. (2013a). Emerging topics in the cell biology of mitogen-activated protein kinases. *Trans. Plant Sci.* 18, 140–148. doi: 10.1016/j.tplants.2012.11.004

Šamajová, O., Plíhal, O., Al-Yousif, M., Hirt, H., and Šamaj, J. (2013b). Improvement of stress tolerance in plants by genetic manipulation of mitogen-activated protein kinases. *Biotech. A.* 31, 118–128. doi: 10.1016/j.biotechadv.2011.12.002

Sander, J. D., Dahlborg, E. J., Goodwin, M. J., Cade, L., Zhang, F., Cifuentes, D., et al. (2011). Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat. Methods* 8, 67–69. doi: 10.1038/nmeth.1542

Sato, S., Nakamura, Y., Kaneko, T., Asamizu, E., Kato, T., Nakao, M., et al. (2008). Genome structure of the legume, *Lotus japonicus*. *DNA Res.* 15, 227–239. doi: 10.1093/dnares/dsn008

Scheben, A., Verpaalen, B., Lawley, C. T., Chan, C. K. K., Bayer, P. E., Batley, J., et al. (2019). CropSNPdb: a database of SNP array data for Brassica crops and hexaploid bread wheat. *Plant J.* 98, 142–152. doi: 10.1111/tpj.14194

Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467–470. doi: 10.1126/science.270.5235.467

Schiml, S., and Puchta, H. (2016). Revolutionizing plant biology: multiple ways of genome engineering by CRISPR/Cas. *Plant Methods* 12:8. doi: 10.1186/s13007-016-0103-0

Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., et al. (2010). Genome sequence of the palaeopolyploid soybean. *Nature* 463, 178–183. doi: 10.1038/nature08670

Schreiber, M., Stein, N., and Mascher, M. (2018). Genomic approaches for studying crop evolution. *Genome Biol.* 19:140. doi: 10.1186/s13059-018-1528-8

Segura, A., Moreno, M., Madueno, F., Molina, A., and Garcia-Olmedo, F. (1999). Snakin-1, a peptide from potato that is active against plant pathogens. *Mol. Plant Microbe Interact.* 12, 16–23. doi: 10.1094/MPMI.1999.12.1.16

Severin, A. J., Cannon, S. B., Graham, M. M., Grant, D., and Shoemaker, R. C. (2011). Changes in twelve homoeologous genomic regions in soybean following three rounds of polyploidy. *Plant Cell* 23, 3129–3136. doi: 10.1105/tpc.111.089573

Shafique, A., Rehman, A., Khan, A., and Kazi, A. G. (2014). "Chapter 1 - Improvement of legume crop production under environmental stresses through biotechnological intervention," in *Emerging Technologies and Management of Crop Stress Tolerance: Volume II - A Sustainable Approach*, eds P. Ahmad and S. Rehman (San Diego: Accademic Press), 1–22. doi: 10.1016/B978-0-12-800875-1.00001-6

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., et al. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol.* 31, 686–688. doi: 10.1038/nbt.2650

Shan, S., Soltis, P. S., Soltis, D. E., and Yang, B. (2020). Considerations in adapting CRISPR/Cas9 in nongenetic model plant systems. *Appl. Plant Sci.* 8, e11314. doi: 10.1002/aps3.11314

Singer, S. D., Hannoufa, A., and Acharya, S. (2018). Molecular improvement of alfalfa for enhanced productivity and adaptability in a changing environment. *Plant Cell Environ.* 41, 1955–1971. doi: 10.1111/pce.13090

Smith, J., Grizot, S., Arnould, S., Duclert, A., Epinat, J. C., Chames, P., et al. (2006). A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. *Nucleic Acids Res.* 34, e149. doi: 10.1093/nar/gkl720

Song, L., Jiang, L., Chen, Y., Shu, Y., Bai, Y., and Guo, C. (2016). Deep-sequencing transcriptome analysis of field-grown *Medicago sativa* L. crown buds acclimated to freezing stress. *Func. Integr. Genomics* 16, 495–511. doi: 10.1007/s10142-016-0500-5

Stefanova, G., Slavov, S., Gecheff, K., Vlahova, M., and Atanassov, A. (2013). Expression of recombinant human lactoferrin in transgenic alfalfa plants. *Biol. Plant.* 57, 457–464. doi: 10.1007/s10535-013-0305-5

Steinert, J., Schiml, S., and Puchta, H. (2016). Homology-based double-strand break-induced genome engineering in plants. *Plant Cell Rep.* 35, 1429–1438. doi: 10.1007/s00299-016-1981-3

Strizhov, N., Keller, M., Mathur, J., Koncz-Kálmán, Z., Bosch, D., Prudovsky, E., et al. (1996). A synthetic *cryIC* gene, encoding a *Bacillus thuringiensis* δ-endotoxin, confers *Spodoptera* resistance in alfalfa and tobacco.ă*Proc. Natl. Acad. Sci. U.S.A.* 93, 15012–15017. doi: 10.1073/pnas.93.26.15012

Stritzler, M., Elba, P., Berini, C., Gomez, C., Ayub, N., and Soto, G. (2018). High-quality forage production under salinity by using a salt-tolerant AtNXH1-expressing transgenic alfalfa combined with a natural stress-resistant nitrogen-fixing bacterium. *J. Biotechnol.* 276, 42–45. doi: 10.1016/j.jbiotec.2018.04.013

Sun, X., Hu, Z., Chen, R., Jiang, Q., Song, G., Zhang, H., et al. (2015). Targeted mutagenesis in soybean using the CRISPR-Cas9 system. *Sci. Rep.* 5:10342. doi: 10.1038/srep10342

Tang, F., Yang, S., Liu, J., and Zhu, H. (2016). Rj4, a gene controlling nodulation specificity in soybeans, encodes a thaumatin-like protein but not the one previously reported. *Plant Physiol.* 170, 26–32. doi: 10.1104/pp.15.01661

Tang, H., Krishnakumar, V., Bidwell, S., Rosen, B., Chan, A., Zhou, S., et al. (2014). An improved genome release (version Mt4. 0) for the model legume *Medicago truncatula*. *BMC genom.* 15:312. doi: 10.1186/1471-2164-15-312

Tang, X., Liu, G. Q., Zhou, J. P., Ren, Q., You, Q., Tian, L., et al. (2018). A large-scale whole-genome sequencing analysis reveals highly specific genome editing by both Cas9 and Cpf1 (Cas12a) nucleases in rice. *Genome Biol.* 19:84. doi: 10.1186/s13059-018-1458-5

Tesfaye, M., Denton, M. D., Samac, D. A., and Vance, C. P. (2005). Transgenic alfalfa secretes a fungal endochitinase protein to the rhizosphere. *Plant Soil* 269, 233–243. doi: 10.1007/s11104-004-0520-0

Tesfaye, M., Liu, J., and Vance, C. P. (2007). Genomic and genetic control of phosphate stress in legumes. *Plant Physiol.* 144, 594–603. doi: 10.1104/pp.107.097386

Toth, E., and Bakheit, B. R. (1983). Results of resistance breeding in alfalfa. II. Resistance to Verticillium wilt. *Acta Biol. Hung.* 32, 78–85.

Triboi, E., and Triboi-Blondel, A. M. (2014). "Towards sustainable, self-supporting agriculture: biological nitrogen factories as a key for future cropping systems," in *Soil as World Heritage*, ed. D. Dent (Dordrecht: Springer), 329–342. doi: 10.1007/978-94-007-6187-2_32

Tripathi, P., Rabara, R. C., Reese, R. N., Miller, M. A., Rohila, J. S., Subramanian, S., et al. (2016). A toolbox of genes, proteins, metabolites and promoters for improving drought tolerance in soybean includes the metabolite coumestrol and stomatal development genes. *BMC Genom.* 17:102. doi: 10.1186/s12864-016-2420-0

Tu, X., Liu, Z., and Zhang, Z. (2018a). Comparative transcriptomic analysis of resistant and susceptible alfalfa cultivars (*Medicago sativa* L.) after thrips infestation. *BMC Genom.* 19:116. doi: 10.1186/s12864-018-4495-2

Tu, X., Zhao, H., and Zhang, Z. (2018b). Transcriptome approach to understand the potential mechanisms of resistant and susceptible alfalfa (*Medicago sativa* L.) cultivars in response to aphid feeding. *J. Integr. Agric.* 17, 2518–2527. doi: 10.1016/S2095-3119(17)61843-4

Valliyodan, B., Ye, H., Song, L., Murphy, M., Shannon, J. G., and Nguyen, H. T. (2017). Genetic diversity and genomic strategies for improving drought and

waterlogging tolerance in soybeans. *J. Exp. Bot.* 68, 1835–1849. doi: 10.1093/jxb/erw433

Van, K., Rastogi, K., Kim, K. H., and Lee, S. H. (2013). Next-generation sequencing technology for crop improvement. *SABRAO J. Breed. Genet.* 45, 84–99. doi: 10.3389/fpls.2014.00367

Varshney, R. K., and Kudapa, H. (2013). Legume biology: the basis for crop improvement. *Funct. Plant Biol.* 40, 5–8. doi: 10.1071/FPv40n12_FO

Volkov, V., Wang, B., Dominy, P. J., Fricke, W., and Amtmann, A. (2004). Thellungiella halophila, a salt-tolerant relative of *Arabidopsis thaliana*, possesses effective mechanisms to discriminate between potassium and sodium. *Plant Cell Environ.* 27, 1–14. doi: 10.1046/j.0016-8025.2003.01116.x

Walter, M. H., Liu, J. W., Wünn, J., and Hess, D. (1996). Bean ribonuclease-like pathogenesis-related protein genes Ypr10 display complex patterns of developmental, dark-induced and exogenous-stimulus-dependent expression. *Eur. J. Biochem.* 239, 281–293. doi: 10.1111/j.1432-1033.1996.0281u.x

Wang, K., Wang, Z., Li, F., Ye, W., and Wang, J. (2012). The draft genome of a diploid cotton *Gossypium raimondii*. *Nature Gen.* 44, 1098–1103. doi: 10.1038/ng.2371

Wang, L., Rubio, M. C., Xin, X., Zhang, B., Fan, Q., Wang, Q., et al. (2019). CRISPR/Cas9 knockout of leghemoglobin genes in *Lotus japonicus* uncovers their synergistic roles in symbiotic nitrogen fixation. *New Phytol.* 224, 818–832. doi: 10.1111/nph.16077

Wang, L., Sun, S., Wu, T., Liu, L., Sun, X., Cai, Y., et al. (2020). Natural variation and CRISPR/Cas9-mediated mutation in *GmPRR37* affect photoperiodic flowering and contribute to regional adaptation of soybean. *Plant Biotechnol. J.* 1–13. doi: 10.1111/pbi.13346

Wang, L., Wang, L., Tan, Q., Fan, Q., Zhu, H., Hong, Z., et al. (2016). Efficient inactivation of symbiotic nitrogen fixation related genes in *Lotus japonicus* using CRISPR-Cas9. *Front. Plant Sci.* 7:1333. doi: 10.3389/fpls.2016.01333

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10, 57–63. doi: 10.1038/nrg2484

Wang, Z., Li, H., Ke, Q., Jeong, J. C., Lee, H. S., Xu, B., et al. (2014). Transgenic alfalfa plants expressing AtNDPK2 exhibit increased growth and tolerance to abiotic stresses. *Plant Physiol. Biochem.* 84, 67–77. doi: 10.1016/j.plaphy.2014.08.025

Watson, B. S., Bedair, M. F., Urbanczyk-Wochniak, E., Huhman, D. V., Yang, D. S., Allen, S. N., et al. (2015). Integrated metabolomics and transcriptomics reveal enhanced specialized metabolism in *Medicago truncatula* root border cells. *Plant Physiol.* 167, 1699–1716. doi: 10.1093/jxb/erx308

Wen, L., Chen, Y., Schnabel, E., Crook, A., and Frugoli, J. (2019). Comparison of efficiency and time to regeneration of Agrobacterium-mediated transformation methods in *Medicago truncatula*. *Plant Met.* 15:20. doi: 10.1186/s13007-019-0404-1

Wiedenheft, B., Sternberg, S. H., and Doudna, J. A. (2012). RNA-guided genetic silencing systems in bacteria and archaea. *Nature* 482, 331–338. doi: 10.1038/nature10886

Wong, C. E., Li, Y., and Moffatt, B. A. (2006). Transcriptional profiling implicates novel interactions between abiotic stress and hormonal responses in *Thellungiella*, a close relative of *Arabidopsis*. *Plant Physiol.* 140, 1437–1450. doi: 10.1104/pp.105.070508

Wright, D. A., Townsend, J. A., Winfrey, R. J. Jr., Irwin, P. A., and Rajagopal, J. (2005). High-frequency homologous recombination in plants mediated by zinc-finger nucleases. *Plant J.* 44, 693–705. doi: 10.1111/j.1365-313X.2005.02551.x

Xia, T., Apse, M. P., Aharon, G. S., and Blumwald, E. (2002). Identification and characterization of a NaCl-inducible vacuolar Na+/H+ antiporter in *Beta vulgaris*. *Physiol. Plant.* 116, 206–212. doi: 10.1034/j.1399-3054.2002.1160210.x

Xie, X., Ma, X., Zhu, Q., Zeng, D., Li, G., and Liu, Y. G. (2017). CRISPR-GE: a convenient software toolkit for CRISPR-based genome editing. *Mol. Plant.* 10, 1246–1249. doi: 10.1016/j.molp.2017.06.004

Xiong, L., Lee, H., Ishitani, M., and Zhu, J. K. (2002). Regulation of osmotic stress-responsive gene expression by theLOS6/ABA1 locus in *Arabidopsis*. *J. Biol. Chem.* 277, 8588–8596. doi: 10.1074/jbc.M109275200

Xu, B., Wang, Y., Zhang, S., Guo, Q., Jin, Y., Chen, J., et al. (2017). Transcriptomic and physiological analyses of *Medicago sativa* L. roots in response to lead stress. *PLoS One* 12:e0175307. doi: 10.1371/journal.pone.0175307

Yacoubi, R., Job, C., Belghazi, M., Chaibi, W., and Job, D. (2011). Toward characterizing seed vigor in alfalfa through proteomic analysis of germination and priming. *J. Proteome Res.* 10, 3891–3903. doi: 10.1021/pr101274f

Yacoubi, R., Job, C., Belghazi, M., Chaibi, W., and Job, D. (2013). Proteomic analysis of the enhancement of seed vigour in osmoprimed alfalfa seeds germinated under salinity stress. *Seed Sci. Res.* 23, 99–110. doi: 10.1017/S0960258513000093

Yang, S., Gao, M., Xu, C., Gao, J., Deshpande, S., Lin, S., et al. (2008). Alfalfa benefits from *Medicago truncatula*: the RCT1 gene from M. truncatula confers broad-spectrum resistance to anthracnose in alfalfa. *Proc. Natl. Acad. Sci. U.S.A.* 105, 12164–12169. doi: 10.1073/pnas.0802518105

Yang, S. S., Tu, Z. J., Cheung, F., Xu, W. W., Lamb, J. F., Jung, H. J. G., et al. (2011). Using RNA-Seq for gene identification, polymorphism detection and transcript profiling in two alfalfa genotypes with divergent cell wall composition in stems. *BMC genom.* 12:199. doi: 10.1186/1471-2164-12

Yang, S. S., Xu, W. W., Tesfaye, M., Lamb, J. F., Jung, H. J. G., VandenBosch, K. A., et al. (2010). Transcript profiling of two alfalfa genotypes with contrasting cell wall composition in stems using a cross-species platform: optimizing analysis by masking biased probes. *BMC genom.* 11:323. doi: 10.1186/1471-2164-11-323

Yin, P., Ma, Q., Wang, H., Feng, D., Wang, X., Pei, Y., et al. (2020). SMALL Leaf and BUSHY1 controls organ size and lateral branching by modulating the stability of BIG SEEDS1 in *Medicago truncatula*. *New Phytol.* [Epub ahead of print]. doi: 10.1111/nph.16449

Young, N. D., Debellé, F., Oldroyd, G. E., Geurts, R., Cannon, S. B., Udvardi, M. K., et al. (2011). The medicago genome provides insight into the evolution of rhizobial symbioses. *Nature* 480, 520–524. doi: 10.1038/nature10625

Yu, L. X., Liu, X., Boge, W., and Liu, X. P. (2016). Genome-wide association study identifies loci for salt tolerance during germination in autotetraploid alfalfa (*Medicago sativa* L.) using genotyping-by-sequencing. *Front. Plant Sci.* 7:956. doi: 10.3389/fpls.2016.00956

Yu, L. X., Zheng, P., Zhang, T., Rodringuez, J., and Main, D. (2017). Genotyping-by-sequencing-based genome-wide association studies on *Verticillium* wilt resistance in autotetraploid alfalfa (*Medicago sativa* L.). *Mol. Plant Pathol.* 18, 187–194. doi: 10.1111/mpp.12389

Zeng, N., Yang, Z., Zhang, Z., Hu, L., and Chen, L. (2019). Comparative transcriptome combined with proteome analyses revealed key factors involved in alfalfa (*Medicago sativa*) response to waterlogging stress. *Int. J. Mol. Sci.* 20:1359. doi: 10.3390/ijms20061359

Zhang, C., and Shi, S. (2018). Physiological and proteomic responses of contrasting alfalfa (*Medicago sativa* L.) varieties to PEG-induced osmotic stress. *Front Plant Sci.* 9:242. doi: 10.3389/fpls.2018.00242

Zhang, H., Zhang, J., and Wei, P. (2014). The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnol. J.* 12, 797–807. doi: 10.1111/pbi.12200

Zhang, J. (2004). *Harvesting Inducible Gene And Promoters In Alfalfa*. Dissertation thesis, University of Guelph, Guelph, ON.

Zhang, L. Q., Niu, Y. D., Huridu, H., Hao, J. F., Qi, Z., and Hasi, A. (2014). Salicornia europaea L. Na+/H+ antiporter gene improves salt tolerance in transgenic alfalfa (*Medicago sativa* L.). *Genet. Mol. Res.* 13, 5350–5360. doi: 10.4238/2014.July.24.14

Zhang, S., Shi, Y., Cheng, N., Du, H., Fan, W., and Wang, C. (2015). *De novo* characterization of fall dormant and nondormant alfalfa (*Medicago sativa L.*) leaf transcriptome and identification of candidate genes related to fall dormancy. *PloS One* 10:e0122170. doi: 10.1371/journal.pone.0122170

Zhang, T., Yu, L. X., Zheng, P., Li, Y., Rivera, M., Main, D., et al. (2015). Identification of loci associated with drought resistance traits in heterozygous autotetraploid alfalfa (*Medicago sativa* L.) using genome-wide association studies with genotyping by sequencing. *PLoS One* 10:e0138931. doi: 10.1371/journal.pone.0138931

Zhang, Y. M., Liu, Z. H., Wen, Z. Y., Zhang, H. M., Yang, F., and Guo, X. L. (2012). The vacuolar Na+- H+ antiport gene TaNHX2 confers salt tolerance on transgenic alfalfa (*Medicago sativa*). *Funct. Plant Biol.* 39, 708–716. doi: 10.1071/FP12095

Zhao, B., Liang, R., Ge, L., Li, W., Xiao, H., Lin, H., et al. (2007). Identification of drought-induced microRNAs in rice. *Biochem. Biophys. Res. Comm.* 354, 585–590. doi: 10.1016/j.bbrc.2007.01.022

Zhou, C., Han, L., Pislariu, C., Nakashima, J., Fu, C., Jiang, Q., et al. (2011). From model to crop: functional analysis of a STAY-GREEN gene in the model legume *Medicago truncatula* and effective use of the gene

for alfalfa improvement. *Plant Physiol.* 157, 1483–1496. doi: 10.1104/pp.111.185140

Zhou, M., and Luo, H. (2013). MicroRNA-mediated gene regulation: potential applications for plant genetic engineering. *Plant Mol. Biol.* 83, 59–75. doi: 10.1007/s11103-013-0089-1

Zhu, J. K. (2001). Plant salt tolerance. *Trends Plant Sci.* 6, 66–71. doi: 10.1016/S1360-1385(00)01838-0

Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. *Ann. Rev. Plant Biol.* 53, 247–273. doi: 10.1146/annurev.arplant.53.091401.143329

Zipfel, C. (2014). Plant pattern-recognition receptors. *Trends Immunol.* 35, 345–351. doi: 10.1016/j.it.2014.05.004

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Supplement II

Research article

Overexpression of alfalfa SIMK promotes root hair growth, nodule clustering and shoot biomass production

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Overexpression of alfalfa SIMK promotes root hair growth, nodule clustering and shoot biomass production

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Summary

Nitrogen-fixing rhizobia and legumes have developed complex mutualistic mechanism that allows to convert atmospheric nitrogen into ammonia. Signalling by mitogen-activated protein kinases (MAPKs) seems to be involved in this symbiotic interaction. Previously, we reported that stress-induced MAPK (SIMK) shows predominantly nuclear localization in alfalfa root epidermal cells. Nevertheless, SIMK is activated and relocalized to the tips of growing root hairs during their development. SIMK kinase (SIMKK) is a well-known upstream activator of SIMK. Here, we characterized production parameters of transgenic alfalfa plants with genetically manipulated SIMK after infection with *Sinorhizobium meliloti*. *SIMKK RNAi* lines, causing strong downregulation of both *SIMKK* and *SIMK*, showed reduced root hair growth and lower capacity to form infection threads and nodules. In contrast, constitutive overexpression of GFP-tagged SIMK promoted root hair growth as well as infection thread and nodule clustering. Moreover, *SIMKK* and *SIMK* downregulation led to decrease, while overexpression of GFP-tagged SIMK led to increase of biomass in above-ground part of plants. These data suggest that genetic manipulations causing downregulation or overexpression of SIMK affect root hair, nodule and shoot formation patterns in alfalfa, and point to the new biotechnological potential of this MAPK.

Introduction

Medicago sativa L., commonly known as alfalfa or 'lucerne', is the world's leading forage legume and a low-input bioenergy crop (Aung *et al*., 2015). The genus Medicago includes both perennial and annual species. Perennial legumes have important roles in providing cheap and widespread forages of high nutritive value, in soil protection, or improvement of nitrogen-limited soils (Radović *et al*., 2009). Due to variable genetic origin, alfalfa can adapt to different environmental conditions (Radović *et al*., 2003).

Mitogen-activated protein kinase (MAPK) pathways represent universal signalling modules in eukaryotes, including yeasts, animals and plants (Ichimura *et al*., 2000; Šamajová *et al*., 2013a). A typical MAPK cascade is organized into three-tiered module composed of MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK (Cristina *et al*., 2010). MAPKs are phosphorylated and thereby activated by MAPKKs via dual phosphorylation of conserved threonine and tyrosine residues at a TXY motif. MAPKKs themselves are activated by MAPKKKs through phosphorylation of two serine/threonine residues in the S/T-X3-5-S/T motif (Chen *et al*., 2017; Jonak *et al*., 2002). Signalling through MAPK cascades can regulate various cellular and developmental processes (Komis *et al*., 2018; Šamajová *et al*., 2013a). MAPKs phosphorylate and regulate broad range of substrates such as other protein kinases, nuclear transcription factors, cytoskeletal components and proteins involved in metabolism and vesicular trafficking (Šamajová *et al*., 2013b; Smékalová *et al*., 2014). Plant MAPKs can be activated by several abiotic stimuli such as cold, drought or salinity (Ovečka *et al*., 2014; Šamajová *et al*., 2013a; Sinha *et al*., 2011) and biotic stimuli such as pathogens, pathogen-derived toxins or generally by microbe-associated molecular patterns (MAMPs; Pitzschke *et al*., 2009; Rasmussen *et al*., 2012).

In alfalfa, stress-induced MAPK (SIMK) was identified as a salt stress- and elicitor-induced MAPK (Cardinale *et al*., 2000, 2002; Munnik *et al*., 1999). Yeast two-hybrid screen and activation studies identified SIMK kinase (SIMKK) as an upstream activator of SIMK (Cardinale *et al*., 2002; Kiegerl *et al*., 2000). Interaction between SIMK and SIMKK upon salt stress is quite specific, because no interaction was observed with three other MAPKs, such as MMK2 (Jonak *et al*., 1995), MMK3 (Bögre *et al*., 1999) and SAMK (Jonak *et al*., 1996). SIMKK is a functional dual-specificity protein kinase that phosphorylates SIMK on both threonine and tyrosine residues of the activation loop (Cardinale *et al*., 2002; Kiegerl *et al*., 2000). Previously, we have shown that SIMK predominantly localizes in nuclei while it is activated and redistributed from nucleus into growing root hair tips (Šamaj *et al*., 2002). In latrunculin B-treated root hairs, SIMK relocated back to the nucleus while after jasplakinolide treatment, SIMK colocalized with thick F-actin cables in the cytoplasm. Thus, these drugs affecting actin cytoskeleton (Baluška *et al*., 2000b; Bubb *et al*., 2000) have a direct impact on the intracellular localization of SIMK (Šamaj *et al*., 2002).

Legumes are able to perform symbiotic interactions with nitrogen-fixing soil bacteria collectively called rhizobia (e.g. *Bradyrhizobium* or *Sinorhizobium*) which can reduce atmospheric dinitrogen (N_2) into ammonium (NH_3) in specialized organs, the so-called root nodules (Carro *et al*., 2018; Wang *et al*., 2018). This type of symbiosis plays an essential role in both agronomical and natural systems (Geurts *et al*., 2016; Oldroyd *et al*., 2011; Ryu *et al*., 2017). It begins with a signal exchange between rhizobia and its host plant (Oldroyd *et al*., 2013). Flavonoid compounds released by legumes represent the signal for bacteria to produce nodulation (Nod) factors. Nod factors are lipochitooligosaccharides (LCOs) that, together with rhizobia, induce specific responses required for nodulation process in legume host plants. Nod factors are responsible for the establishment of pre-infection thread structures, while Nod factors together with rhizobia are directly involved in the formation of infection threads (ITs), thin tubular structures filled with rhizobia and penetrating several root cell layers towards target cells in newly developing nodules (van Brussel *et al*., 1992; Jones *et al*., 2007; Perret *et al*., 2000; Remigi *et al*., 2016). Rhizobia released from ITs, enfolded by a membrane of plant origin, are transformed into bacteroids that are able to fix nitrogen (Jones *et al*., 2007; Oldroyd *et al*., 2011; Wang *et al*., 2018). Negative and positive regulatory pathways control the number of nodules on the legume root system (Caetano-Anollés and Bauer, 1988). Plant hormones linked to stress and defence responses, such as salicylic acid (SA), abscisic acid (ABA), jasmonate (JA) and ethylene, are negative regulators of Nod factor signalling (Roy *et al*., 2020; Ryu *et al*., 2012). Moreover, these hormones are also common activators of MAPK cascades in various plants (Cristina *et al*., 2010; Ryu *et al*., 2017). Upon infection, rhizobia activate MAPK cascade and stress-related responses early during infection (Lopez-Gomez *et al*., 2012).

In this study, we generated stable transgenic alfalfa plants overexpressing GFP-tagged SIMK. These plant lines with overexpressed and activated GFP-SIMK showed longer root hairs, more ITs, clustered nodules and increase in above-ground biomass production. In addition, we employed *SIMKK RNAi* lines showing downregulation of both *SIMKK* and *SIMK* accompanied by shorter root hairs, less ITs and nodules, and lower biomass of above-ground plant parts. These data suggest that genetic manipulation of *SIMK* affects root hair, IT, nodule and biomass production in alfalfa.

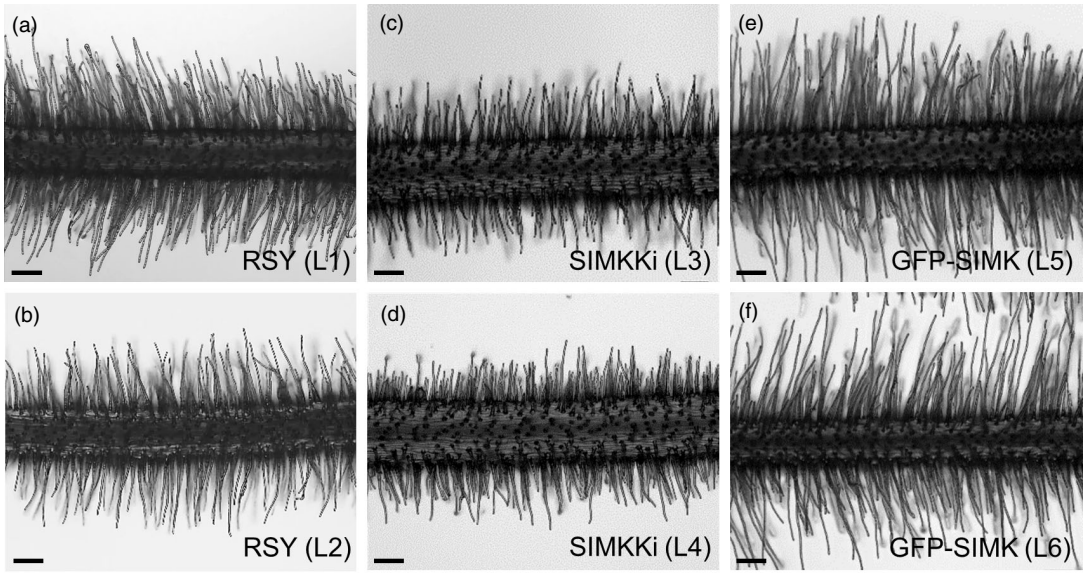
Results

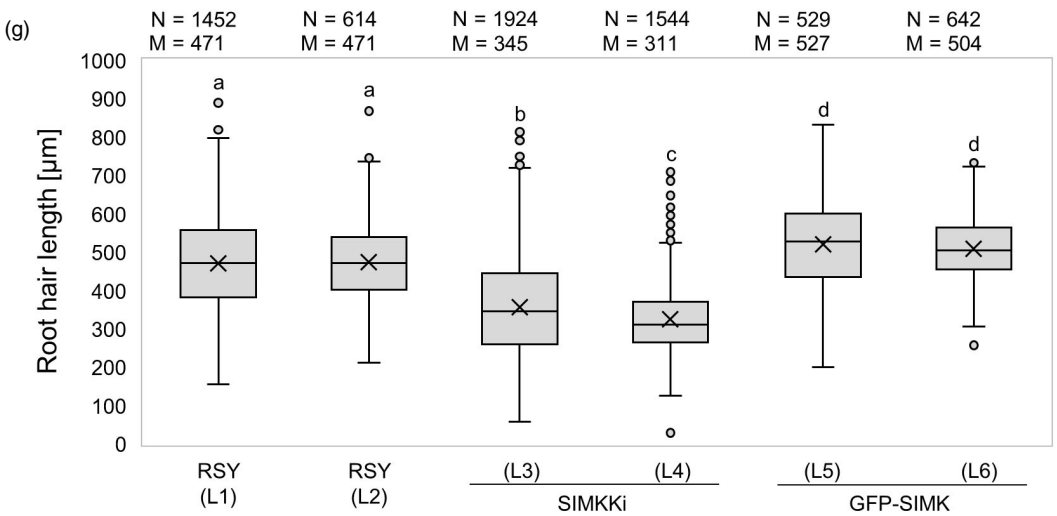
SIMK-dependent root hair phenotypes

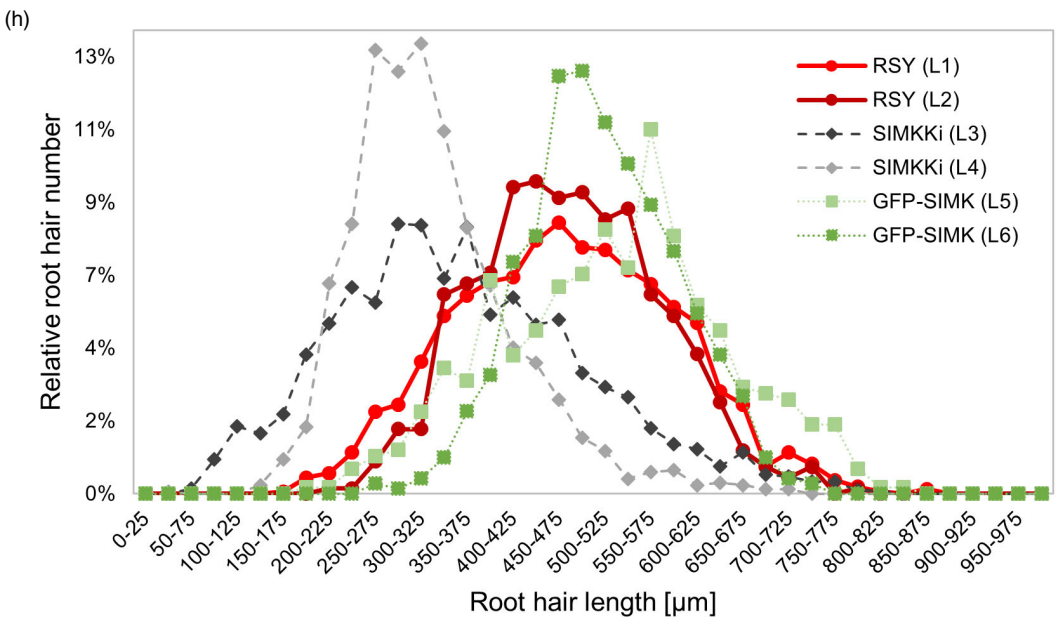
Root hair phenotypes were examined in stable transformed alfalfa lines with downregulated or upregulated *SIMK,* using *SIMKK RNAi* or overexpression (both under constitutive *35S* promoter) approaches, respectively. For evaluation of root hair growth efficiency, the appropriate parameter of root hair length in mature parts of the root was measured in these lines. In control wild-type plants, root hair length median value in both analyzed lines (RSY, lines L1 and L2) was 471 μm (Figure 1a,b,g). In transgenic lines carrying *SIMKK RNAi* construct (annotated as SIMKKi, lines L3 and L4), showing strong downregulation of *SIMKK* and *SIMK* transcripts (Figure 2a) and SIMK protein (Figure 2b,d), root hair length median decreased to 345 μm and 311 μm, respectively (Figure 1c,d,g). In contrast, transgenic lines overexpressing *35S::GFP:SIMK* in wild-type RSY background (annotated as GFP-SIMK, lines L5 and L6) showed an increase of root hair length median to 527 μm and 504 μm, respectively (Figure 1e,f,g). Root hair phenotypes of alfalfa lines presented in the form of contingency graph with 25 μm intervals (Figure 1h) showed a relative root hair number (%) found within each root hair length interval. In SIMKKi lines (L3, L4), the root hair distribution pattern was shifted to the left (Figure 1h) in comparison with RSY (L1, L2), which means an earlier cessation of root hair tip growth. In contrast, the distribution of root hairs in GFP-SIMK lines (L5, L6) was shifted to the right while distribution curves showed higher values in the range of longer root hairs (Figure 1h), which means later cessation of the tip growth and higher proportion of longer root hairs.

Quantitative RT-PCR of native *SIMKK*, native *SIMK* (*SIMKe*) and total *SIMK* (meaning the sum of *GFP-SIMK* and *SIMKe* levels) transcripts was performed in order to gain insight into transcriptional regulation of selected genes in transgenic lines as compared to RSY. This analysis revealed downregulation of *SIMKK* gene in SIMKKi line L4 to approximately 63% compared to *SIMKK* transcript level in RSY L1. Simultaneously, SIMKKi line L4 showed downregulation of native *SIMKe* transcript level to approximately 30-40% (screened by two different sets of primers) compared to *SIMK* transcript level in RSY L1 (Figure 2a). In GFP-SIMK line L5, total *SIMK* transcript level was upregulated approximately 3.14 times due to overexpression of *GFP-SIMK*, while native *SIMKe* transcript level was downregulated to approximately 65% compared to *SIMK* transcript level in RSY L1 (Figure 2a). Western blot analysis for quantification of SIMK protein level (Figure 2b,d) and phosphorylated SIMK (pSIMK) protein level (Figure 2c,e) was performed in order to explain previously obtained phenotypical results at the level of protein abundance and activity. Endogenous SIMK protein with molecular mass around 46 kDa and recombinant GFP-SIMK protein with molecular mass around 72 kDa (Figure 2b) were quantified (Figure 2d). Relative SIMK abundance was strongly decreased in SIMKKi line L4 to approximately 1 % (Figure 2b,d). Relative GFP-SIMK abundance was strongly increased in *35S::GFP:SIMK* line L5 to approximately 6.48 times (Figure 2b,d) showing upregulation

Figure 1 Root hair phenotypes in alfalfa RSY, *SIMKK RNAi* (SIMKKi) lines and lines overexpressing GFP-SIMK. (a,b) Representative images of root hair phenotypes of plants from two independent lines (L1, L2) of control wild-type RSY, (c,d) two independent transgenic lines with *SIMKK RNAi* construct (SIMKKi L3, L4) and (e,f) two independent transgenic lines expressing *35S::GFP:SIMK* in wild-type RSY background (GFP-SIMK L5, L6). (g) Box plot graph depicting comparison in root hair length in indicated lines, number of observations N and median value M. Statistics was calculated in SigmaPlot11.0 using Kruskal–Wallis one-way analysis of variance on ranks (Dunn's method) and is based on $N = 529–1924$. The numbers of root hairs observed were 1452 (RSY L1), 614 (RSY L2), 1924 (SIMKKi L3), 1544 (SIMKKi L4), 529 (GFP-SIMK L5) and 642 (GFP-SIMK L6). Different lower case letters indicate statistical significance between treatments ($P < 0.05$). (h) Relative distribution of root hair lengths in indicated alfalfa lines. Normalized root hair number was evaluated using 25 μm intervals distribution. Transgenic lines show different distribution pattern of root hair lengths as compared to RSY wild-type lines. Scale bar: (a–f) 200 μm.


(a)
RSY (L1)
(c)
SIMKKi (L3)
(e)
GFP-SIMK (L5)
(b)
RSY (L2)
(d)
SIMKKi (L4)
(f)
GFP-SIMK (L6)


(g)
N = 1452
M = 471
N = 614
M = 471
N = 1924
M = 345
N = 1544
M = 311
N = 529
M = 527
N = 642
M = 504
Root hair length [µm]
1000
900
800
700
600
500
400
300
200
100
0
a
a
b
c
d
d
RSY
(L1)
RSY
(L2)
(L3)
(L4)
SIMKKi
(L5)
(L6)
GFP-SIMK


(h)
Relative root hair number
13%
11%
9%
7%
4%
2%
0%
RSY (L1)
RSY (L2)
SIMKKi (L3)
SIMKKi (L4)
GFP-SIMK (L5)
GFP-SIMK (L6)
0-25
50-75
100-125
150-175
200-225
250-275
300-325
350-375
400-425
450-475
500-525
550-575
600-625
650-675
700-725
750-775
800-825
850-875
900-925
950-975
Root hair length [µm]

similarly to relative transcript level (Figure 2a), while relative abundance of endogenous SIMK showed a decrease to approximately 49 % (Figure 2b,d) similarly to reduced relative transcript level (Figure 2a). These results are consistent with the root hair length phenotypes and indicate that relative SIMK abundance in above-mentioned lines correlates with effectiveness of the root hair tip growth.

Phospho-specific antibody was used to check out activity status of respective proteins. Endogenous phosphorylated pSIMK protein with molecular mass around 46 kDa and also phosphorylated GFP-pSIMK with molecular mass around 72 kDa (Figure 2c) were quantified (Figure 2e). Relative level of pSIMK was considerably decreased in SIMKKi line L4 to approximately 12 % (Figure 2c,e) while relative level of GFP-pSIMK was strongly increased in *35S::GFP:SIMK* line L5 to approximately 8.21 times and relative level of endogenous pSIMK level showed non-significant change compared to RSY line L1 (Figure 2c,e). These results are also consistent with root hair length phenotypic results. Moreover, they fit with previously published results showing that root hair growth requires activated SIMK (Šamaj *et al*., 2002).

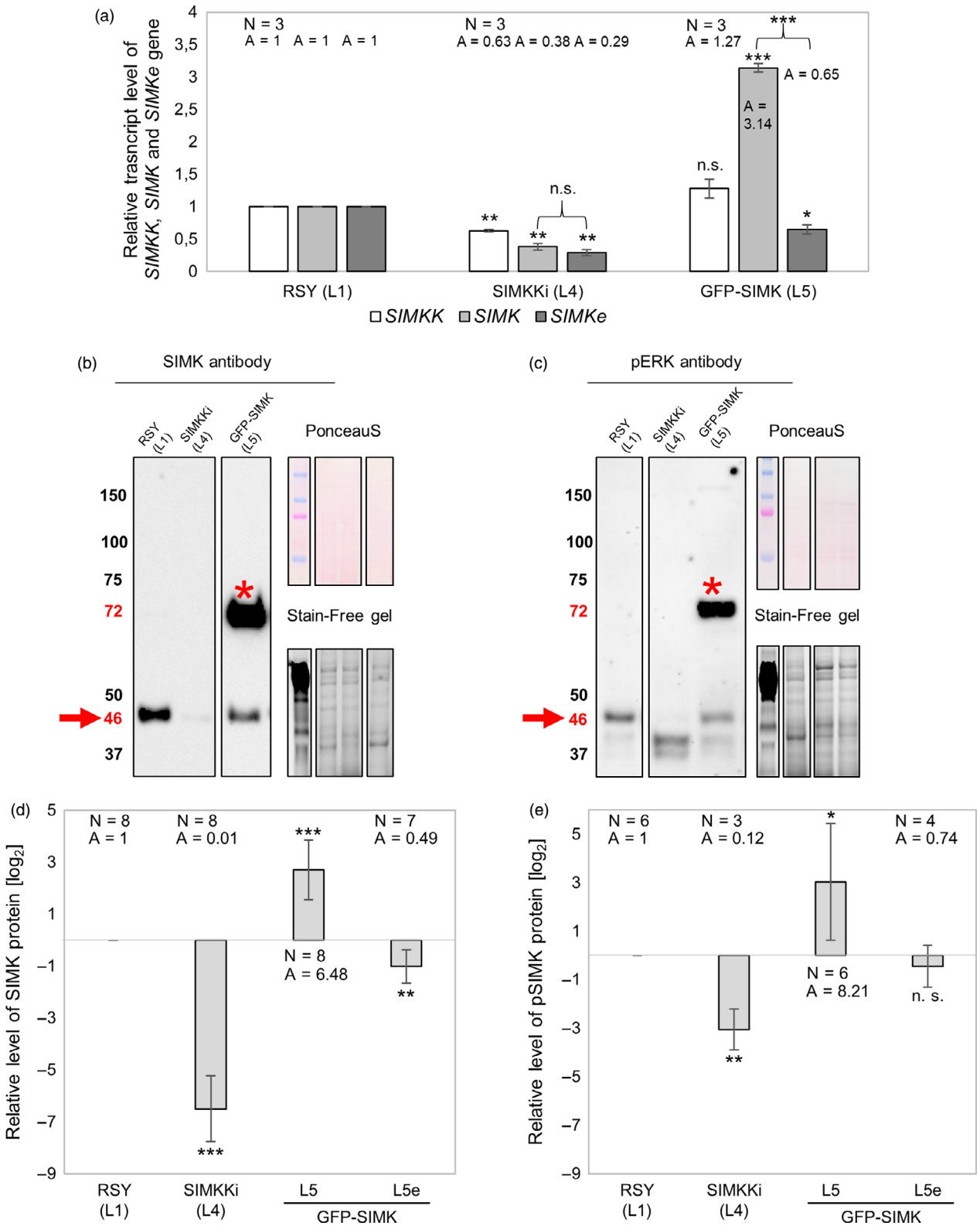
Impact of overexpressed GFP-SIMK on infection thread and nodule formation

To get further insight into the possible involvement of SIMK in alfalfa symbiosis with beneficial microbes, we examined infection thread (IT) and nodule formation (Figure 3) after inoculation with *S. meliloti* (Sm2011 strain) labelled with monomeric red fluorescent protein (mRFP). Evaluation of ITs was performed 10-day post-inoculation (10 dpi) per whole root system in alfalfa RSY plants L1 (Figure 3a), transgenic SIMKKi plants L4 (Figure 3b) and GFP-SIMK plants L5 (Figure 3c). Transgenic lines were compared to RSY and between each other. GFP-SIMK line L5 showed increased IT clustering (Figure 3c-e) and these ITs seem to be longer, consistently with previously observed long root hair phenotype. Quantitative analysis showed that most of ITs in RSY L1 (76.34 %) and SIMKKi L4 (83.61 %) developed individually, while only 45.74 % of ITs was spatially separated in GFP-SIMK L5 (Figure 3d). The rest, 54.26 % of ITs in GFP-SIMK L5 line was present in clusters. Portion of ITs in clusters was only 23.66 % in RSY L1 and 16.39 % in SIMKKi L4 (Figure 3d). In RSY L1 and SIMKKi L4, most of the clusters contained two or three ITs, while in GFP-SIMK L5 there was a significant amount of clusters possessing also four or five ITs. In 5.7 % of clusters in GFP-SIMK L5, we found more than five ITs; it occurred in only 4.5 % of clusters in RSY L1, while it was absent completely in SIMKKi L4 (Figure 3e).

Together with previously published data that activated SIMK is required for root hair growth (Šamaj *et al*., 2002), SIMK can be involved also in the symbiosis with *S. meliloti*. Thus, more efficient formation of ITs in clusters may be directly induced by SIMK overexpression. To this point, we characterized association of SIMK with IT formation by localization of GFP-SIMK in transgenic line L5 using live cell imaging. Alternatively, we detected SIMK also in RSY line L1 using immunolocalization. Both methods revealed association of SIMK with *S. meliloti* internalization sites in root hairs, such as specific accumulation of GFP-SIMK in infection pockets (Figure S1a-d) and in growing ITs (Figure S1e-i). Immunostaining of phosphorylated MAPKs using phospho-specific pERK 44/42 antibody showed colocalization with SIMK-specific signal in ITs (Figure S1i-l), indicating IT-specific presence of SIMK in activated form.

When infection threads reach the nodule primordium, rhizobia are released into host cells by an endocytosis, which allows to form functional nitrogen-fixing bacteroids within infected plant cells of the root nodule. Evaluation of nodule formation was performed 15 dpi per whole root system in alfalfa RSY plants of L1 (Figure 3f, S2a,d), transgenic SIMKKi plants of L4 (Figure 3g, S2b,e) and GFP-SIMK plants of L5 (Figure 3h, S2c,f). SIMKKi line L4 formed significantly less nodules than RSY L1, while GFP-SIMK line L5 produced similar number of nodules as RSY line L1 (Figure S2g). Interestingly, GFP-SIMK line L5 often produced nodules in clusters (Figure 3h, S2c,f), which was less frequent in RSY line L1 and in SIMKKi line L4 (Figure S2a,b,d,e). Analysis of nodule clustering showed that 89.8 % of nodules in RSY L1, 95 % in SIMKKi L4 and 87.4 % in GFP-SIMK L5 developed individually (Figure 3i). However, clusters in transgenic GFP-SIMK L5 line contained much higher number of nodules in comparison with RSY L1 and SIMKKi L4 (Figure 3j). Detailed analysis revealed that 27.3 % of clusters in GFP-SIMK line L5 possessed more than five/ six and more nodules while in RSY L1 it was only in 2.9 % of clusters (Figure 3j) and SIMKKi line L4 did not form clusters with more than five nodules (Figure 3j). On the contrary, RSY line L1 and SIMKKi line L4 had 62.9 % and 61.5 % of clusters formed from two nodules only, respectively, as compared to 18.2 % of such clusters in GFP-SIMK line L5 (Figure 3j). It is resembling IT clustering where the ratio of clusters with two ITs represented 54.5 % and 60 % in RSY line L1 and SIMKKi line L4, respectively, but it was only 32.9 % in GFP-SIMK line L5 (Figure 3e). These data suggest that GFP-SIMK line L5 is very effectively able to produce ITs and nodules spatially organized in bigger clusters (Figure 3c,h). To confirm the nodule phenotype of characterized lines also *in vivo*, the root systems of alfalfa plants were documented after extraction from soil (Figure S3). Thorough surface examination

Figure 2 Expression analysis of *SIMKK* and *SIMK* genes by quantitative real-time (qRT-PCR) and immunoblotting analysis of total endogenous SIMK, active endogenous SIMK and both total and active GFP-SIMK. (a) Deregulated transcript levels of *SIMKK,* total (endogenous native *SIMKe* + GFP-tagged) *SIMK* and endogenous native *SIMKe* gene in SIMKKi L4 and GFP-SIMK L5 transgenic lines of alfalfa. (b) Western blot detection of SIMK and GFP-SIMK bands using SIMK antibody and (c) detection of active amount of respective proteins pSIMK and GFP-pSIMK bands using pERK antibody in root tissue of control and transgenic alfalfa plants of SIMKKi (L4) and expressing *35S::GFP:SIMK* (L5). Arrows point to the 46 kDa which corresponds to (b) endogenous SIMK and (c) endogenous pSIMK, while asterisks show bands around 72 kDa which corresponds to (b) GFP-SIMK and (c) GFP-pSIMK. (d,e) Log_2 graphs depicting comparison of protein levels in respective lines (SIMKKi L4, GFP-SIMK L5) relative to RSY L1, number of observations N and average value A (presented as inversed log_2 values). GFP-SIMK L5e refer to endogenous level of protein, while GFP-SIMK L5 refers to GFP-SIMK level. (d) Relative SIMK protein level in roots of control and transgenic plants (RSY L1, SIMKKi L4, GFP-SIMK L5). (e) Relative pSIMK protein level in roots of control and transgenic plants (RSY L1, SIMKKi L4, GFP-SIMK L5). (a,d,e) Statistics was calculated in Microsoft Excel using *t*-test and is based on $N = 3–8$. Error bars show ± SD. Asterisks indicate statistical significance between treatments, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n. s. indicates no statistical significance.



revealed that RSY line L1 (Figure S3a), SIMKKi line L4 (Figure S3b) and GFP-SIMK line L5 (Figure S3c) formed nodules in soil. In GFP-SIMK line L5, formation of nodules in clusters has been confirmed (Figure S3c, inset image). Interestingly, development of the root system did not show considerable differences among lines (Figure S3). Analyzed plants were 9 months after transfer to soil, and the same plants were used also for above-ground biomass analysis.

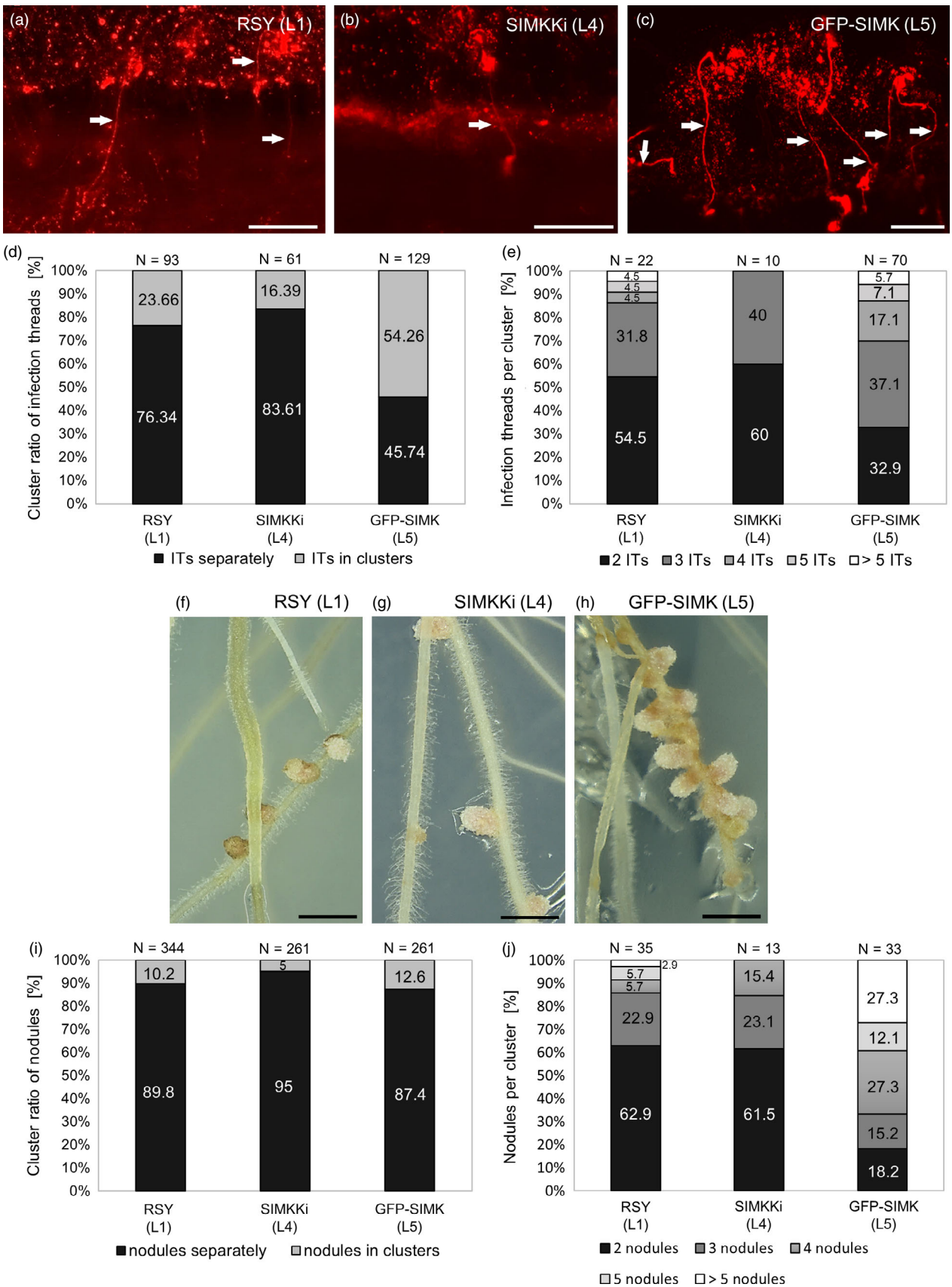

(a)
RSY (L1)
(b)
SIMKKi (L4)
(c)
GFP-SIMK (L5)
(d)
Cluster ratio of infection threads [%]
N = 93
N = 61
N = 129
23.66
76.34
16.39
83.61
54.26
45.74
RSY (L1)
SIMKKi (L4)
GFP-SIMK (L5)
ITs separately
ITs in clusters
(e)
Infection threads per cluster [%]
N = 22
N = 10
N = 70
4.5
4.5
4.5
31.8
54.5
40
60
5.7
7.1
17.1
37.1
32.9
2 ITs
3 ITs
4 ITs
5 ITs
> 5 ITs
(f)
RSY (L1)
(g)
SIMKKi (L4)
(h)
GFP-SIMK (L5)
(i)
Cluster ratio of nodules [%]
N = 344
N = 261
N = 261
10.2
89.8
5
95
12.6
87.4
nodules separately
nodules in clusters
(j)
Nodules per cluster [%]
N = 35
N = 13
N = 33
5.7
5.7
22.9
62.9
2.9
15.4
23.1
61.5
27.3
12.1
27.3
15.2
18.2
2 nodules
3 nodules
4 nodules
5 nodules
> 5 nodules

Figure 3 Infection thread and nodule formation in alfalfa roots inoculated with *Sinorhizobium meliloti*-mRFP. (a–c) Overview of the infection threads containing *S. meliloti* with mRFP (white arrows) in roots of (a) wild-type RSY line L1, (b) in transgenic SIMKKi line L4 and (c) in transgenic GFP-SIMK line L5 at 10 dpi. (d) Ratio of individual/clustered infection threads (in %) at 10 dpi. (e) Number of infection threads per cluster (in %) at 10 dpi. (f–h) Representative images of root nodules formed in respective alfalfa lines, (f) control RSY line L1, (g) SIMKKi line L4 and (h) GFP-SIMK line L5 inoculated with *S. meliloti*-mRFP 15 dpi on Fåhreus medium. (i) Ratio of individual/clustered nodules (in %) at 15 dpi. (j) Number of nodules per cluster (in %) at 15 dpi. *N* = number of observations. Scale bar: (a–c) 100 μm, (f–h) 1 cm. Dpi = day post-inoculation.

Impact of overexpressed GFP-SIMK on shoot biomass and leaf formation

Besides the documentation of root hair phenotypes and subsequent alfalfa-*Sinorhizobium* symbiotic interaction, we analyzed also impact of SIMK abundance and activity on alfalfa above-ground biomass production (Figure 4) and on leaf development (Figure S4). Formation and regrowth of new individual shoots in alfalfa can be induced and synchronized by cutting off the green part. Documentation of plants growing in pots in the soil 60 days after cutting revealed smaller and thinner habitus of above-ground parts in SIMKKi line L4 (Figure 4b) in comparison with RSY line L1 (Figure 4a). However, above-ground parts in GFP-SIMK line L5 showed more robust and bushy habitus (Figure 4c). In quantitative terms, GFP-SIMK line L5 produced significantly longer shoots (Figure 4d) with significantly higher weight (Figure 4e) in comparison with both RSY line L1 and SIMKKi L4 plants. Consistently with the qualitative analysis (Figure 4a-c), SIMKKi line L4 developed significantly lower number of shoots per plant in comparison with both RSY L1 and GFP-SIMK L5 (Figure 4f). Taking together, the weight of above-ground biomass was significantly decreased in SIMKKi L4 plants, but significantly increased in GFP-SIMK L5 plants in comparison to control RSY L1 (Figure 4g). This results indicate that, in addition to the process of alfalfa-*Sinorhizobium* symbiotic interactions, SIMK likely plays also an important role in the regulation of shoot development and green biomass production.

Individual shoots of RSY line L1 regrown on plants 15 days after cutting off the green part contained shoot apical meristem with leaf primordia and already formed trifoliate compound leaves. They developed from nodes, interconnected by elongated internodes (Figure S4a). The size and shape of leaves of SIMKKi line L4, however, were considerably affected (Figure S4b). Leaves were smaller, narrower and slightly curled in SIMKKi line L4 in comparison with RSY line L1 (Figure S4b). SIMK overexpression in GFP-SIMK line L5 led to enhanced development of shoots (Figure 4c-e), represented mainly by formation of large leaves with long petioles (Figure S4c). Size and shape of leaves were analyzed in more details. Five representative individual shoots from analyzed plants (from 5 plants per line) were selected, and first three fully developed leaves beneath the shoot apex were dissected, photographed and measured. Among leaves of the individual shoot, regardless of alfalfa line being analyzed there were no visible morphological differences, only the smallest size of the first leaf in comparison with second and third leaf according to the leaf developmental sequence within the shoot (Figure S4d-f). Regarding the shape, SIMKKi line L4 showed much narrow leaves and less notched at the apex, while GFP-SIMK line L5 showed longer and broader leaves in comparison with more oval leaves of RSY line L1 (Figure S4d-f). Comparing size of analyzed leaves, SIMKKi line L4 contained always the smallest leaves, which was demonstrated in all types (first, second and third leaf, Figure S4g). Interestingly, the third leaves of GFP-SIMK line L5 showed the largest leaf area (Figure S4g). These phenotypical differences were corroborated by length of leaf petioles that were significantly shorter in all leaves of SIMKKi line L4, but significantly longer in third leaves of GFP-SIMK line L5 (Figure S4h). These data indicate that SIMK plays pleiotropic roles in developmental programmes not only in alfalfa roots, but also in above-ground organs. This is documented by reduced shoot and leaf development when SIMK is downregulated in SIMKKi L4 line. However, SIMK overproduction in GFP-SIMK line L5 leads to enhanced shoot biomass production, leaf and petiole development, which may represent potentially valuable biotechnological trait of alfalfa as an important forage crop.

Subcellular localization of GFP-SIMK

In order to observe subcellular localization of SIMK in alfalfa plants, we tagged it with GFP marker and overexpressed such fusion protein under the control of *CaMV 35S* promoter. Subcellular localization of GFP-SIMK was performed using confocal laser scanning microscopy (CLSM) and Airyscan CLSM. First, transient transformation of *Nicotiana benthamiana* leaves was performed to test expression of *35S::GFP:SIMK* construct. GFP-SIMK was preferentially localized to the nuclei (except nucleoli), and it was also dispersed in cytoplasmic structures (Figure S5). In stably transformed alfalfa plants, GFP-SIMK preferentially accumulated in the nucleus and less in the cytoplasmic structures of epidermal cells and stomata in leaf (Figure S6a) and hypocotyl (Figure S6b). Maximum intensity projection provided overview of root tip and revealed nuclear and cytoplasmic GFP-SIMK localization (Figure S6c) with depleted signal in nucleoli. Similar subcellular localization was found also in root hairs (Figure S6d) and epidermal cells of lateral roots (Figure S6e). In growing root hairs, GFP-SIMK was mostly localized in nuclei and in the cytoplasm at the root hair tips (Figure S6d). This pattern of subcellular localization in root cells was confirmed in GFP-SIMK line L5 by using whole mount immunofluorescence co-labelling (Tichá *et al*., 2020) with GFP-specific (see Materials and Methods) and phospho-specific (anti-phospho-p44/42) antibodies (Figure S7). Imaging of co-immunolabelled samples using Airyscan CLSM revealed that GFP-SIMK is localized in distinct spot-like structures in the nucleoplasm and in cytoplasmic structures, preferentially in activated form (Figure S7). Live cell imaging of GFP-tagged SIMK, as well as SIMK immunolocalization in root hairs of alfalfa plants inoculated with *S. meliloti* revealed presence of SIMK along ITs and its accumulation in infection pockets (Figure S1).

Subcellular localization of SIMK in root nodules

To analyse the expression level and localization of GFP-SIMK in root nodules of L5 line (Figure 5) inoculated with mRFP-marked *S. meliloti*, nodules were harvested 10 dpi (Figure 5a) and 20 dpi (Figure 5b) and analyzed by CLSM live cell imaging with the appropriate settings of lasers for GFP and mRFP channels. GFP-SIMK was expressed in young nodules (Figure 5a) and also in

mature nodules, including meristematic (I), infection (II) and symbiotic (III) zones (Figure 5b).

Next, root nodules developed on alfalfa root system 15 dpi with *S. meliloti* were characterized using immunofluorescence localization microscopy. Hand-made median nodule sections were used for visualization of cell nuclei (Figure 6a), for immunolocalization of SIMK using anti-SIMK-specific antibody (Figure 6b,d,e) and for immunolocalization of activated pool of MAPKs using anti-phospho-p44/42 antibody (Figure 6c-e). The pattern of SIMK and activated MAPK localization was documented in infection (II), symbiotic (III) and senescent (IV) nodule zones (Figure 6f). Importantly, DAPI staining of the DNA nuclear content in alfalfa root nodule cells stained also *S. meliloti* (Figure 6g,j,m). In the infection zone (II), there were clearly visible ITs among the nodule cells, forming terminal branches inside of the cells, from which bacteria were released (Figure 6g). SIMK was localized mainly around ITs in the form of prominent spots (Figure 6h). Labelling with anti-phospho-p44/42 antibody showed colocalization with SIMK-specific signal, suggesting that MAPKs present in these spots were phosphorylated (Figures 6i). Waste majority of cells in the symbiotic zone (III) contained bacteroids already differentiated in the cytoplasm, although several ITs were still present in this zone (Figure 6j). SIMK was localized mainly in cortical layer of symbiotic cells and in some prominent spots in the cytoplasm (Figure 6k). Labelling pattern with anti-phospho-p44/42 antibody showed high colocalization

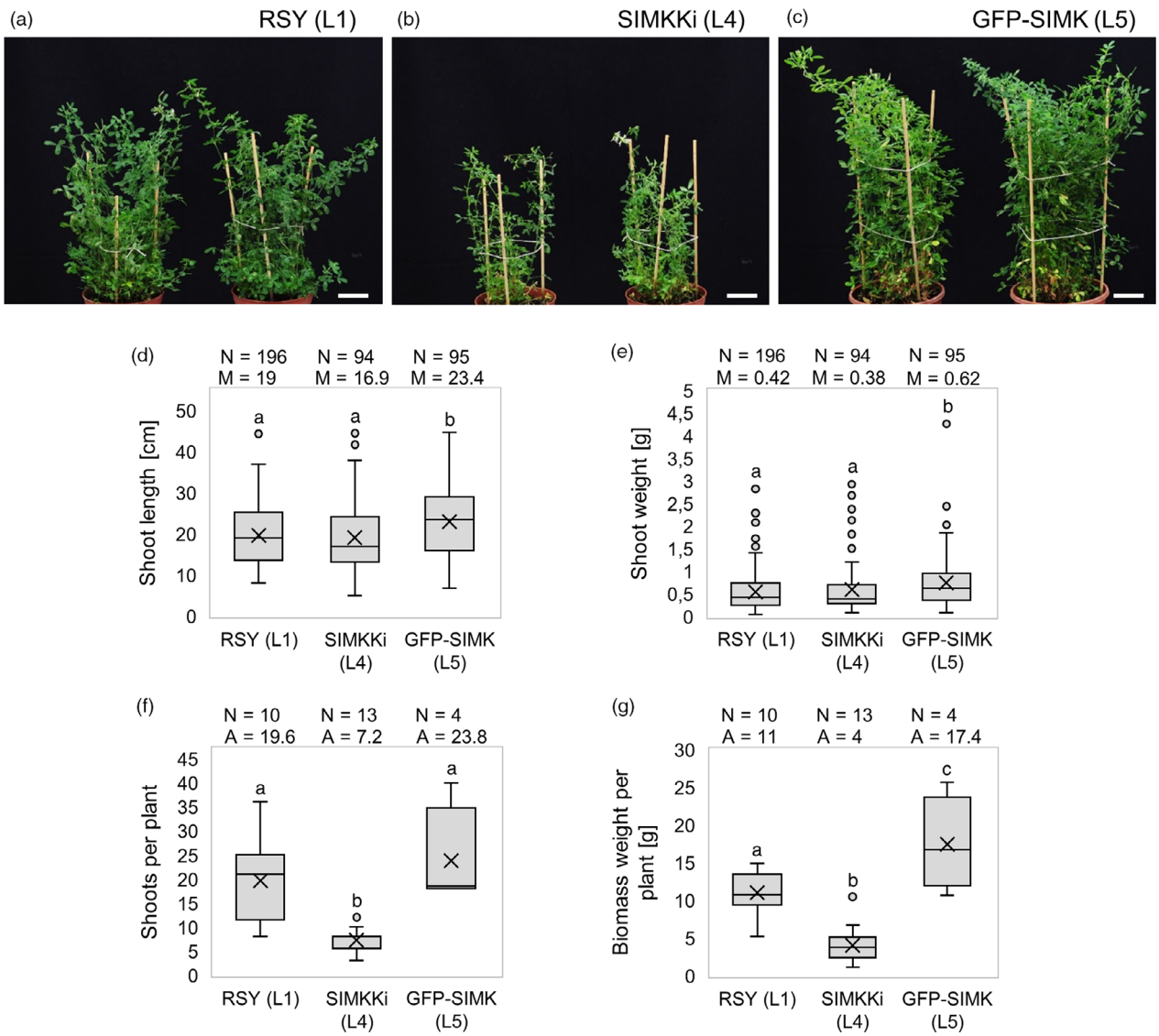


Figure 4 Shoot biomass production in transgenic alfalfa plants grown *in vivo*. (a–c) Representative images of above-ground parts of mature plants grown in pots in control RSY L1 (a), SIMKKi L4 (b) and GFP-SIMK L5 (c). Regrown plants were documented 60 days after cutting the shoots. (d) Box plot graph depicting comparison in shoot length of indicated lines, number of observations N and median value M. (e) Box plot graph depicting comparison in shoot weight of indicated lines, number of observations N and median value M. (f) Box blot graph depicting comparison in number of shoots per plant of indicated lines, number of observations N and average value A. (g) Box plot graph depicting comparison in biomass weight per plant of indicated lines, number of observations N and average value A. Statistics was calculated in SigmaPlot11.0 using Kruskal–Wallis one-way analysis of variance on ranks (Dunn's method) (d, e), or using one-way analysis of variance (Holm–Sidak method) (f, g) and is based on (d, e) N = 94–196 and (f, g) N = 4–13. Different lower case letters indicate statistical significance between treatments ($P < 0.05$). Scale bar: (a–c) 4 cm.

with SIMK signal (Figure 6l). In the senescent zone of nodules (IV), symbiotic cells contained bacteroids and showed positive reaction to immunolabelling, while nodule cells entering the senescence stage were massively enriched with bacteroids (overstained with DAPI) and vacuolated, while their immunoreactivity to MAPK-specific antibodies was abolished (Figure 6m-o). Remnants of ITs were still present; however, SIMK, unlike the situation in cells of the infection zone (Figure 6h), was not associated with them (Figure 6n). Rather, SIMK was massively localized to cell cortex and to some spots in the cytoplasm (Figure 6n). Colocalization with anti-phospho-p44/42 antibody (Figure 6o) indicated that SIMK in these locations was activated.

Detailed analyses revealed that ITs invade cells in the infection zone (II) and terminate by branched finger-like extensions from which bacteria were released in the form of infection droplets (Figure 7a). SIMK was accumulated in these subcellular domains (faint red signal in Figure 7b,d), but particularly finger-like extensions and released bacteria in the cytoplasm were decorated by prominent spot-like structures, highly positive for both SIMK (Figure 7b,d) and phosphorylated MAPKs (Figure 7c,d). In the symbiotic zone (III), bacteria were already internalized in differentiated bacteroids (Figure 7e), which changed dramatically also SIMK localization pattern. Both SIMK (Figure 7f) and phosphorylated MAPKs (Figure 7g) were located in nuclei, in cytoplasm among differentiated bacteroids, and in bright spot-like structures around the nuclei or in the nuclei. In all these places, signal of both SIMK and phosphorylated MAPKs colocalized (Figure 7h). In the senescent zone (IV), both DAPI signal intensity (Figure 7i) and immunofluorescence detection (Figure 7j-l) clearly discriminated between still active and senescing cells. In active cells, SIMK (Figure 7j) and phosphorylated MAPKs (Figure 7k) were located in cytoplasm among bacteroids and in spot-like structures, and both colocalized (Figure 7l). SIMK was located in small spots close to differentiated bacteroids (Figure 7j,l) and topologically similar localization signal was achieved using anti-phospho-p44/42 antibody (Figure 7k,l), leading to high degree of colocalization with SIMK (Figure 7l). To determine quantitatively degree of colocalization of SIMK signal with phospho-p44/42 antibody, we performed colocalization analysis in certain regions of interests (ROIs) in cells of infection zone (II, Figure S8, mean Mander's coefficient 0.90), symbiotic zone (III, Figure S10, mean Mander's coefficient 0.82) and senescent zone (IV, Figure S12, mean Mander's coefficient 0.87). These data indicated that overall colocalization rate of SIMK with phosphorylated pool of MAPKs is rather high in cells of all developmental zones of root nodules. Next, we analyzed degree of colocalization of SIMK with phosphorylated pool of MAPKs in subcellular spot-like structures, closely associated with bacteroids. In cells of infection zone (II), mean Mander's coefficient reached 0.94 (Figure S9); in cells of symbiotic zone (III), it was 0.91 (Figure S11); and in cells of senescent zone (IV), it was 0.95 (Figure S13). The comparison of mean Mander's coefficients between large areas (ROIs) of the cells and individual spots clearly showed significantly higher degree of colocalization between SIMK and phosphorylated pool of MAPKs in spot-like structures (Figure 7m). This analysis revealed that within the infection zone, SIMK is located mainly in spots close to released bacteria and is activated. In cells of symbiotic and senescent zones, it is located also in other places in nucleus and cytoplasm, nevertheless, SIMK presence in spots close to bacteroids is still high, and it is always activated. Collectively, SIMK was located in all places related to internalization of symbiotic bacteria into host cells of functional root nodules, and in later stages of symbiotic process, SIMK was closely associated with bacteroids. Simultaneous immunolocalization of phosphorylated MAPK motives clearly indicated that SIMK was activated in all these locations.

Discussion

Leguminous plant species are important members of the agricultural ecosystems and are widely utilized also in nutritional production industry. They are able to grow in soils deficient for nitrogen by performing symbiotic interaction with rhizobia in root nodules, specialized organs for atmospheric nitrogen fixation. Alfalfa is an important legume crop in agronomy, especially for forage or silage production. Both initial and later interactions between legumes and rhizobia require exchange of different signals and activation of signal transduction pathways. Protein phosphorylation in general is one of the major signalling mechanisms controlling cellular responses to external stimuli. In particular, MAPK-dependent signal transduction cascades

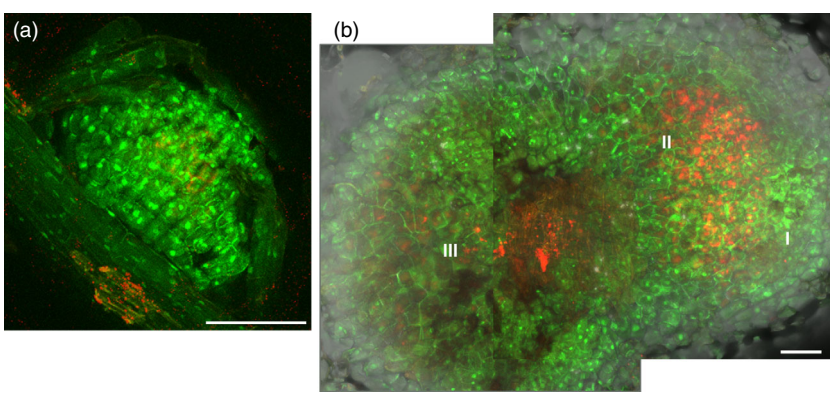
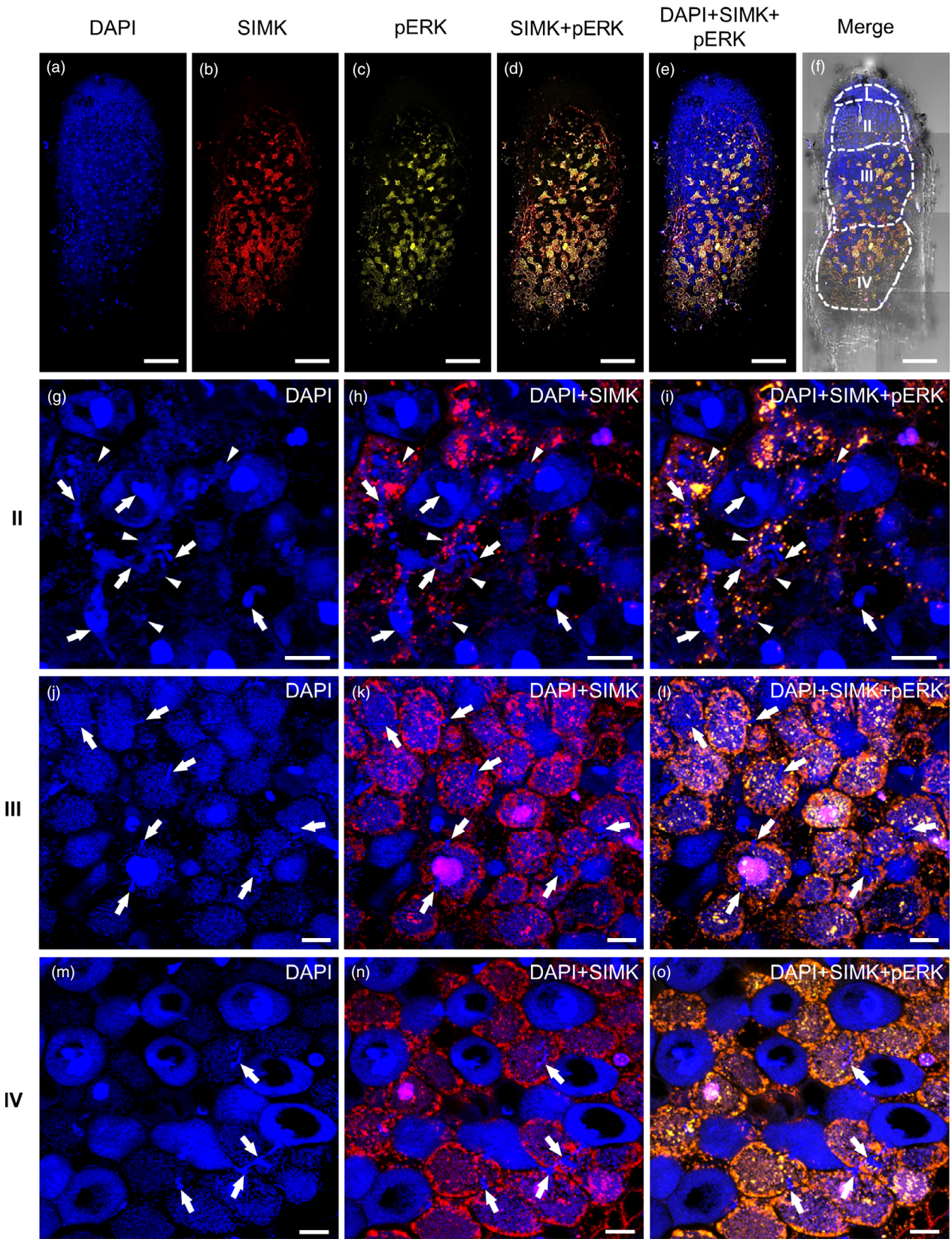


Figure 5 Localization of GFP-SIMK in alfalfa root nodules. Examples of nodule (a) at the early stage of development and (b) at the late stage of development observed by CLSM. Localization of fused GFP-SIMK protein (green) in root nodules induced after inoculation with *S. meliloti*-mRFP (red) on plants of GFP-SIMK line L5 at 10 dpi (a) and 20 dpi (b). A composite image of two consequential frames is shown in (b). Tissue organization of the late nodule: I, meristematic zone; II, infection and differentiation zone; III, symbiotic zone. Scale bar: (a) 100 μm; (b) 200 μm.



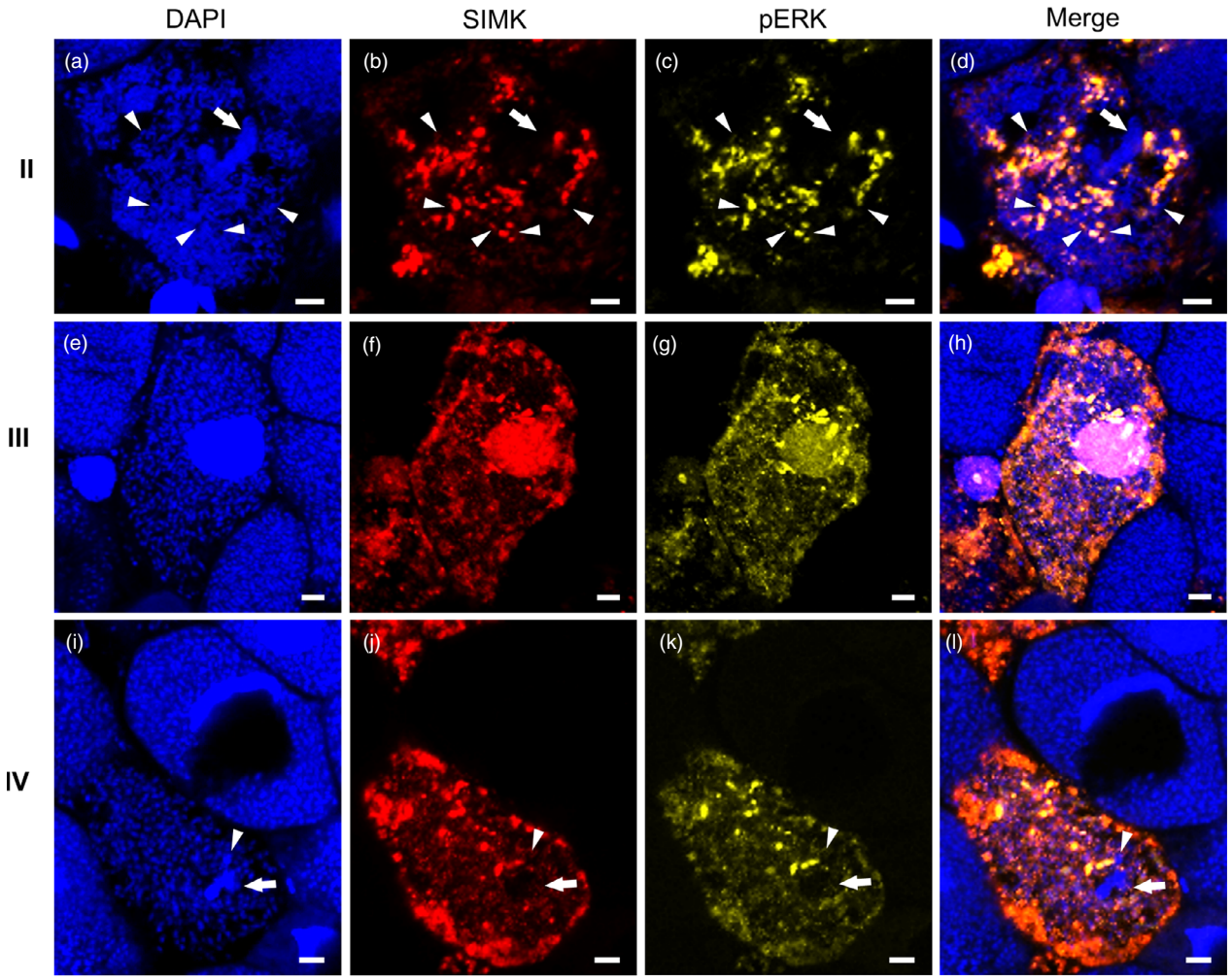
regulate many developmental and cellular processes in plants (Komis *et al*., 2018). Systemic approaches indicated that legume–rhizobia interactions and subsequent root nodule development involve activity of various protein kinases (Grimsrud *et al*., 2010; Roy *et al*., 2020). Main effort in research of symbiotic nitrogen fixation is conducted in legumes important for food production

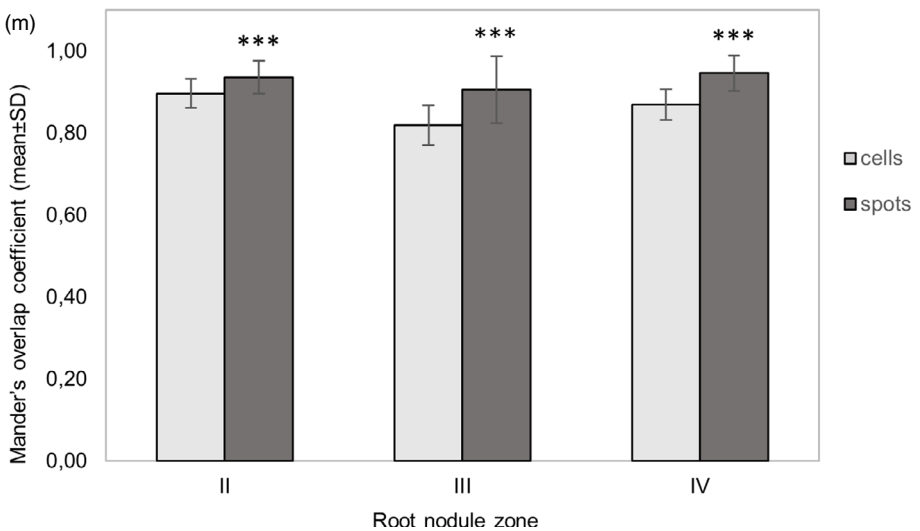
Figure 6 Overview of SIMK localization and distribution in root nodules induced after *Sinorhizobium meliloti* inoculation of alfalfa control RSY L1 plants using immunofluorescence localization microscopy. (a–f) Overview of the representative root nodule. This overview was mounted as a composite image out of eight consequential frames. (a) Hand-sectioned root nodules were stained for DNA using DAPI, and (b) immunostained for SIMK using anti-AtMPK6 antibody and (c) for phosphorylated MAPKs using phospho-specific pERK 44/42 antibody. (d) Overlay of SIMK and phosphorylated MAPKs, and (e) distribution of cell nuclei in SIMK-phosphorylated MAPKs overlay. (f) Bright-field image of the same nodule with overlaid fluorescence channels schematically depicts distribution of individual developmental zones, namely meristematic (I), infection (II), symbiotic (III) and senescent (IV) zones. (g-o) Representative images of cells from different nodule developmental zones: (II, g–i) the infection zone, (III, j–l) the symbiotic zone and (IV, m–o) the senescent zone. (g,j,m) Blue channels represent DAPI staining, (h,k,n) red channels (overlaid with DAPI channel) represent SIMK immunolocalization, and (i, l,o) yellow channels (overlaid with DAPI channel) represent colocalization (in yellow colour) of SIMK with phosphorylated MAPKs. Note specific staining of bacteria with DAPI. Infection threads are pointed by arrows, and releases of bacteria from branched infection threads in the form of infection droplets are pointed by arrowheads. Scale bars: (a–f) 10 mm, (g–o) 20 μm.

such as bean (Ge *et al*., 2016), soybean (Hyoungseok *et al*., 2008), pea (Stracke *et al*., 2002) or non-crop model *Medicago truncatula* (Ryu *et al*., 2017). However, the regulation of symbiotic interactions, nodule development and nitrogen fixation, including possible involvement of MAPK signalling, is much less clear in alfalfa. Here, we studied effects of both SIMK downregulation and overexpression in alfalfa using genetically modified transgenic lines. We characterized parameters such as length of root hairs, phenotype of above-ground plant parts and size of leaves, but also addressed possible involvement of SIMK in the efficiency of root nodulation, through determination of clustering of ITs and nodules. To achieve this goal, we analyzed transgenic alfalfa plants. In order to decrease SIMK functions, we prepared two independent RNAi transgenic lines downregulating SIMKK, an upstream activator of SIMK (SIMKKi lines). We confirmed that SIMK expression was strongly downregulated in these lines. In order to enhance SIMK functions, we cloned N-terminal fusion construct of enhanced *GFP* (*eGFP*) with *SIMK* driven under constitutive *35S* promoter (*35S::GFP:SIMK*) and stably transformed this construct into alfalfa plants. Using these transgenic lines, we were able to describe subcellular localization of GFP-SIMK fusion protein. Our observations from live cell microscopy revealed GFP-SIMK localization predominantly to the nucleus and cytoplasm in cells of diverse organs, which is consistent with previously published data on SIMK immunolocalization in alfalfa roots (Baluška *et al*., 2000a; Ovečka *et al*., 2014). The GFP-SIMK was also localized in tips of growing root hairs in agreement with previously published SIMK localization pattern using immunofluorescence microscopy (Šamaj *et al*., 2002). It is known that during root hair formation, SIMK is activated and redistributed from nucleus into growing root hair tips. Moreover, the positive role of SIMK in root hair tip growth was confirmed by overexpression of gain-of-function SIMK in transgenic plants of tobacco showing longer root hairs (Šamaj *et al*., 2002). The question remained whether genetically based downregulation or overexpression of SIMK might have an effect on the root hair growth in homologous alfalfa species. We addressed this question and report here about earlier cessation of root hair tip growth leading to short root hairs in SIMKKi lines with strongly downregulated SIMK, while GFP-SIMK overexpressing lines showed an opposite phenotype manifested by later cessation of the tip growth and longer root hairs. Effectivity in the root hair tip growth is an important aspect affecting early stages of plant–rhizobia interaction.

Major task of this study was to find out whether SIMK is involved in the regulation of root nodulation. Publications reporting involvement of MAPK signalling cascades in nodule development are rather scarce. A comparative study confirmed that MAPK signalling cascade and stress-related responses are activated early upon plant infection with symbiotic rhizobia (Lopez-Gomez *et al*., 2012). It has been shown that SIP2 is a MAPKK in *Lotus japonicus,* which interacts with symbiosis receptor-like kinase SymRK (Chen *et al*., 2012). Recent study demonstrated that phosphorylation target of SIP2 is LjMPK6 (Yin *et al*., 2019). Thus, signalling module SymRK-SIP2-MPK6 is required for nodulation, playing a positive role in nodule formation and organogenesis in *L. japonicus*. The orthologue of SIP2 has been identified in *M. truncatula* as MtMAPKK4 (Chen *et al*., 2017). It is involved in the regulation of different plant developmental processes and also mediates root nodule formation. Downstream interacting partners of MtMKK4 are MtMPK3 and MtMPK6 (Chen *et al*., 2017). Moreover, another *M. truncatula* MAPKK, namely MtMKK5, also interacts with MtMPK3 and MtMPK6 in alternative signalling pathway, having a negative role in the symbiotic nodule formation (Ryu *et al*., 2017). In alfalfa, SIMKK is specific upstream activator of SIMK under salt stress (Kiegerl *et al*., 2000). Interestingly, SIMKK shares 88% amino acid similarity with LjSIP2 (Chen *et al*., 2012) and by its amino acid sequence is highly similar also to MtMKK4 (Chen *et al*., 2017). Previously, Bekešová *et al*. (2015) showed decreased accumulation of phosphorylated SIMK in SIMKKi lines which is confirmed also in this study. SIMKKi transgenic line exhibited strong downregulation of *SIMKK* and *SIMK* transcripts and SIMK protein and showed shorter root hairs and less nodules. Such decreases in root hair growth and formation of ITs and nodules in the SIMKKi line but enhanced clustering of ITs and nodules in overexpression GFP-SIMK line support a positive role of SIMK in the alfalfa nodulation.

Clustering of ITs after inoculation with *S. meliloti* in overexpressor GFP-SIMK line is an interesting finding. Moreover, clustered development of ITs correlated well with clustered formation of fully developed and equally growing root nodules. This may represent important aspect of root nodule formation, since appropriate number of nodules developed in whole root system is tightly regulated by the plant and depends on overall physiological conditions. It has been observed that legumes tend to maintain development of the minimal number of nodules that are required for optimal growth at given growth conditions (Mortier *et al*., 2012). Control of the nodule number is a feedback mechanism that, at the level of the whole root system, may originate in early nodules that hinder further nodule development, a phenomenon called 'autoregulation of nodulation' (Delves *et al*., 1986), or may involve long-distance communication and control from the shoots (Sasaki *et al*., 2014). This mechanism is regulated by local and systemic endogenous signals. Locally, the number of developing nodules is controlled through ethylene signalling pathway, restricting the initiation of nodule primordia





to cortical cells close to xylem poles (Heidstra *et al*., 1997), and through nitrate-induced signalling peptides of the CLAVATA (CLV)/EMBRYO SURROUNDING REGION (ESR)-RELATED PROTEIN (CLE) (Mortier *et al*., 2010; Saur *et al*., 2011). A particular class of these small signalling CLE peptides, induced by rhizobia infection, controls also systemic regulation of nodulation (Concha and Doerner, 2020; Djordjevic *et al*., 2015; Mortier *et al*., 2010). CLE peptides move as a long-distance signals from roots to shoots where specifically interact with shoot receptors, such as leucine-rich-repeat receptor SUPER NUMERIC NODULES (SUNN) in *M. truncatula* (Schnabel *et al*., 2005), and negatively autoregulate the nodule number. On the other hand, root competence for

Figure 7 Detailed SIMK localization in root nodule cells induced by *Sinorhizobium meliloti* on alfalfa control RSY L1 plants using immunofluorescence localization microscopy. (a–d) Cell of the infection zone (II) with branched infection thread (arrow) during the release of bacteria in the form of infection droplets (arrowheads). (e–h) Cells of the symbiotic zone (III) with developed bacteroids. (i–l) Cells of the senescent zone (IV) with developed bacteroids. (a,e, i) Nuclei and bacteria are stained with DAPI, (b,f,j) SIMK (in red) is immunostained with anti-AtMPK6 antibody and (c,g,k) phosphorylated MAPKs (in yellow) are immunostained with phospho-specific pERK 44/42 antibody. (d,h,l) Overlay of DAPI, SIMK and phosphorylated MAPKs. Note specific staining of bacteria with DAPI. Infection threads are pointed by arrows, and releases of bacteria from branched infection threads in the form of infection droplets are pointed by arrowheads. (m) Averaged Mander's overlap coefficients from quantitative colocalization of SIMK with phosphorylated MAPKs evaluated in defined ROIs in each zone (cells), and in particular subcellular structures (spots) of the infection zone (II, $N = 23$ ROIs in cells and $N = 84$ in spots), the symbiotic zone (III, $N = 23$ ROIs in cells and $N = 83$ in spots) and the senescent zone (IV, $N = 15$ ROIs in cells and $N = 32$ in spots). Quantitative colocalization analysis is presented in Figures S8–S13. Statistics was calculated in Microsoft Excel using *t*-test. Error bars show ± SD. Asterisks indicate statistical significance between treatments (***$P < 0.001$). Scale bar: (a–l) 5 μm.

nodulation is controlled also by the microRNAs such as miR2111, which is produced upon activity of the receptor Compact Root Architecture 2 (CRA2) in shoots, and affecting positively root nodulation as systemic regulation signal (Gautrat *et al*., 2020). This feedback mechanism is controlled by the number, the activity and the age of early-developed nodules (Caetano-Anollés *et al*., 1991; Pierce and Bauer, 1983). Formation of IT and nodule clusters in GFP-SIMK lines may indicate a new SIMK role in spatial control of nodule formation on the root system. Possible explanation of increased nodule numbers developing close to each other and forming clusters might require SIMK involvement in IT formation and viability, as less infection events might abort in early stages of development. This unique aspect of nodulation process and mode of its regulation within the whole root system certainly deserves further detailed study.

Possible scenario how SIMK may be involved in alfalfa nodulation, and symbiosis with *S. meliloti* can be anticipated from its subcellular localization pattern. Originally, we observed during root hair tip growth that SIMK relocates from nucleus to the tip of growing root hairs (Šamaj *et al*., 2002). Upon salt stress, both SIMKK and SIMK became activated and relocated from nucleus to cytoplasm, where they accumulated in spot-like structures (Ovečka *et al*., 2014). Importantly, subcellular localization of SIMK in root nodules using immunofluorescence detection revealed close association of SIMK with terminal branching of ITs that were invading cells within the infection zone of root nodules. These branched finger-like extensions represented the intracellular places of rhizobia release from ITs in the form of infection droplets. We found SIMK prominently accumulated in such subcellular domains, in the form of spot-like structures decorating finger-like extensions of the ITs and released bacteria. Activated state of SIMK in such locations was confirmed by colocalization using anti-SIMK and anti-phospho-p44/42-specific antibodies. In the symbiotic zone of root nodules, activated SIMK was located in nuclei and in cytoplasm. The specific pattern of SIMK localization in the cytoplasm, in the form of small spots, was closely related to distribution of differentiated bacteroids, particularly in cells of the symbiotic and senescent zones of root nodules. Colocalization with anti-phospho-p44/42-specific antibody again revealed that SIMK was activated in all these locations within different nodule zones. Thus, local release of rhizobia from ITs and their accommodation in nodule cells up to differentiation of functional bacteroids involve active form of SIMK.

Another aspect interesting from the biotechnological point of view is the development and production of above-ground plant parts. Particularly in alfalfa, an important forage crop, total leaf surface area and shoot biomass are agronomical parameters of interest. In this respect, genetic manipulation of SIMK brought interesting and potentially promising results. Downregulation of *SIMKK* and *SIMK* genes in SIMKKi lines influenced negatively the development of above-ground plant parts, leading to formation of smaller leaves and production of less shoot biomass per plant. SIMK overexpression in GFP-SIMK lines, on the other hand, resulted in higher shoot biomass per plant, production of bigger size of analyzed leaves and their better distribution in shoots due to the longer petioles. This result may support a general effort of alfalfa biotechnological improvement as a forage crop. Nowadays, genetic, genomic and recombinant DNA technology approaches are widely utilized in alfalfa improvements, including leaf production parameters and biomass yield (Biazzi *et al*., 2017; Lei *et al*., 2017). Some physiological shoot and leaf characteristics of alfalfa were improved by transgenic approach. For example, genetic modification of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 8* (*MsSPL8*) gene in alfalfa significantly altered shoot architecture. Knock-down of *MsSPL8* significantly increased shoot branching and biomass yield; however, shoot branching was suppressed, and biomass yield was reduced by *MsSPL8* overexpression (Gou *et al*., 2018). Overexpression of *WXP1*, a gene encoding AP2 domain-containing putative transcription factor from *M. truncatula* under the control of the *35S* promoter in alfalfa resulted in excessive formation of cuticular wax layer on leaves. Such leaves were more resistant to water loss making these plants more resistant against drought stress (Zhang *et al*., 2005). Alfalfa develops typical compound leaves (Efroni *et al*., 2010) and increase of total leaf surface area, together with shoot biomass increase that we observed in SIMK overexpressing lines, may eventually influence also other production parameters including light acquisition efficiency.

Conclusively, we show that SIMK plays a positive role in alfalfa nodulation process and has a positive impact also on some other important agronomical factors, such as shoot biomass production, petiole and leaf development. SIMK is connected with internalization of symbiotic bacteria into host cells in root nodules and later on, closely associates with bacteroids. SIMKK-SIMK signalling module thus represents potentially new regulatory pathway required for the establishment of symbiotic interaction between rhizobia and alfalfa. This study opens a new door for alfalfa biotechnological improvement by genetic manipulation of MAPK signalling.

Experimental procedures

Plant and bacterial material

Somatic embryos of *M. sativa* cv. Regen-SY carrying *35S::GFP:SIMK* construct or *SIMKK RNAi* carrying pHellsgate12 plasmid driven under *35S* promoter (obtained from CSIRO Plant Industry, Australia) with well-developed root poles were separated,

individually transferred and inserted into root and plant development medium (MMS) or Fåhreus medium without nitrogen (FAH-N_2; Fåhreus, 1957). Plants were inoculated with *S. meliloti* SM2011 (Casse *et al*., 1979).

Cloning of *35S::GFP:SIMK* and stable transformation of alfalfa

To obtain stable transformed line of alfalfa with N-terminal fusion construct of enhanced GFP (*eGFP*) with *SIMK* driven under *35S* promoter (*35S::GFP:SIMK*), leaves of mature plants were transformed with *Agrobacterium tumefaciens* carrying *35S::GFP:SIMK* in pB7m34GW,0 expression plasmid (Karimi *et al*., 2005). Construction of *35S::GFP:SIMK* in pB7m34GW,0 was performed by MultiSite Gateway® Three-fragment vector construction kit, using pEN-L4-2-R1^{TM} carrying *p35S* sequence (Karimi *et al*., 2007), pEN-L1-F-L2^{TM} carrying *eGFP* sequence (Karimi *et al*., 2007) and pDONR^{TM}P2R-P3 carrying *SIMK* cDNA sequence (https://www.thermofisher.com). In the first step, 1188 bp *SIMK* PCR fragment was amplified using specific primers listed in Table S1 and total cDNA of alfalfa as a template. Donor and destination vectors were transformed in *Escherichia coli* strain TOP10 during construct preparation. Final destination vector was used for *A. tumefaciens* strain GV3101 transformation.

Kanamycin (50 μg/ml) resistance was used for selection of donor vectors, and spectinomycin (100 μg/ml) was used for selection of destination vector, and phosphinothricin (50 μg/ml) as the selection marker in planta together with ticarcillin (500 μg/ml) to inhibit *Agrobacterium* growth after transformation. Stable alfalfa transformation was performed by co-cultivation method with *Agrobacterium* described by Samac and Austin-Phillips, 2006. Leaves from well-developed plant nodes were surface sterilized, cut in half cross-wise with sterile scalpel blade, incubated with overnight *Agrobacterium* culture showing cell density between 0.6 and 0.8 at A_{600} nm for 30 minutes. Induction of callogenesis from leaf explants, production of somatic embryos from calli, development of shoots and somatic embryo rooting were performed on the appropriate media in the culture chamber at 22°C, 70% humidity, light intensity 100 $\mu mol.m^{-2}s^{-1}$ and 16/8 h light/dark photoperiod. Regenerated plants were maintained on media with phosphinothricin selection and tested for the presence of GFP-SIMK protein using molecular genotyping or fluorescent microscope. Transgenic alfalfa plants were further propagated in sterile culture via somatic embryogenesis.

Phenotypic analysis and plant inoculation with *S. meliloti*

Wild-type plants of alfalfa RSY (two independent lines L1 and L2), transgenic plants with *SIMKK RNAi* (SIMKKi, two independent transgenic lines L3 and L4) and transgenic plants carrying *35S::GFP:SIMK* construct (GFP-SIMK, two independent transgenic lines L5 and L6) were used for phenotype analysis of root hairs. Plants 18-day-old originating from somatic embryos were transferred to Petri dishes with FAH-N_2 medium containing 13 g/L micro agar. These plants were used for root hair imaging with Axio Zoom.V16 (Carl Zeiss, Germany) in light conditions. Statistics was calculated in SigmaPlot11.0 using Kruskal–Wallis one-way analysis of variance on ranks (Dunn's method) and is based on N = 529-1924. Different lower case letters indicate statistical significance between treatments ($p < 0.05$). Plants were then inoculated with bacteria *S. meliloti* strain Sm2011 labelled with mRFP with OD_{600} = 0.5 (Boivin *et al*., 1990). After 10-day post-inoculation (10 dpi), the infection threads were counted using Axio Zoom.V16 (Carl Zeiss, Germany) with excitation laser lines at 590 nm for mRFP and plants were scanned 5, 10, 15 and 20 dpi for counting of nodules.

Pictures of above-ground parts of alfalfa plants of RSY line L1, SIMKKi line L4 and GFP-SIMK line L5 regrown in pots 60 days after shoot cutting (Gou *et al*., 2018) were acquired by digital camera (Nikon D5000, Japan). Individual shoots were detached from the plants and shoot length (in cm), shoot weight (in g), number of shoots per plant and biomass weight per plant (in g) were recorded. Quantitative analysis was performed in SigmaPlot11.0 using Kruskal–Wallis one-way analysis of variance on ranks (Dunn's method) or using one-way analysis of variance (Holm–Sidak method) and was based on N = 94-196 shoots (for shoot length and weight) and N = 4-13 plants (for number of shoots per plant and biomass weight). Pictures of fresh shoots from alfalfa plants RSY line L1, SIMKKi line L4 and GFP-SIMK line L5 and images of first three developed leaves beneath the shoot apex from mature plants of RSY line L1, SIMKKi line L4 and GFP-SIMK line L5 grown in pots 15 days after cutting of the above-ground part were taken by digital camera (Nikon D5000, Japan). Quantitative analysis of total leaf area (area of left, right and apical leaflet together) and full length of the petioles were performed on 1st, 2nd and 3rd leaf of one shoot. In total, leaves of 5 independent shoots from 5 independent plants (maximal N = 25 for each 1st, 2nd and 3rd trifoliate leaf) of each line were analyzed. Total leaf area and length of the petioles were measured using measurement functions of ImageJ (http://rsb.info.nih.gov/ij/) and statistically evaluated in SigmaPlot11.0 using two-way analysis of variance (Holm–Sidak method) based on N = 11-25 (leaf area) and N = 25 (petiole length). Different lower case letters indicate statistical significance between treatments ($p < 0.05$).

Quantitative analysis of transcript levels by RT-qPCR

Total RNA was isolated from the roots of wild-type plants of alfalfa RSY line L1, transgenic plants with *SIMKK RNAi* (SIMKKi L4) and transgenic plants carrying *35S::GFP:SIMK* construct (GFP-SIMK L5), powdered in liquid nitrogen, using phenol–chloroform extraction (Sigma-Aldrich, USA). RNA concentration and purity were determined before DNaseI digestion with a NanoDrop Lite (Thermo Scientific, USA). The template-primer mix for reverse transcription was composed of 1 μl oligo(dT) primers, 1 μl RNasin Plus RNase inhibitor (Promega, USA), 1 μg RNA and PCR H_2O in a total volume of 14 μl. The mixture was denaturated at 70°C for 10 min. The following components were added: 4 μl M-MLV reverse transcriptase 5x reaction buffer (Promega, USA), 1 μl deoxynucleotide mix (10 mM each) and 1 μl M-MLV reverse transcriptase (Promega, USA) in a total volume of 6 μl. Reverse transcriptions were performed under the following conditions: 42°C for 50 min and 65°C for 15 min for inactivation of the reverse transcriptase. qRT-PCR was performed in a 96-well plate with the StepOnePlus Real-Time PCR system (Applied Biosystems, USA) using SYBR Green to monitor dsDNA synthesis. The reaction contained 5 μl Power SYBR Green PCR Master mix (Life Technologies, USA), 2.5 μl cDNA and 2.5 μl gene-specific primers (0.5 μM). The following standard thermal profile was used for all PCRs: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Experiments were run in three biological replicates. The transcription data were normalized to the transcription of *ACT2*

as a reference gene, and relative gene transcription was calculated by the 2∧(−ΔΔCt) method. Relative transcript levels were calculated as a ratio to control RSY L1, and thus, RSY level was always one without dispersion of variation. Statistics was calculated in Microsoft Excel using t-test and was based on N = 3. Error bars represent ± SD. Asterisks indicated statistical significance between treatments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n. s. did not indicate statistical significance). The primers are listed in Table S1. Primers for detection of total (endogenous native *SIMKe* + GFP-tagged) *SIMK* transcript were specific for third exon of *SIMK* gene. Primers for endogenous native *SIMKe* transcript were specific for 5′ UTR sequence and first exon of *SIMK* gene.

Immunoblotting analysis

Immunoblotting analysis was performed as described in Takáč *et al*. (2017). Plants of 20-day-old alfalfa RSY L1, transgenic plants with *SIMKK RNAi* construct (SIMKKi L4) and transgenic plants carrying *35S::GFP:SIMK* construct (GFP-SIMK L5) were used for immunoblotting analysis. Roots from 20-day-old plants of alfalfa were homogenized using liquid nitrogen to fine powder, and the proteins were extracted in E-buffer [50 mM HEPES (pH 7.5), 75 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM NaF, 10% (v/v) glycerol, Complete™ EDTA-free protease inhibitor and PhosSTOP™ phosphatase inhibitor cocktails (both from Roche, Basel, Switzerland)]. After centrifugation, supernatants were mixed with Laemmli buffer [final concentration 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 300 mM 2-mercaptoethanol]. After protein concentration measurement using Bradford assay, equal protein amounts (10 ng) were separated on 12% TGX Stain-Free™ (Bio-Rad) gels (Bio-Rad). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes in a wet tank unit (Bio-Rad) overnight at 24 V and 4°C using the Tris-glycine-methanol transfer buffer. Membranes were blocked in 4% (w/v) bovine serum albumin in Tris-buffered-saline (TBS, 100 mM Tris-HCl; 150 mM NaCl; pH 7.4) at 4°C overnight. Following washing step with TBS-T (TBS, 0.1% Tween-20) and incubation with polyclonal anti-AtMPK6 antibody (Sigma, Life Science, USA), highly specific for SIMK detection (Bekešová *et al*., 2015), diluted 1:15000 in TBST-T containing 1% (w/v) BSA and anti-Phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204) antibody (Cell Signaling, Netherlands) diluted 1:1000 in TBS-T containing 1% (w/v) BSA at 4°C overnight. Following five washing steps in TBS-T and incubation with a horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody (diluted 1:5000) in the both cases of anti-AtMPK6 and anti-phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204) primary antibody. The signals were developed using Clarity Western ECL substrate (Bio-Rad, Hercules, CA) and detected on Chemidoc MP documentation system (Bio-Rad). In total, eight immunoblots were performed from three biological samples representing different lines. Arbitrary units measured from immunoblotting using software Image Lab (Bio-Rad, USA) were normalized according to Stain-Free gels for corrections of imbalanced loading. Relative protein levels were after that calculated as a ratio to control RSY L1; thus, RSY level is one (zero in log_2 graphs) without dispersion of variation. Statistics was calculated in Microsoft Excel using t-test and was based on N = 3-8. Error bars represent ± SD. Asterisks indicated statistical significance between treatments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n. s. did not indicate statistical significance.

Live cell subcellular localization of GFP-SIMK

Agrobacteria carrying *35S::GFP:SIMK* construct were used for transient transformation of *N. benthamiana* leaves and for further stable transformation into alfalfa RSY L1 plants. Transgenic plants were regenerated through somatic embryogenesis and cultivated in the culture chamber at above-described conditions. Fluorescence signals were observed in *N. benthamiana* epidermal leaf cells and in alfalfa plant organs using Confocal Laser Scanning Microscope LSM710 (Carl Zeiss, Germany) equipped with Plan-Apochromat 20×/0.8 (Carl Zeiss, Germany), and Confocal Laser Scanning Microscope LSM880 with Airyscan (Carl Zeiss, Germany) equipped with Plan-Apochromat 20×/0.8 (Carl Zeiss, Germany). Samples were imaged with 488 nm excitation laser line and appropriate detection range for GFP emission. Image post-processing was done using ZEN 2010 software.

Fixation and immunolabelling of SIMK and phosphorylated MAPKs in fixed alfalfa roots and nodule sections

Immunolocalization of GFP-tagged SIMK and pERK in root whole mounts of transgenic plants carrying *35S::GFP:SIMK* construct (GFP-SIMK L5) was done as described previously (Tichá *et al*., 2020). For root tip samples, a double-immunolabelling with mouse anti-GFP (Abcam) and rabbit anti-phospho-p44/42 (Cell Signaling, Netherlands) primary antibodies at 1:100 and 1:400 dilution, respectively, in 2.5% (w/v) BSA in PBS was performed. To improve antibody penetration, vacuum pump was used (3 × 5 min), followed by incubation at 4°C overnight. Samples were then sequentially incubated with secondary antibody solutions. Firstly, Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen, USA) diluted 1:500 in 2.5% (w/v) BSA in PBS was used for incubation for 2h at 37°C. After extensively washing with PBS and subsequent blocking [5% (w/v) BSA in PBS for 20 min], samples were incubated with Alexa Fluor 555 goat anti-rabbit secondary antibody (Abcam, UK) diluted 1:500 in 2.5% (w/v) BSA in PBS. Nuclei were counterstained with 1 μg/ml DAPI.

For immunolabelling of nodule sections, mature nodules were excised from alfalfa RSY L1 roots, transferred sequentially to small glass Petri dish with fixative solution (composition described previously by Kitaeva *et al*., 2016) and cut into several thin longitudinal sections with sharp razor blade. Hand-cut nodules were transferred to plastic baskets with mesh in well plate containing fresh fixative solution and fixed using vacuum pump (6 × 15 min). Nodule sections were subsequently incubated in fixative solution for 1h at RT, followed by incubation at 4°C overnight. Nodule sections were washed (3 × 20 min) in microtubule stabilizing buffer [MTSB; 50 mM PIPES, 5 mM $MgSO_4 \times 7H_2O$ and 5 mM EGTA, pH 6.9] and 2 × 10 min in phosphate-buffered saline [PBS; 140 mM NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4 \times 2H_2O$, 1.5 mM KH_2PO_4, pH 7.3]. Sections were incubated (3 × 5 min) in reduction solution [0.05 % (w/v) sodium borohydride ($NaBH_4$) in PBS] and washed in PBS (3 × 5 min). To minimize unspecific antibody binding, sections were incubated for 1h at RT in blocking solution [5% (w/v) bovine serum albumin (BSA) in PBS]. Sections were simultaneously incubated in 2.5% (w/v) BSA in PBS with rabbit anti-AtMPK6 (Sigma, Life Science, USA) primary antibody at 1:750 dilution for SIMK localization and mouse anti-phospho-p44/42 (Cell Signaling, Netherlands) primary antibody at 1:400 dilution. To improve primary antibodies penetration, air was pumped out (3 × 5 min)

using vacuum pump, followed by incubation at 4°C overnight. Next day, sections were put for 1h at 37°C. The excess of primary antibodies was rinsed out with PBS (5 × 10 min), followed by sections blocking (5% (w/v) BSA in PBS for 20 min at RT). Sections were then sequentially incubated in 2.5% (w/v) BSA in PBS with appropriate Alexa Fluor conjugated secondary antibody. Firstly, sections were incubated with Alexa Fluor 647 rabbit anti-mouse secondary antibody (Abcam) at 1:500 dilution for 2h at 37°C. Sections were washed in PBS (5 × 10 min) and blocked [5% (w/v) BSA in PBS for 20 min at RT]. Sections were then incubated with Alexa Fluor 555 goat anti-rabbit (Abcam) secondary antibody at 1:500 dilution for 2h at 37°C. Sections were washed in PBS (5 × 10 min) and stained with 1 μg/ml DAPI in PBS for nuclei and bacteria visualization.

Immunolabelled samples of roots and nodules were mounted in antifade mounting medium [0.1% (w/v) paraphenylenediamine in 90% (v/v) glycerol buffered with 10% (v/v) PBS at pH 8.2 - 8.6] and used for microscopy. Microscopic analysis was performed with a Zeiss LSM710 platform (Carl Zeiss, Germany) or Zeiss LSM880 Airyscan equipped with a 32 GaAsP detector (Carl Zeiss, Germany), using excitation laser lines at 405 nm for DAPI, 488 nm for Alexa Fluor 488, 561 nm for Alexa Fluor 555 and 631 nm for Alexa Fluor 647. The image post-processing was done using ZEN 2014 software, and final figure plates were obtained using Photoshop 6.0/CS and Microsoft PowerPoint software.

Quantitative colocalization analysis

Quantitative colocalization analysis of SIMK and phosphorylated MAPKs was performed on immunolabelled hand-cut nodules of alfalfa control RSY L1 plants induced by inoculation with *S. meliloti*. Images used for colocalization analysis were acquired at the same imaging conditions by confocal laser scanning microscope LSM710 (Carl Zeiss, Germany) with Plan-Apochromat 40×/1.4 Oil DIC M27 objective, operated by Zeiss ZEN 2011 software (Black version). Images were acquired with the same laser attenuation values for both laser lines, and the thickness of individual optical sections was optimized according to Nyquist criteria in ZEN software. The pinhole sizes for both pseudocolored red (555/565 nm for excitation/emission) and pseudocolored yellow (652/668 nm for excitation/emission) channels were matched, and range of detection was appropriately adjusted to ensure separation of both emission wavelengths. Colocalization of SIMK with phosphorylated MAPKs in cells of infection, symbiotic and senescent nodule zones was strictly analyzed from single confocal optical sections, and in total, three independent optical sections per zone were analyzed using the colocalization tool of Zen 2011 software (Black version). Background threshold was adjusted by crosshairs setting according to Costes *et al*. (2004). Colocalization data were calculated from specific regions of interests (ROIs) selected manually by the drawing rectangle in cells of infection, symbiotic and senescent nodule zones, and from particular spots in the same cells outlined manually using the closed Bezier tool of the ZEN 2011 software (Black version). Data were displayed in intensity-corrected scatter plot diagrams. Intensity correlation of colocalizing pixels was expressed by Mander's overlap coefficient according to Manders *et al*. (1993).

Statistical analysis

All statistical parameters of the performed experiments are included in the figures or figure legends, number of samples (N), type of statistical tests and methods used, statistical significance denoted by lowercase letters or stars. Statistics was calculated in SigmaPlot11.0 using Kruskal–Wallis one-way analysis of variance on ranks (Dunn's method) if normality and/or equal variance failed or using one-way analysis of variance (Duncan's method) or two-way analysis of variance (Holm–Sidak method) if normality and equal variance passed. Different lowercase letters indicate statistical significance between treatments ($p < 0.05$). Statistical analysis using t-test was done in Microsoft Excel, and statistical significance between treatments is indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Data (and software) accessibility

The data supporting the findings of this study are available within the paper and its Supplementary Information files.

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Conflicts of interest

The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J.Š. (jozef.samaj@upol.cz).

Author contributions

M.H., I.L., K.H., P.D., O.Š., M.T. and M.O. performed experiments. M.H., I.L., K.H., P.D., D.N., H.B. and K.N. evaluated data, and I.L. performed statistical analyses. J.Š. provided infrastructure and coordinated the whole project. J.Š. and M.O. helped to plan experiments and contributed to data interpretation. M.H. and I.L. drafted the manuscript which was finally edited by J.Š. and M.O.

References

Aung, B., Gruber, M.Y., Amyot, L., Omari, K., Bertrand, A. and Hannoufa, A. (2015) Micro RNA 156 as a promising tool for alfalfa improvement. *Plant Biotechnol. J.* **13**, 779–790.

Baluška, F., Ovečka, M. and Hirt, H. (2000a) Salt stress induces changes in amounts and localization of the mitogen-activated protein kinase SIMK in alfalfa roots. *Protoplasma*, **212**, 262–267.

Baluška, F., Salaj, J., Mathur, J., Braun, M., Jasper, F., Šamaj, J., Chua, N. H. *et al*. (2000b) Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Dev. Biol.* **227**, 618–632.

Bekešová, S., Komis, G., Křenek, P., Vyplelová, P., Ovečka, M., Luptovčiak, I. and Šamaj, J. (2015) Monitoring protein phosphorylation by acrylamide pendant Phos-Tag™ in various plants. *Front. Plant Sci.* **6**, 336.

Biazzi, E., Nazzicari, N., Pecetti, L., Brummer, E.C., Palmonari, A., Tava, A. and Annicchiarico, P. (2017) Genome-wide association mapping and genomic selection for alfalfa (*Medicago sativa*) forage quality traits. *PLoS One*, **12**, e0169234.

Bögre, L., Calderini, O., Binarova, P., Mattauch, M., Till, S., Kiegerl, S., Jonak, C. *et al*. (1999) A MAP kinase is activated late in plant mitosis and becomes localized to the plane of cell division. *Plant Cell*, **11**, 101–113.

Boivin, C., Camut, S., Malpica, C.A., Truchet, G. and Rosenberg, C. (1990) *Rhizobium meliloti* genes encoding catabolism of trigonelline are induced under symbiotic conditions. *Plant Cell*, **2**, 1157–1170.

van Brussel, A.A., Bakhuizen, R., van Spronsen, P.C., Spaink, H.P., Tak, T., Lugtenberg, B.J. and Kijne, J.W. (1992) Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium*. *Science*, **257**, 70–72.

Bubb, M.R., Spector, I., Beyer, B.B. and Fosen, K.M. (2000) Effects of jasplakinolide on the kinetics of actin polymerization an explanation for certain *in vivo* observations. *J. Biol. Chem.* **275**, 5163–5170.

Caetano-Anollés, G. and Bauer, W.D. (1988) Feedback regulation of nodule formation in alfalfa. *Planta* **175**, 546–557.

Caetano-Anollés, G., Paparozzi, E.T. and Gresshoff, P.M. (1991) Mature nodules and root tips control nodulation in soybean. *J. Plant Physiol.* **137**, 389–396.

Cardinale, F., Jonak, C., Ligterink, W., Niehaus, K., Boller, T. and Hirt, H. (2000) Differential activation of four specific MAPK pathways by distinct elicitors. *J. Biol. Chem.* **275**, 36734–36740.

Cardinale, F., Meskiene, I., Ouaked, F. and Hirt, H. (2002) Convergence and divergence of stress-induced mitogen-activated protein kinase signaling pathways at the level of two distinct mitogen-activated protein kinase kinases. *Plant Cell*, **14**, 703–711.

Carro, L., Veyisoglu, A., Riesco, R., Spröer, C., Klenk, H.P., Sahin, N. and Trujillo, M.E. (2018) *Micromonospora phytophila* sp. nov. and *Micromonospora luteiviridis* sp. nov., isolated as natural inhabitants of plant nodules. *Int. J. Syst. Evol. Microbiol.* **68**, 248–253.

Casse, F., Boucher, C., Julliot, J.S., Michel, M. and Denarie, J. (1979) Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. *Microbiology* **113**, 229–242.

Chen, T., Zhou, B., Duan, L., Zhu, H. and Zhang, Z. (2017) *MtMAPKK4* is an essential gene for growth and reproduction of *Medicago truncatula*. *Physiol. Plant.* **159**, 492–503.

Chen, T., Zhu, H., Ke, D., Cai, K., Wang, C., Gou, H., Hong, Z. *et al*. (2012) A MAP kinase kinase interacts with SymRK and regulates nodule organogenesis in *Lotus japonicus*. *Plant Cell*, **24**, 823–838.

Concha, C. and Doerner, P. (2020) The impact of the rhizobia–legume symbiosis on host root system architecture. *J. Exp. Bot.* **71**, 3902–3921.

Costes, S.V., Daelemans, D., Cho, E.H., Dobbin, Z., Pavlakis, G. and Lockett, S. (2004) Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophys. J* . **86**, 3993–4003.

Cristina, M.S., Petersen, M. and Mundy, J. (2010) Mitogen-activated protein kinase signaling in plants. *Annu. Rev. Plant Biol.* **61**, 621–649.

Delves, A.C., Mathews, A., Day, D.A., Carter, A.S., Carroll, B.J. and Gresshoff, P.M. (1986) Regulation of the soybean-*Rhizobium* nodule symbiosis by shoot and root factors. *Plant Physiol.* **82**, 588–590.

Djordjevic, M.A., Mohd-Radzman, N.A. and Imin, N. (2015) Small-peptide signals that control root nodule number, development, and symbiosis. *J. Exp. Bot.* **66**, 5171–5181.

Efroni, I., Eshed, Y. and Lifschitz, E. (2010) Morphogenesis of simple and compound leaves: A critical review. *Plant Cell*, **22**, 1019–1032.

Fåhreus, G. (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *Microbiology*, **16**, 374–381.

Gautrat, P., Laffont, C. and Frugier, F. (2020) Compact Root Architecture 2 promotes root competence for nodulation through the miR2111 systemic effector. *Curr. Biol.* **30**, 1339–1345.

Ge, Y.Y., Xiang, Q.W., Wagner, C., Zhang, D., Xie, Z.P. and Staehelin, C. (2016) The type 3 effector NopL of *Sinorhizobium* sp. strain NGR234 is a mitogen-activated protein kinase substrate. *J. Exp. Bot.* **67**, 2483–2494.

Geurts, R., Xiao, T.T. and Reinhold-Hurek, B. (2016) What does it take to evolve a nitrogen-fixing endosymbiosis? *Trends Plant Sci.* **21**, 199–208.

Gou, J., Debnath, S., Sun, L., Flanagan, A., Tang, Y., Jiang, Q. and Wang, Z.Y. (2018) From model to crop: functional characterization of *SPL8* in *M. truncatula* led to genetic improvement of biomass yield and abiotic stress tolerance in alfalfa. *Plant Biotechnol. J.* **16**, 951–962.

Grimsrud, P.A., den Os, D., Wenger, C.D., Swaney, D.L., Schwartz, D., Sussman, M.R., Ané, J.M. *et al*. (2010) Large-scale phosphoprotein analysis in *Medicago truncatula* roots provides insight into in vivo kinase activity in legumes. *Plant Physiol.* **152**, 19–28.

Heidstra, R., Yang, W.C., Yalcin, Y., Peck, S., Emons, A.M., van Kammen, A. and Bisseling, T. (1997) Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in *Rhizobium*–legume interaction. *Development* **124**, 1781–1787.

Hyoungseok, L., Jitae, K., Jong, H.I., Ho, B.K., Chang, J.O. and Chung, S.A. (2008) Mitogen-Activated Protein Kinase Is Involved in the Symbiotic Interaction between *Bradyrhizobium japonicum* USDA110 and Soybean. *J. Plant Biol.* **51**, 291–296.

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T. and Shinozaki, K. (2000) Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. *Plant J.* **24**, 655–665.

Jonak, C., Kiegerl, S., Hirt, H., Lloyd, C. and Chan, J. (1995) MMK2, a novel alfalfa MAP kinase, specifically complements the yeast MPK1 function. *Mol Gen Genet*. **248**, 686–694.

Jonak, C., Kiegerl, S., Ligterink, W., Barker, P.J., Huskisson, N.S. and Hirt, H. (1996) Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Natl Acad. Sci.* **93**, 11274–11279.

Jonak, C., Okresz, L., Bögre, L. and Hirt, H. (2002) Complexity, cross talk and integration of plant MAP kinase signalling. *Curr. Opin. Plant Biol.* **5**, 415–424.

Jones, K.M., Kobayashi, H., Davies, B.W., Taga, M.E. and Walker, G.C. (2007) How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat. Rev. Microbiol.* **5**, 619–633.

Karimi, M., Bleys, A., Vanderhaeghen, R. and Hilson, P. (2007) Building blocks for plant gene assembly. *Plant Physiol.* **145**, 1183–1191.

Karimi, M., De Meyer, B. and Hilson, P. (2005) Modular cloning and expression of tagged fluorescent protein in plant cells. *Trends Plant Sci.* **10**, 103–105.

Kiegerl, S., Cardinale, F., Siligan, C., Gross, A., Baudouin, E. and Liwosz, A. (2000) SIMKK, a mitogen-activated protein kinase (MAPK) kinase, is a specific activator of the salt stress-induced MAPK, SIMK. *Plant Cell* **12**, 2247–2258.

Kitaeva, A.B., Demchenko, K.N., Tikhonovich, I.A., Timmers, A.C. and Tsyganov, V.E. (2016) Comparative analysis of the tubulin cytoskeleton organization in nodules of *Medicago truncatula* and *Pisum sativum*: bacterial release and bacteroid positioning correlate with characteristic microtubule rearrangements. *New Phytol.* **210**, 168–183.

Komis, G., Novák, D., Ovečka, M., Šamajová, O. and Šamaj, J. (2018) Advances in imaging plant cell dynamics. *Plant Physiol.* **176**, 80–93.

Lei, Y., Hannoufa, A. and Yu, P. (2017) The use of gene modification and advanced molecular structure analyses towards improving alfalfa forage. *Int. J. Mol. Sci.* **18**, 298.

Lopez-Gomez, M., Sandal, N., Stougaard, J. and Boller, T. (2012) Interplay of flg22-induced defence responses and nodulation in *Lotus japonicus*. *J. Exp. Bot.* **63**, 393–401.

Manders, E.M.M., Verbeek, F.J. and Aten, J.A. (1993) Measurement of co-localization of objects in dual-colour confocal images. *J. Microsc.* **169**, 375–382.

Mortier, V., Den Herder, G., Whitford, R., Van de Velde, W., Rombauts, S., D'Haeseleer, K., Holsters, M. *et al*. (2010) CLE peptides control *Medicago truncatula* nodulation locally and systemically. *Plant Physiol.* **153**, 222–237.

Mortier, V., Holsters, M. and Goormachtig, S. (2012) Never too many? How legumes control nodule numbers. *Plant Cell Environ.* **35**, 245–258.

Munnik, T., Ligterink, W., Meskiene, I., Calderini, O., Beyerly, J., Musgrave, A. and Hirt, H. (1999) Distinct osmo-sensing protein kinase pathways are involved in signalling moderate and severe hyper-osmotic stress. *Plant J.* **20**, 381–388.

Oldroyd, G.E. (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nat. Rev. Microbiol.* **11**, 252–264.

Oldroyd, G.E., Murray, J.D., Poole, P.S. and Downie, J.A. (2011) The rules of engagement in the legume-rhizobial symbiosis. *Annu. Rev. Genet.* **45**, 119–144.

Ovečka, M., Takáč, T., Komis, G., Vadovič, P., Bekešová, S., Doskočilová, A., Smékalová, V. *et al*. (2014) Salt-induced subcellular kinase relocation and seedling susceptibility caused by overexpression of *Medicago* SIMKK in *Arabidopsis*. *J. Exp. Bot.* **65**, 2335–2350.

Perret, X., Staehelin, C. and Broughton, W.J. (2000) Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* **64**, 180–201.

Pierce, M. and Bauer, W.D. (1983) A rapid regulatory response governing nodulation in soybean. *Plant Physiol.* **73**, 286–290.

Pitzschke, A., Schikora, A. and Hirt, H. (2009) MAPK cascade signaling networks in plant defence. *Curr. Opin. Plant Biol.* **12**, 421–426.

Radović, J., Lugić, Z., Jevtić, G., Mrfat-Vukelić, S. and Ignjatović, S. (2003) Variability for production and morfological traits of alfalfa (*Medicago sativa* L.). *Czech J. Genet. Plant Breeding*, **39**, A1-A3. (Special issue).

Radović, J., Sokolović, D. and Marković, J. (2009) Alfalfa-most important perennial forage legume in animal husbandry. *Biotechnol. Anim. Husband.* **25**, 465–475.

Rasmussen, M.W., Roux, M., Petersen, M. and Mundy, J. (2012) MAP kinase cascades in *Arabidopsis* innate immunity *Front. Plant Sci.* **3**, 169.

Remigi, P., Zhu, J., Young, J.P. and Masson-Boivin, C. (2016) Symbiosis within Symbiosis: Evolving Nitrogen-Fixing Legume Symbionts. *Trends Microbiol.* **24**, 63–75.

Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., Frugoli, J. *et al*. (2020) Celebrating 20 years of genetic discoveries in legume nodulation and symbiotic nitrogen fixation. *Plant Cell*, **32**, 15–41.

Ryu, H., Cho, H., Choi, D. and Hwang, I. (2012) Plant hormonal regulation of nitrogen-fixing nodule organogenesis. *Mol. Cells*, **34**, 117–126.

Ryu, H., Laffont, C., Frugier, F. and Hwang, I. (2017) MAP kinase-mediated negative regulation of symbiotic nodule formation in *Medicago truncatula*. *Mol. Cells*, **40**, 17–23.

Samac, D.A. and Austin-Phillips, S. (2006) Alfalfa (*Medicago sativa* L.). In *Agrobacterium Protocols. Methods Mol Biol*. (Wang, K., ed) **343**, pp, 301–312. Clifton, N.J.: Humana Press.

Šamaj, J., Ovečka, M., Hlavačka, A., Lecourieux, F., Meskiene, I., Lichtscheidl, I., Baluška, F. *et al.*, (2002) Involvement of the mitogen-activated protein kinase SIMK in regulation of root hair tip growth. *EMBO J.* **21**, 3296–3306.

Šamajová, O., Komis, G. and Šamaj, J. (2013b) Emerging topics in the cell biology of mitogen-activated protein kinases. *Trends Plant Sci.* **18**, 140–148.

Šamajová, O., Plíhal, O., Al-Yousif, M., Hirt, H. and Šamaj, J. (2013a) Improvement of stress tolerance in plants by genetic manipulation of mitogen-activated protein kinases. *Biotechnol. Adv.* **31**, 118–128.

Sasaki, T., Suzaki, T., Soyano, T., Kojima, M., Sakakibara, H. and Kawaguchi, M. (2014) Shoot-derived cytokinins systemically regulate root nodulation. *Nat. Commun.* **5**, 4983.

Saur, I., Oakes, M., Djordjevic, M.A. and Imin, N. (2011) Crosstalk between the nodulation signaling pathway and the autoregulation of nodulation in *Medicago truncatula*. *New Phytol.* **190**, 865–874.

Schnabel, E., Journet, E.P., de Carvalho-Niebel, F., Duc, G. and Frugoli, J. (2005) The *Medicago truncatula SUNN* gene encodes a CLV1-like leucine-rich repeat receptor kinase that regulates nodule number and root length. *Plant Mol. Biol.* **58**, 809–822.

Sinha, A.K., Jaggi, M., Raghuram, B. and Tuteja, N. (2011) Mitogen-activated protein kinase signaling in plants under abiotic stress. *Plant Signal. Behav.* **6**, 196–203.

Smékalová, V., Doskočilová, A., Komis, G. and Šamaj, J. (2014) Crosstalk between secondary messengers, hormones and MAPK modules during abiotic stress signalling in plants. *Biotechnol. Adv.* **32**, 2–11.

Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., Tabata, S. *et al*. (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. *Nature*, **417**, 959–962.

Takáč, T., Šamajová, O., Luptovčiak, I., Pechan, T. and Šamaj, J. (2017) Feedback microtubule control and microtubule-actin cross-talk in *Arabidopsis* revealed by integrative proteomic and cell biology analysis of *KATANIN 1* mutants. *Mol. Cell. Proteomics*, **16**, 1591–1609.

Tichá, M., Hlaváčková, K., Hrbáčková, M., Ovečka, M., Šamajová, O. and Šamaj, J. (2020) Super-resolution imaging of microtubules in *Medicago sativa*. *Methods Cell Biol.* **160**, 237–251.

Wang, Q., Liu, J. and Zhu, H. (2018) Genetic and molecular mechanisms underlying symbiotic specificity in legume-rhizobium interactions. *Front. Plant Sci.* **9**, 313.

Yin, J., Guan, X., Zhang, H., Wang, L., Li, H., Zhang, Q., Chen, T. *et al*. (2019) An MAP kinase interacts with LHK1 and regulates nodule organogenesis in *Lotus japonicus*. *Sci. China Life Sci.* **62**, 1–15.

Zhang, J.Y., Broeckling, C.D., Blancaflor, E.B., Sledge, M.K., Sumner, L.W. and Wang, Z.Y. (2005) Overexpression of *WXP1*, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). *Plant J.* **42**, 689–707.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Localization of SIMK during infection thread formation.

Figure S2 Nodule formation in alfalfa plants after inoculation with *Sinorhizobium meliloti*-mRFP.

Figure S3 Root system development and nodulation in plants of alfalfa lines grown in soil.

Figure S4 Shoot and leaf phenotypes in plants of alfalfa lines grown in soil.

Figure S5 Expression pattern of *35S::GFP:SIMK* construct after transient transformation of *Nicotiana benthamiana* leaf epidermal cells.

Figure S6 Subcellular localization of GFP-SIMK in stable transformed alfalfa plants using confocal and Airyscan CLSM.

Figure S7 Whole mount immunofluorescence localization of GFP-SIMK in stable transformed root tip of alfalfa by Airyscan CLSM.

Figure S8 Quantitative colocalization analysis of SIMK and phosphorylated MAPKs in cells of the infection zone in immunolabeled hand-sectioned nodule, induced after *Sinorhizobium meliloti* inoculation of alfalfa control RSY L1 plants.

Figure S9 Quantitative colocalization analysis of SIMK and phosphorylated MAPKs in cells of the infection zone in immunolabeled hand-sectioned nodule, induced after *Sinorhizobium meliloti* inoculation of alfalfa control RSY L1 plants.

Figure S10 Quantitative colocalization analysis of SIMK and phosphorylated MAPKs in cells of the symbiotic zone in immunolabeled hand-sectioned nodule, induced after *Sinorhizobium meliloti* inoculation of alfalfa control RSY L1 plants.

Figure S11 Quantitative colocalization analysis of SIMK and phosphorylated MAPKs in cells of the symbiotic zone in immunolabeled hand-sectioned nodule, induced after *Sinorhizobium meliloti* inoculation of alfalfa control RSY L1 plants.

Figure S12 Quantitative colocalization analysis of SIMK and phosphorylated MAPKs in cells of the senescent zone in immunolabeled hand-sectioned nodule, induced after *Sinorhizobium meliloti* inoculation of alfalfa control RSY L1 plants.

Figure S13 Quantitative colocalization analysis of SIMK and phosphorylated MAPKs in cells of the senescent zone in immunolabeled hand-sectioned nodule, induced after *Sinorhizobium meliloti* inoculation of alfalfa control RSY L1 plants.

Table S1 Primers used for MultiSite GateWay® cloning and quantitative real-time qPCR.

Supplement III

Ph.D. Thesis Summary.

PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

Department of Biochemistry

Preparation and characterization of MAP kinase transgenic alfalfa lines

Ph.D. Thesis Summary

P1406 Biochemistry

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Olomouc

2021

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The summary of the Ph.D. thesis has been sent out on …….
The oral defence will take place at the Faculty of Science, Šlechtitelů 27, Olomouc on ……
The Ph.D. thesis is available at the Library of the Biological Centre, Šlechtitelů 27, Olomouc

Prof. Mgr. Marek Petřivalský, Dr.
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Content

1 Abstract

Legumes are able to perform symbiotic interactions with rhizobia that are able to convert atmospheric nitrogen into ammonia, which is assimilated by the host plant. Signaling by mitogen-activated protein kinases (MAPKs) seems to be involved in this symbiotic interaction. MAPK cascades are one of the most conserved and best characterized protein kinase signaling pathways. In alfalfa, SIMK was identified as a salt stress- and elicitor-induced MAPK. SIMKK is an upstream activator of SIMK during alfalfa response to the salt stress. One of the main aims of this Ph.D. thesis was to prepare and transform constructs with fluorescently-tagged SIMK and SIMKK to plant cells. Advanced microscopy techniques were used for live-cell imaging and immunolabeling of SIMK in alfalfa tissues. Next, role of overexpressed SIMK was studied in root hair growth, infection thread formation, nodule clustering and shoot biomass production.

The first part of the thesis is devoted to the crop *Medicago sativa*. It summarizes the current knowledge on this plant and its biotechnological potential. This chapter of thesis also describes legume-rhizobia interactions and MAPKs identified in alfalfa and model plant *Arabidopsis thaliana*.

The following chapter deals with the cloning and characterization of fusion proteins, GFP-tagged SIMK and tagRFP-tagged SIMKK. Expression of fluorescently-tagged SIMK and SIMKK was checked using transient transformation of *Nicotiana benthamiana* leaves. New stably transformed alfalfa lines have been prepared by the improved and efficient transformation protocol using *Agrobacterium tumefaciens* and somatic embryogenesis.

The last part of the thesis is dedicated to the characterization of production parameters of transgenic alfalfa plants with genetically engineered SIMK after infection with *Sinorhizobium meliloti*. *SIMKK RNAi* lines, showing strong downregulation of both *SIMKK* and *SIMK* genes, revealed reduced root hair growth and lower capacity to form infection threads and nodules. In contrast, constitutive overexpression of GFP-tagged SIMK promoted root hair growth as well as infection thread and nodule clustering. Moreover, *SIMKK* and *SIMK* downregulation led to decrease, while overexpression of GFP-tagged SIMK promoted shoot biomass production. These data suggest that gene engineering of SIMK expression levels affects root hair, nodule and shoot formation patterns in alfalfa. It highlights new biotechnological potential of this protein kinase.

Key words:	*Medicago sativa*, alfalfa, SIMK, SIMKK, root hair, *Sinorhizobium meliloti*, infection thread, nodule, CLSM, immunofluorescent labelling
Number of pages:	56
Number of supplements:	1
Language:	English

2 Objectives

1. Summary of the recent knowledge and biotechnological potential of alfalfa, legume-rhizobia interactions, and MAPKs.
2. Cloning and transformation of fluorescently tagged MAPKs of alfalfa. Live-cell imaging and immunolabeling of SIMK in alfalfa tissues.
3. Role of overexpressed SIMK in root hair growth, nodule clustering and shoot biomass production.

3 Part I – General introduction

3.1 Basic characteristics of *Medicago sativa*

Medicago sativa L., also known as alfalfa, "Queen of Forage", or "lucerne", belongs to Fabaceae. Its first cultivated form most likely originates from western Persia. Alfalfa then spread into many regions in Europe, Asia and America (Rashmi *et al.*, 1997; Samac and Temple, 2004). Alfalfa is a highly valuable perennial deep-rooted forage legume (Radović *et al.*, 2009).

Generally, legumes are important forage crops worldwide because they are capable of producing high yields of high quality forage (Albrecht and Beauchemin, 2003). It can be argued that no other family of plants provides a better balance of protein, energy, and minerals in the form of forage for high-producing livestock than the legumes (Beever and Thorp, 1996). Perennial legumes have been used historically as a hay or pasture. They provide continuous groundcover for several years, reducing opportunities for water runoff from hillsides and potential loss of soil, N, and P from fields and into the surface water (Albrecht and Beauchemin, 2003; Zemenchik *et al.*, 1997). Moreover, perennial legumes have higher crude protein concentrations than either perennial or annual grasses. They contain relatively high concentrations of organic acids (Albrecht and Beauchemin, 2003; Playne and McDonald, 1966).

Alfalfa is one the most important legume forage crops in the world, mostly because of its high biomass yield, good forage quality, and palatability for ruminants (Lei *et al.*, 2017; Radović *et al.*, 2009). It has relatively vigorous and deep rooting systems helping to prevent soil erosion and reduce contamination of surface and ground water (Radović *et al.*, 2009). It serves as an animal and livestock feed, but also is used for biofuel, soil conservation, natural bioremediation, or for the production of pharmaceutical compounds and industrial enzymes (Kineman *et al.*, 2010; Kumar *et al.*, 2018). Alfalfa is able to fix a large amount of atmospheric nitrogen due to the symbiosis with rhizobia (Doyle and Luckow, 2003; Putnam *et al.*, 2001).

Alfalfa has the obligate outcrossing and autotetraploid character (2n = 4X = 32) resulting in a large genetic diversity. So far, it has been quite intensively studied, including abiotic and biotic stress responses as well as by employing modern genomic, proteomic and metabolomics approaches (reviewed by Hrbáčková *et al.*, 2020).

3.2 Biotechnological perspectives of genomic approaches in alfalfa

For several decades, researchers are working to develop improved major crops with better adaptability and tolerance to environmental stresses. Abiotic and biotic stresses are main factors limiting legume production, however, alfalfa (*M. sativa* L.) shows relatively high level of tolerance to drought and salt stress. The identification of genes that affect legume crop production represents an important aim of current genomic studies (Bevan *et al.*, 2017), and this requires knowledge of their full genomic sequences. The use of modern biotechnology tools is facilitated in alfalfa and its close relative barrel medic (*M. truncatula* Gaertn.), since full genomes were released (Tang *et al.*, 2014). Technologies for sequencing DNA and RNA have undergone revolutionary improvements (Ari and Arikan, 2016). It is known that early after split between monocots and eudicots during the evolution, several whole-genome duplications and triplications occurred in legumes (Severin *et al.*, 2011; Masonbrink *et al.*, 2017). New NGS platforms, such as the Roche/454 system (Margulies *et al.*, 2005), Illumina platform (Wang *et al.*, 2012), real-time DNA sequencing by Pacific Biosciences (Eid *et al.*, 2009), Oxford Nanopore system (Lu *et al.*, 2016), and Ion Torrent system (Rothberg *et al.*, 2011), were used for sequencing crop and legume genomes. They have had a major impact on plant research, since they help to understand the genome complexity.

3.2.1 Next generation sequencing for genomics and transcriptomics

Several NGS methods have been developed that allowed the examination of global transcriptional changes. The most used ones are the hybridization of cDNAs (DNA microarrays) and the deep sequencing of cDNA (RNA-Seq; Schena *et al.*, 1995; Lardi and Pessi, 2018). RNA-Seq is a massive parallel sequencing method for transcriptome analysis (Wang *et al.*, 2009). Transcriptomic studies analyze only the transcribed portion of the genome and provides in-depth sequencing coverage and additional qualitative information such as isoform-specific expression (Abdelrahman *et al.*, 2018). In contrast to microarrays, ribosomal RNA (rRNA) does not hybridize to the chip, as homologous probes are not present. In RNA-Seq, the abundant rRNA is removed (Lardi and Pessi, 2018). Originally, transcriptomic studies were based on Sanger sequencing of expressed sequence tags (ESTs) or microarrays, which was used in alfalfa and barrel medic (Cheung *et al.*, 2006; Yang *et al.*, 2010).

3.2.2 Transcriptomic approaches and gene expression modifications

3.2.2.1 Resistance to abiotic stress

Salinity stress interferes with plant growth because it causes two main stresses on plants: hyperosmotic pressure and ion toxicity, especially due to Na^+ (Volkov *et al.*, 2004). High

salinity often triggers an increase in cytosolic Ca^{2+}, reactive oxygen species (ROS), abscisic acid (ABA) and mitogen activated protein kinase (MAPK) signaling (Ovečka *et al.,* 2014; Mittler and Blumwald, 2015). These activated signal molecules affect plant transcriptomes by regulating transcription factors (Zhu, 2002). One of the basic strategies in plant stress responses is the accumulation of water-soluble compounds of low molecular weight, such as betaines, polyols, sugars and amino acids (Chen and Murata, 2002). These compounds accumulate to high concentrations under water or salt stress and protect plants via ROS detoxification and membrane integrity maintenance (Bohnert and Jensen, 1996).

Functional and structural genomics studies are fundamental for the understanding of plant biology. Access to high-quality genome and transcriptome sequences is important to perform studies of this kind. Recently, the third-generation sequencing technology PacBio RSII has emerged as a unique method for constructing full-length transcripts (Dong *et al.,* 2015; Nakano *et al.,* 2017). PacBio RSII is an ideal tool for whole genome sequencing, targeted sequencing, RNA-Seq, and epigenetic characterization. This technique allows the sequencing of single DNA molecules in real-time (SMRT) without amplification by PCR (Dong *et al.,* 2015). Using PacBio RSII, Luo *et al.* (2019) studied salt stress as a major environmental factor that affects alfalfa development and production. They have constructed the first full-length transcriptome database of alfalfa root tips treated with mannitol (a non-ionic osmotic stress) and NaCl (an ionic osmotic stress), which provided evidence that the response to salinity stress includes both osmotic and ionic components. They have found 8,016 mannitol-regulated DEGs and 8,861 NaCl-regulated DEGs. These DEGs are involved in signal transduction, transcriptional regulation, anti-oxidative defense, and signal perceptions (Luo *et al.,* 2019).

3.2.2.2 Resistance to biotic stress

Plants are subjected to a wide range of environmental stresses. Attacks by various pathogens, such as bacteria, fungi, oomycetes, nematodes, or herbivores are classified as biotic stresses. Plants have no choice to escape, so they developed various mechanisms protecting them against environmental cues (Gull *et al.,* 2019). Many pathogens can be problematic for alfalfa production.

Transcriptomic studies contributed to the knowledge of alfalfa resistance to nematodes, aphids and strips. Alfalfa is the host of several important nematode species, especially stem nematode (*Ditylenchus dipsaci*), cyst nematode (*Heterodera* spp.), root lesion nematode (*Pratylenchus* spp.) and root-knot nematode (*Meloidogyne* spp.). These nematodes are an

economic thread to alfalfa production (Griffin, 1990; Hafez and Sundararaj, 2009). Postnikova *et al.* (2015) have used RNA-sequencing to study alfalfa-root-knot nematode interactions. They have performed root transcriptomic analysis of resistant and susceptible alfalfa cultivars infected by root-knot nematode *Meloidogyne incognita.* Aphids are major insect pests of alfalfa, consuming sap from phloem tissue, leading to plant death. Aphids are highly mobile, have high reproductive rates and are able to damage plants. Tu *et al.* (2018) performed a transcriptomic analysis of two alfalfa cultivars differing in aphid resistance. Genes involved in salicylic acid biosynthesis represented an important defense mechanism in both cultivars. The alfalfa resistance against aphids was mainly determined by induction of genes involved in linoleic acid synthesis important for jasmonic acid and flavonoid biosynthesis (Tu *et al.,* 2018).

Generally, the most frequently appearing pathogens are bacteria and fungi belonging to Ascomycetes and Basidiomycetes. Considerable declines in alfalfa production have been observed mostly due to root infections leading to root diseases caused by the bacterial wilt (*Clavibacter michiganensis* subsp. *insidiosus*), Verticillium wilt (*Verticillium alfalfae*), Phytophthora root rot (*Phytophthora medicaginis*), Fusarium wilt (*Fusarium oxysporum*), and the foliar disease anthracnose caused by *Colletotrichum trifolii* (Annicchiarico *et al.,* 2015; Singer *et al.,* 2018). There are also other diseases caused by *Phoma sclerotioides*, *Phoma medicaginis varieties*, and *Sclerotinia trifoliorum* (Rubiales *et al.,* 2015). Alfalfa varieties resistant to these diseases have been obtained by common breeding methods over decades (Toth and Bakheit, 1983; Pratt and Rowe, 2002). García *et al.* (2014) proposed that the use of SNAKIN-1 PEPTIDE (SN1) could improve alfalfa tolerance to virulent fungal pathogens. It is cysteine-rich peptide, which could be a component of constitutive defense barriers (Segura *et al.,* 1999). Three independent transgenic lines carrying the *CaMV35S:MsSN1* construct showed significantly lower amounts of infected leaves than wild type plants when treated by *C. trifolii* and with the oomycete *P. medicaginis* (García *et al.,* 2014).

3.3 Legume-rhizobial symbiosis

Many plant species are able to manage beneficial interactions with a wide range of microorganisms. The most and best-studied symbiotic interactions are those between legumes and nitrogen-fixing rhizobial bacteria, and between plants and arbuscular mycorrhizal fungi (Oldroyd, 2013). Nitrogen is a major essential element for all living organisms. It is a main constituent of cellular macromolecules, such as nucleic acids and proteins, and part of low-molecular mass compounds like amines, vitamins and chlorophylls (Stambulska and Bayliak, 2020).

Diverse plant species of *Fabaceae* family developed during their evolution symbiotic interactions with nitrogen-fixing soil bacteria collectively called rhizobia (e.g. *Bradyrhizobium* or *Sinorhizobium*) which can reduce atmospheric dinitrogen (N_2) into ammonium (NH_3) in specialized organs, the so called root nodules (Wang *et al.*, 2018). This relationship provides nutrient benefits for both partners, plant provides carbon sources for bacteria, and bacteria supply NH_3 for plant growth (Clúa *et al.*, 2018; Halbleib and Ludden, 2000). The successful establishment of legume-rhizobial symbiosis can increase plant biomass and crop yield and help to enrich nitrogen in the soil (Dupont *et al.*, 2012). There are several agronomically important legumes performing symbiosis with rhizobia, such as common bean (*P. vulgaris*), pea (*Pisum sativum*), alfalfa (*M. sativa*), soybean (*G. max*) or lentil (*Lend culinaris*) (Clúa *et al.*, 2018; Dupont *et al.*, 2012).

3.3.1 Nod factors

Nod factors are specific rhizobial lipochitooligosaccharides and *nod* genes control their production. They are signaling molecules essential for bacterial invasion and initiation of the nodule formation in the root cortex (Dupont *et al.*, 2012; Gourion *et al.*, 2015; Long, 2001). Without them rhizobia cannot enter legume roots (Cooper, 2007). Different rhizobial species produce various Nod factors. For example, each species of *Rhizobium* has a specific set of *nod* genes that make the Nod factors specific to the host plant (Cooper, 2007). Rhizobia possess common and specific *nod* genes. The group of *nod* genes (*nod*ABC) encodes the core Nod structure typical for all rhizobial species (Bonaldi *et al.*, 2010). *NodA* gene encodes an acetyltransferase, *nodB* encodes a deacetylase, and *nodC* encodes an *N*-acetyl-glucosaminyltransferase (Bonaldi *et al.*, 2010). Another group of *nod* genes (*nod*PQ, *nod*EF, *nod*X, *nod*H) shows a strong species specifity (Franche *et al.*, 2009). Genes encoding enzymes involved in the Nod factor synthesis and genes of symbiotic nitrogen fixation are called *nif* and *fix* genes (Franche *et al.*, 2009; Janczarek *et al.*, 2014).

3.3.2 Flavonoids as infection signals

Flavonoids are low-molecular weight secondary metabolites that are produced by plants. They possess fifteen-carbon skeleton consisting of two benzene rings biosynthesized by phenylpropanol pathway (Liu and Murray, 2016). Secondary modifications like methylation, hydroxylation and glycosylation can strongly effect flavonoid function, mobility and solubility in the plant and the soil (Dixon and Steele, 1999; Winkel-Shirley, 2001). Isoflavonoids are typical for legumes and only specific subsets have function in nodulation. Roots secrete nodulation-specific flavonoids into the rhizosphere, at the root surface and inside infection

threads, where they induce expression of *nod* genes (Kape *et al.,* 1992; Redmond *et al.,* 1986; Subramanian *et al.,* 2007). Flavonoids serve as chemoattractants for compatible species of rhizobia and primary plant signals that regulate expression of several rhizobial genes (Liu and Murray, 2016; Oldroyd *et al.,* 2011). Different flavonoids can have distinct roles in the nodulation process of *M. truncatula* inoculated by *S. meliloti* (Zhang *et al.,* 2009). Rhizobia can degrade plant flavonoids to derivatives and phenolic metabolites (Brencic and Winans, 2005; Janczarek *et al.,* 2015).

3.3.3 Legume inoculation

Symbiosis between rhizobium and legume plants requires a mutual recognition of both partners. Recognition starts when flavonoids are recognized as specific inducers of *NOD* genes in rhizobia (Subramanian *et al.* 2007). The early steps in the invasion of barrel medic (*M. truncatula*) and alfalfa roots by *S. meliloti* are characterized by the mutual exchange of signals that allow the bacteria enter the plant root hair cells (Jones *et al.,* 2007).

Alfalfa derived flavonoid luteolin stimulates binding of an active form of NodD1 to an *S. meliloti NOD*-box promoter, and thereby activation of downstream *NOD* genes transcription (Peck *et al.,* 2006). Genome of *S. meliloti* contains two other NodD proteins – NodD2, which is activated by as-yet-unpurified plant compounds, and NodD3, which does not require plant-derived compounds for activation of genes from *NOD* box promoters (Perret *et al.,* 2000). Any of these NodD proteins can start nodulation of alfalfa with *S. meliloti* and flavonoids from non-host plants can inhibit the transcription of *NOD* genes (Peck *et al.,* 2006). One of the earliest plant responses to the matching Nod factor structure is an increase of intracellular Ca^{2+} in root hairs, followed by strong calcium oscillations (Oldroyd and Downie, 2008), and modifications of root hair cytoskeleton (Sieberer *et al.,* 2005; Timmers *et al.,* 1999). Calcium spiking and transcript induction are dependent on phospholipid signaling pathway (Charron *et al.,* 2004). Microtubules and actin filaments work together in coordinated manner. Actin cytoskeleton is important for root hair growth (Ringli *et al.,* 2002). Morphologically, polarized root hair growth shows deformation, such as swelling of the root hair tip caused by Nod factors (**Figure 1**; de Ruijter *et al.* 1998). Curled root hairs entrap rhizobia (Esseling *et al.,* 2003; Gage, 2004) which enter root hair via specific thin tubular structure called infection thread (IT). Nuclei of root hairs are relocalized, which is accompanied by changes of cytoskeletal organization (Genre *et al.,* 2005). The ITs elongate into the root cortex of plant host (**Figure 1**). Bacteria in ITs are actively dividing (Gage, 2004). *S. meliloti* produces the exopolysaccharides succinoglycan (also known

as exopolysaccharide I, EPSI) and galactoglucan (EPSII), which promote IT formation (Glazebrook and Walker, 1989; Pellock *et al.*, 2000).

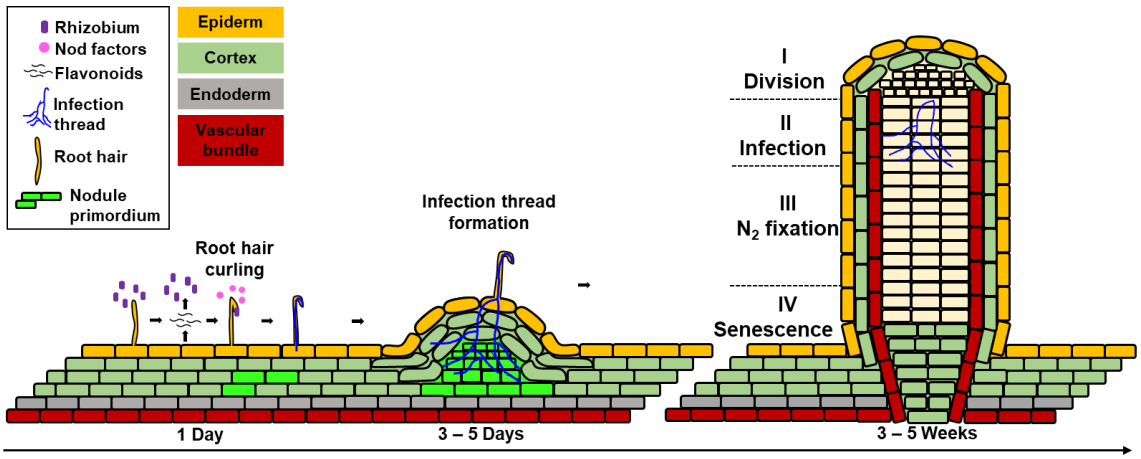

Figure 1 Symbiotic signaling in the legume-rhizobial interaction. The process of bacterial infection and nodule development. A mature indeterminate nodule contains: I – a division zone, II – an infection zone, III – a nitrogen fixing zone and IV – a senescent zone. Adapted from Berger *et al.* (2019) and Wang *et al.* (2018).

Cortical cells initiate cell divisions and form nodule primordia. ITs grow toward the nodule primordia and release rhizobia into the dividing cells in the nodule (Foucher and Kondorosi, 2000). Internalized rhizobia are surrounded by a plant membrane and perform nitrogen fixation inside nodules (Oldroyd and Downie 2008). In legume plants, indeterminate or determinate type of nodules can be formed. Indeterminate nodules initiate from inner cortex and pericycle and they have a sustained nodule meristem originating from the third cortical layer. Barrel medic and alfalfa are examples of indeterminate legumes, which form nodules with such persistent meristem (Xiao *et al.*, 2014). Mature indeterminate nodules have different nodule zones: I – the apical meristem, II – the invasion zone, III – the nitrogen-fixing zone and IV – the senescence zone (**Figure 1**; Timmers *et al.*, 1999). Determinate nodules initiate from the outermost one or two layers of cortical cells and do not maintain a nodule meristem. Such nodules usually proliferate, differentiate and senesce synchronously (Mergaert *et al.*, 2006). Example of determinate legume is *L. japonicus* (Calvert *et al.*, 1984). Development of both types of nodules is controlled by plant hormones (Oldroyd, 2013).

Nodule development is locally regulated by feedback between plant and rhizobia. The total number of nodules on roots is controlled by a systemic mechanism called autoregulation of nodulation (Lin *et al.*, 2020) or control by long-distance communication from shoots. Both the local and the systemic mechanisms are regulated by phytohormones. The host plant is affected by two mechanisms including direct synthesis of phytohormones by rhizobia or

indirect manipulation of the phytohormonal balance in the plant by bacterial Nod factors (Ferguson and Mathesius, 2014).

3.4 Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are one of the largest group of phospho-transferases, catalyzing phosphorylation of appropriate protein substrates on serine and threonine residues. MAPK pathways represent universal signal transduction modules in all eukaryotes, including mammals, plants and yeasts (Ichimura *et al.,* 2002; Zanke *et al.,* 1996; Xu C. *et al.,* 2017). These protein phosphorylation cascades mediate the intracellular transmission and amplification of extracellular signals for induction of cellular responses. A typical MAPK cascade consists of at least three sequentially acting serine/threonine kinases, namely MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK, each subsequently phosphorylating, and hence activating, downstream kinase (Raja *et al.,* 2017; Šamajová *et al.,* 2013). MAPKs are mostly present in the cytoplasm and nucleus (Wang Z. *et al.,* 2015).

3.4.1 MAPKs in alfalfa

In alfalfa, stress-induced MAPK (SIMK) was identified as a 46 kDa salt stress- and elicitor-induced MAPK (Munnik *et al.,* 1999; Cardinale *et al.,* 2002). Yeast two-hybrid screen and activation studies identified SIMK kinase (SIMKK) as an upstream activator of SIMK (Kiegerl *et al.,* 2000; Cardinale *et al.,* 2002). Interaction between SIMKK and SIMK upon salt stress is quite specific, because no interaction was observed with three other MAPKs, such as MMK2 (Jonak *et al.,* 1996), MMK3 (Bögre *et al.,* 1999) and SAMK (Jonak *et al.,* 1996). Another MAPKK identified in alfalfa is PRKK. PRKK together with SIMKK showed strongest interaction with SIMK, but PRKK required activation by an upstream-activated MAPKK kinase. SIMKK has function in pathogen and elicitor signaling. In addition, MMK2 and MMK3 are involved in cell growth and division (Bögre *et al.,* 1999). MMK2 is one of the first alfalfa MAPKs identified as being involved in cytoskeletal regulation. Calderini and co-workers (1998) and subsequently Bögre and co-workers (1999) identified MMK3 in alfalfa and $p43^{NTF6}$ in tobacco, two MAPKs that become activated in a microtubule-dependent manner during late anaphase/early telophase and localize at the phragmoplast midzone (Otegui and Staehelin, 2000). SAMK is stress-activated MAPK identified as kinase inducible by osmotic stress (Munnik *et al.,* 1999), wounding (Bögre *et al.,* 1999) and by various fungal elicitors (Cardinale *et al.,* 2000). SIMKK is a functional dual-specificity protein kinase that phosphorylates SIMK on both threonine and tyrosine residues of the activation loop (Kiegerl *et al.,* 2000; Cardinale

et al., 2002). SIMKK shares 88% amino acid similarity with SIP2 (MAPKK in *L. japonicus;* Chen *et al.,* 2012) and is highly similar to MKK4 (MAPKK4 in *M. truncatula*; Chen *et al.,* 2017). Previously, it was shown that SIMK predominantly localizes in nuclei of root hair while it is activated and redistributed from nucleus into growing root hair tips (Šamaj *et al.,* 2002). In latrunculin B-treated root hairs, SIMK relocated back to the nucleus while after jasplakinolide treatment, SIMK co-localized with thick F-actin cables in the cytoplasm. Thus, these drugs affecting actin cytoskeleton (Baluška *et al.,* 2000), have a direct impact on the intracellular localization of SIMK (Šamaj *et al.,* 2002). In order to enhance SIMK expression, we cloned N-terminal fusion construct of enhanced *GFP* (*eGFP*) with *SIMK* driven under constitutive *35S* promoter (*35S::GFP:SIMK*) and stably transformed this construct into alfalfa plants (Hrbáčková *et al.,* 2021). Live cell microscopy imaging revealed GFP-SIMK localization predominantly to the nucleus and cytoplasm in cells of diverse organs such as roots and leaves. This was consistent with previously published data on SIMK immunolocalization in alfalfa roots (Baluška *et al.,* 2000; Ovečka *et al.,* 2014). The GFP-SIMK was also localized in tips of growing root hairs in agreement with previously published SIMK localization pattern using immunofluorescence microscopy (Šamaj *et al.,* 2002).

Previously, Bekešová *et al.* (2015) showed decreased accumulation of phosphorylated SIMK in SIMKKi lines. In alfalfa, representing an important forage crop, total leaf surface area and shoot biomass are agronomical parameters of interest. In this respect, genetic manipulation of SIMK brought interesting and potentially promising results. Downregulation of *SIMKK* and *SIMK* genes in SIMKKi lines influenced negatively the development of above ground plant parts, leading to formation of smaller leaves and production of less shoot biomass per plant. Importantly, SIMK overexpression in GFP-SIMK lines, on the other hand, resulted in higher shoot biomass per plant, production of bigger size of analyzed leaves and their better distribution in shoots due to the longer petioles. This result may support a general effort on alfalfa biotechnological improvement as a forage crop (Hrbáčková *et al.,* 2021).

4 Part II - Preparation and transformation of fluorescently-tagged MAPKs of *Medicago sativa*

4.1 Material and methods

4.1.1 Plant material

Somatic embryos of *M. sativa* cv. Regen-SY (RSY; Bingham, 1991) wild-type lines (RSY L1, L2), *M. sativa* cv. Regen-SY carrying *35S::GFP:SIMK* construct (GFP-SIMK L5, L6) with well-developed root poles were separated, individually transferred on root and plant development medium (MMS) or MS medium for long-term cultivation of complete plants in controlled environmental chambers. Plants were grown in the environmental chamber at 21 °C and 70% humidity in a 16-h light/8-h dark cycle. Illumination intensity was 150 μmol $m^{-2}s^{-1}$.

4.1.2 Cloning of fluorescently-tagged MAPKs

Cloning of constructs for expression of N-terminal GFP-tagged SIMK or tagRFP-tagged SIMKK driven under *p35S* promoter (*35S::GFP:SIMK*, *35S::tagRFP:SIMKK*) was performed in pB7m34GW,0 by MultiSite Gateway® Three-fragment vector construction kit. For construction of *35S::GFP:SIMK* vector were have used pEN-L4-2-R1™ plasmid carrying *p35S* sequence (Karimi *et al.*, 2007), pEN-L1-F-L2™ carrying *eGFP* sequence (Karimi *et al.*, 2007) and pDONR™P2R-P3 carrying *SIMK* cDNA sequence (https://www.thermofisher.com). For construction of *35S::tagRFP:SIMKK* vector were employed pEN-L4-2-R1™ plasmid carrying *p35S* sequence, pGEM®-T Easy plasmid containing *tagRFP* sequence (obtained from Department of Molecular Biology, CRH, Olomouc) and pDONR™P2R-P3 carrying *SIMKK* cDNA sequence (https://www.thermofisher.com). In the first step, 1190 bp *SIMK* and 1133 bp *SIMKK* PCR fragments were amplified using iProof™ High-Fidelity DNA Polymerase (Bio-Rad, USA) with specific primers and total cDNA of alfalfa as a template. Donor and destination vectors were transformed in *Escherichia coli* strain TOP10. Colonies were tested for presence of required construct by cleavage with PstI (for *35S::GFP:SIMK*) and SacI (for *35S::tagRFP:SIMKK*) restriction enzymes (Thermo Fisher Scientific, USA) at 37°C for 2 hours. Positive colonies were tested by sequencing (SEQme, Czech Republic). Plasmid DNA was isolated by QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany) according recommended protocol. Final destination vector was used for *A. tumefaciens* strain GV3101 cold-shock transformation.

4.1.3 Transient transformation of *Nicotiana benthamiana* leaves

LB medium (5 ml) containing appropriate selection antibiotics was inoculated with *A. tumefaciens* GV3101 strain transformed with vectors encoding GFP-SIMK and tagRFP-SIMKK fusion proteins. Bacterial cultures grown at 28 °C, 200 rpm to OD_{600} 0.7 were pelleted at 3500 g, 4 °C for 15 min. Pellets were suspended in 2 ml buffer containing 10 mM $MgCl_2$, 10 mM MES (pH 5.6) and 150 μM acetosyringone, and subsequently incubated at room temperature in the dark for 2 h. Bacterial cultures containing *35S::GFP:SIMK* and/or *35S::tagRFP:SIMKK* constructs were infiltrated into six-week-old *N. bethamiana* leaves using syringe. Leaves were infiltrated by single construct or simultaneously by two constructs, and eventually treated by 500 mM NaCl for 10 min. Infiltration buffer without any construct was used as a negative control. After infiltration, plants were covered with transparent plastic bags and maintained in fytotron for 24 h, than were uncovered. After 48 h, transformed epidermal cells were observed with CLSM (LSM710, Axio Imager2, Carl Zeiss, Germany) equipped with Plan-Apochromat 20x/0.8 objective (Carl Zeiss, Germany). Samples were imaged with 488 nm excitation laser line and appropriate detection range for GFP emission. Image post- processing was done using ZEN 2010 software.

4.1.4 Stable transformation of *M. sativa*

To obtain stable transformed line of *M. sativa* with N-terminal fusion construct of enhanced GFP (*eGFP*) with *SIMK* driven under *35S* promoter (*35S::GFP:SIMK*), leaves of mature plants were transformed with *A. tumefaciens* GV3101 carrying *35S::GFP:SIMK* in pB7m34GW,0 expression plasmid. The transformation procedure was performed according to protocol for efficient transformation of alfalfa described by Samac and Austin-Phillips (2006). Leaves from well-developed plant nodes were surface sterilized, cut in half and wounded on the surface with sterile scalpel blade, incubated with overnight *Agrobacterium* culture showing cell density between 0.6 and 0.8 at A_{600} nm for 30 minutes. Leaves were dried of the bacterial solution and transferred on appropriate cultivation media (called B5H medium). Induction of callogenesis from leaf explants, production of somatic embryos from calli (B50 medium), development of shoots and somatic embryo rooting were performed on the appropriate media (MMS medium) in the culture chamber at 22 °C, 70% humidity, light intensity 100 $\mu mol.m^{-2}s^{-1}$ and 16/8 h light/dark photoperiod. Regenerated plants were maintained and selected on media (MS medium) with phosphinothricin (50 μg/ml) and tested for the presence of GFP-SIMK fusion protein using molecular genotyping or fluorescent microscope. Transgenic alfalfa plants marked GFP-SIMK L5, L6 were propagated in sterile culture via somatic embryogenesis.

Somatic embryos stably expressing *35S::GFP:SIMK* construct were used in further experiments.

Obtaining stable transformed line of alfalfa with N-terminal fusion construct of tagRFP-tagged *SIMKK* driven under *35S* promoter (*35S::tagRFP:SIMKK*) was not successful. Plants contained *35S::tagRFP:SIMKK* construct verified by PCR-based genotyping, but without fluorescent signal in the microscope.

4.1.5 Live-cell subcellular localization of fluorescently-tagged SIMK and SIMKK

Agrobacteria carrying *35S::GFP:SIMK* and *35S::tagRFP:SIMKK* constructs were used for transient transformation of *N. benthamiana* leaves and *Agrobacterium* carrying *35S::GFP:SIMK* for stable transformation of alfalfa RSY L1 plants. Transgenic alfalfa lines (GFP-SIMK L5, L6) were regenerated through somatic embryogenesis and cultivated in the culture chamber at above-described conditions. Fluorescence signals were observed in transformed *N. benthamiana* epidermal leaf cells and in alfalfa lines using confocal laser scanning microscopes LSM 710 (Carl Zeiss, Germany) equipped with Plan-Apochromat 20×/0.8 (Carl Zeiss, Germany), and LSM880 with Airyscan (Carl Zeiss, Germany) equipped with Plan-Apochromat 20×/0.8 (Carl Zeiss, Germany). Samples were imaged with 488 nm excitation laser line with emission filter 493 – 598 nm for GFP and 561 nm excitation laser line with emission filter 558 – 583 nm for tagRFP. Image post-processing was done using ZEN 2010 software.

4.1.6 Fixation and immunolabeling of SIMK and phosphorylated MAPKs in fixed alfalfa roots

Immunolocalization of GFP-tagged SIMK and pERK in root whole-mounts of transgenic plants carrying *35S::GFP:SIMK* construct (GFP-SIMK L5) was done as described previously (Tichá *et al.*, 2020a). A double-immunolabeling with mouse anti-GFP (Abcam, UK) and rabbit anti-phospho-p44/42 (Cell Signaling, Netherlands) primary antibodies diluted 1:100 in 2.5% (w/v) BSA dissolved in PBS was performed on root tip samples. Vacuum pump was used (3×5 min) to improve antibody penetration, followed by incubation at 4°C overnight. Samples were sequentially incubated with secondary antibody solutions. First, Alexa-Fluor 488 goat anti-mouse secondary antibody (Invitrogen, USA) diluted 1:500 in 2.5% (w/v) BSA in PBS was used for incubation at 37 °C for 2 h. After extensive washing with PBS and subsequent blocking [5 % (w/v) BSA in PBS for 20 min] samples were incubated with Alexa-Fluor 555 goat anti-rabbit secondary antibody (Abcam) diluted 1:500 in 2.5% (w/v) BSA in PBS at 37 °C

for 2 h. Nuclei were counterstained with DAPI. Immunolabeled root samples were mounted in anti-fade mounting medium [0.1 % (w/v) paraphenylenediamine in 90% (v/v) glycerol buffered with 10% (v/v) PBS at pH 8.2 - 8.6] and used for microscopy. Microscopic analysis was performed with a Zeiss 710 CLSM platform (Carl Zeiss) or Zeiss LSM880 Airyscan equipped with a 32 GaAsP detector, using excitation laser lines at 405 nm for DAPI, 561 nm for Alexa-Fluor 555 and 631 nm for Alexa-Fluor 647. The image post-processing was done using ZEN 2014 software and final figure plates were prepared using Photoshop 6.0/CS and Microsoft PowerPoint software. Kateřina Hlaváčková and Olga Šamajová performed fixation and immunolabeling of SIMK.

4.2 Results

4.2.1 Cloning of *GFP*-tagged *SIMK* and *tagRFP*-tagged *SIMKK* under *35S* promoter

Two constructs *35S::GFP:SIMK* and *35S::tagRFP:SIMKK* were prepared using MultiSite Gateway® cloning system. High-Fidelity Polymerase (iProof™) was used for PCR amplification from cDNA sequences. Due to the complexity of Gateway primers (50 bp fused with ATT sequences for recombination with 25 bp incorporated in primers), the PCR additives DMSO and/or betaine were used to suppress primer secondary structure formation like hairpins or dimers. The restriction enzymes NdeI, DpnI, PvuI and XbaI were employed for restriction analyses of entry clones, and PstI and SacI for final destination vectors (**Figure 2**). Entry clones were verified by sequencing.

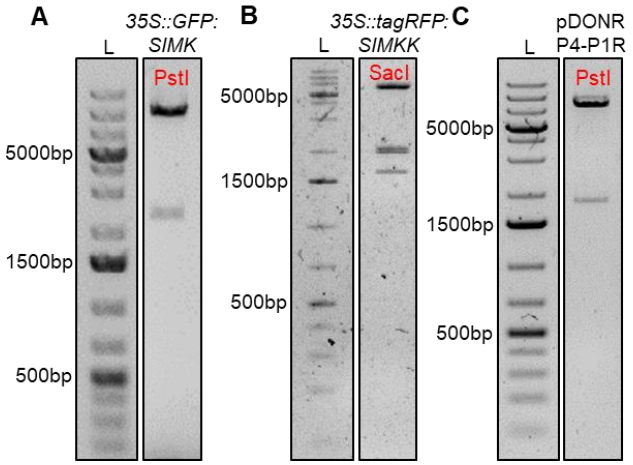

Figure 2 Representative electrophoretic images of destination vectors for *GFP*-fused *SIMK* and *tagRFP*-fused *SIMKK* genes under *35S* promoter. **(A-C)** Enzymatic digestions of destination vectors carrying *GFP*-fused *SIMK* and *tagRFP*-fused *SIMKK* genes under *35S* promoter prepared by Multisite Gateway® LR recombination reaction in pG7m34GW,0. **(A)** Destination vector *35S::GFP:SIMK* digested by PstI (8965 bp + 2287 bp). **(B)** Destination vector *35S::tagRFP:SIMKK* digested by SacI (5755 bp + 1975 bp + 1945 bp +1561 bp). **(C)** Empty destination vector pDONR P4-P1R digested by PstI (8081bp + 1864 bp) used as a control of enzymatic digestion and successful LR recombination. L – DNA ladder.

4.2.2 Transient expression of GFP-SIMK and tagRFP-SIMKK fusion proteins in *N. benthamiana* leaves

Transient transformation of *N. benthamiana* leaves was performed in order to test *35S::GFP:SIMK* and *35S::tagRFP:SIMKK* constructs (**Figure 3**). This experiment proved fluorescent properties of GFP-SIMK (**Figure 3A**) and tagRFP-SIMKK (**Figure 3B**) fusion proteins. Both GFP-SIMK and tagRFP-SIMKK were preferentially localized in the nucleus (except nucleoli) and in the cytoplasm (**Figure 3A,B**). The expression of the GFP-SIMK fusion protein was corroborated by immunoblot analysis of protein extracts isolated from the fluorescent areas of transformed *N. benthamiana* leaves. The presence of *35S::tagRFP:SIMKK* construct was supported by PCR-based genotyping using transformed areas of *N. benthamiana* leaves.

In order to perform colocalization studies, SIMK and SIMKK were tagged with different fluorescent proteins (GFP and tagRFP, respectively) and transiently co-expressed in epidermal cells of *N. benthamiana* leaves. Under control conditions, both GFP-tagged SIMK and tagRFP-tagged SIMKK preferentially localized to the nuclei, but they were also dispersed throughout the cytoplasm (**Figure 3**). In contrast, both GFP-SIMK and tagRFP-SIMKK relocated to cytoplasmic punctate compartments where they colocalized after 10 min of 500 mM NaCl treatment (**Figure 4**). These data suggested that salt treatment triggered tagRFP-SIMKK mediated relocation of GFP-SIMK, which was associated with colocalization of both fluorescently tagged kinases in cytoplasmic spot-like compartments. Simultaneous relocation and colocalization of both GFP-SIMK and tagRFP-SIMKK to these cytoplasmic compartments suggested that both kinases were targeted to these structures in a coordinated manner.

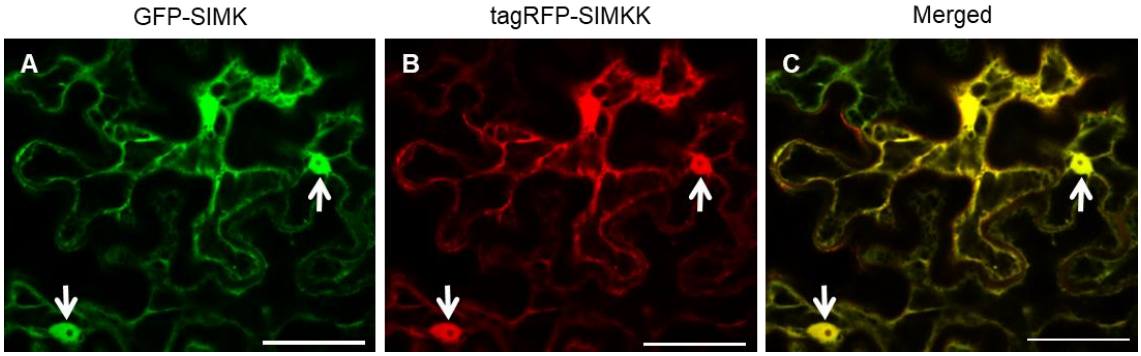

Figure 3 Co-localization of GFP-SIMK and tagRFP-SIMKK in transiently co-transformed leaf epidermal cells of *N. benthamiana*. **(A-C)** GFP-tagged SIMK **(A)** and tagRFP-SIMKK **(B)** colocalize **(C)** in *N. benthamiana* epidermal leaf cells. Both fusion proteins showed predominantly nuclear (arrows) and cytoplasmic localization (merged image shown in **C**). Scale bar: 50 μm.

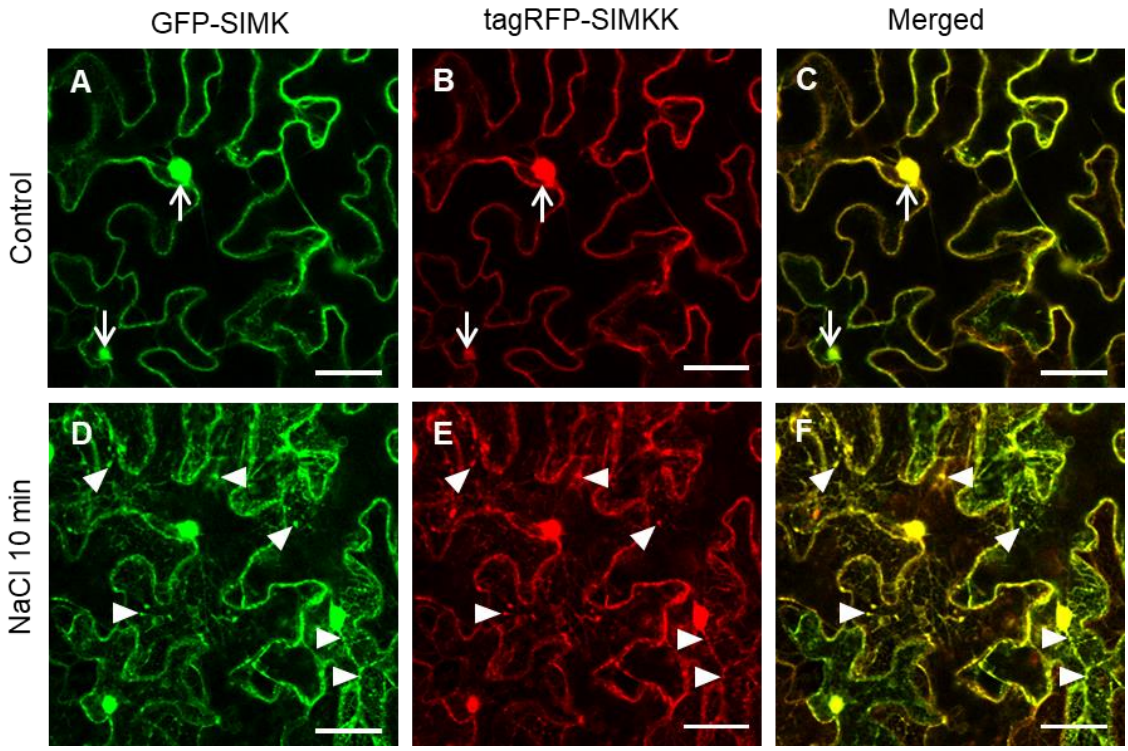

Figure 4 Co-localization of GFP-SIMK and tagRFP-SIMKK in transiently co-transformed leaf epidermal cells of *N. benthamiana* before and after salt (500 mM NaCl) treatment. **(A-C)** GFP-tagged SIMK **(A)** and tagRFP-SIMKK **(B)** colocalize **(C)** in *N. benthamiana* epidermal leaf cells. Both fusion proteins showed predominantly nuclear (arrows) and cytoplasmic localization (merged image shown in C). **(D-F)** After salt treatment (500 mM NaCl for 10 min), both GFP-SIMK **(D)** and tagRFP-SIMKK **(E)** simultaneously relocated to the cytoplasm where they colocalized on cytoplasmic punctate compartments (merged image shown in **F**, indicated by arrowheads). Scale bar: 100 µm.

4.2.3 Stable transformation of *M. sativa* leaf explants using somatic embryogenesis

Surface-sterilized leaves of alfalfa RSY L1 plants were transformed with *A. tumefaciens* strain GV3101 carrying *35S::GFP:SIMK* construct and plants regenerated by somatic embryogenesis (**Figure 5**). Indirect somatic embryogenesis was induced from young developing leaves with three leaflets (**Figure 5A**) that were gently surface-sterilized and cut into parts (**Figure 5B**). It passed through the stage of callus formation which was induced by the application of the appropriate combination of exogenous phytohormones (**Figure 5C**). Subsequently, differentiated calli were cultivated on the culture medium lacking phytohormones leading to the induction of embryogenic calli and somatic embryos (**Figure 5D**). Fully developed somatic embryos showed apical-basal polarity and were promoted to form roots and shoots (**Figure 5E**) up to the regeneration of complete plants developed and maintained *in vitro* (**Figure 5F**). Mature plants obtained by this way were fertile and able to form flowers, produce seeds and the next generation of plants from germinated seeds.

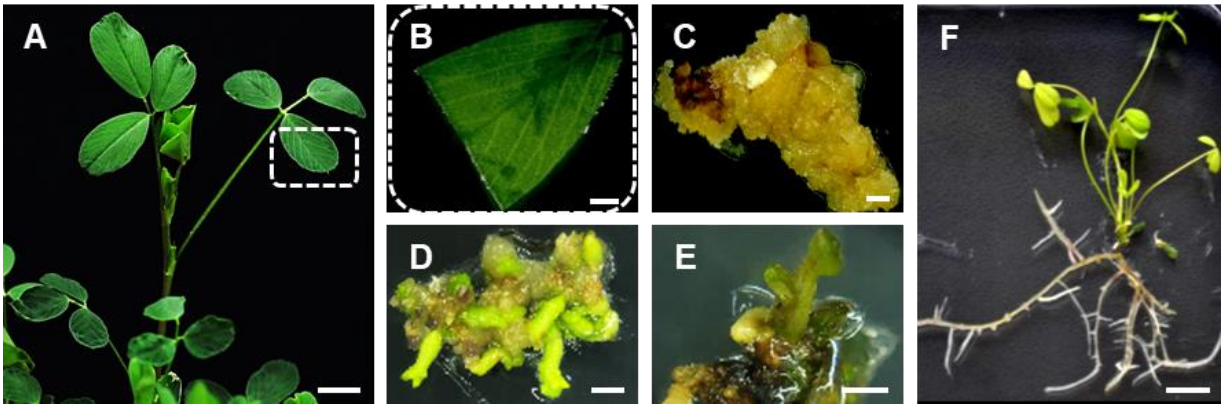

Figure 5 Representative overview showing *in vitro* propagation, multiplication and regeneration of transgenic alfalfa plants using somatic embryogenesis. **(A)** The process is induced from young leaves that are placed into *Agrobacterium* culture containing *35S::GFP:SIMK* construct and transferred to **(B)** appropriate culture medium (B5H medium). **(C)** Subsequently, the massive callogenesis occurs on the leaf explant on the selective antibiotics-containing culture medium followed by the formation of embryogenic calli (B50 medium). **(D)** The next steps of the procedure involve culture media rich in amino acids and vitamins, and for the germination of somatic embryos (MMS medium) regenerating to the **(E)** complete plants on MS medium. **(F)** Regenerated plantlets are fully viable. Scale bar: 10 cm in **(A)**, 0.5 mm in **(B)**, 1 cm in **(C, F)** and 2 cm in **(D, E)**.

4.2.4 Subcellular localization of GFP-SIMK

In order to observe subcellular localization of SIMK in alfalfa plants, SIMK was tagged with GFP marker and overexpressed under *35S* promoter. Subcellular localization of GFP-SIMK fusion protein was performed using confocal laser scanning microscopy (CLSM) and Airyscan CLSM (**Figure 6**). Maximum intensity projection provided overview of alfalfa root tip and revealed nuclear and cytoplasmic GFP-SIMK localization (**Figure 6A**) with lower signal in nucleoli. GFP-SIMK preferentially accumulated in the nucleus and less in the cytoplasmic structures of hypocotyl cells (**Figure 6B**) in stably transformed alfalfa plants. Similar subcellular localization was found also in root hairs (**Figure 6C**), leaf epidermal cells and stomata (**Figure 6D**), and in root border cells detached from lateral root cap (**Figure 6E**). In growing root hairs, GFP-SIMK was mostly localized in nuclei and in the cytoplasm at the root hair tips (**Figure 6C**).

The pattern of subcellular localization in root cells was confirmed in GFP-SIMK transgenic line by using whole-mount immunofluorescence co-immunolabeling (Tichá *et al.*, 2020a) with GFP-specific (see Materials and Methods) and phospho-specific (anti-phospho-p44/42) antibodies (**Figure 7**). Imaging of co-immunolabeled samples with Airyscan CLSM revealed that GFP-SIMK is localized in distinct spot-like structures in the nucleoplasm and in cytoplasmic structures, preferentially in activated form (**Figure 7A-D**). Moreover, GFP-SIMK was localized in activated form in the root hair tip (**Figure 7E-H**).

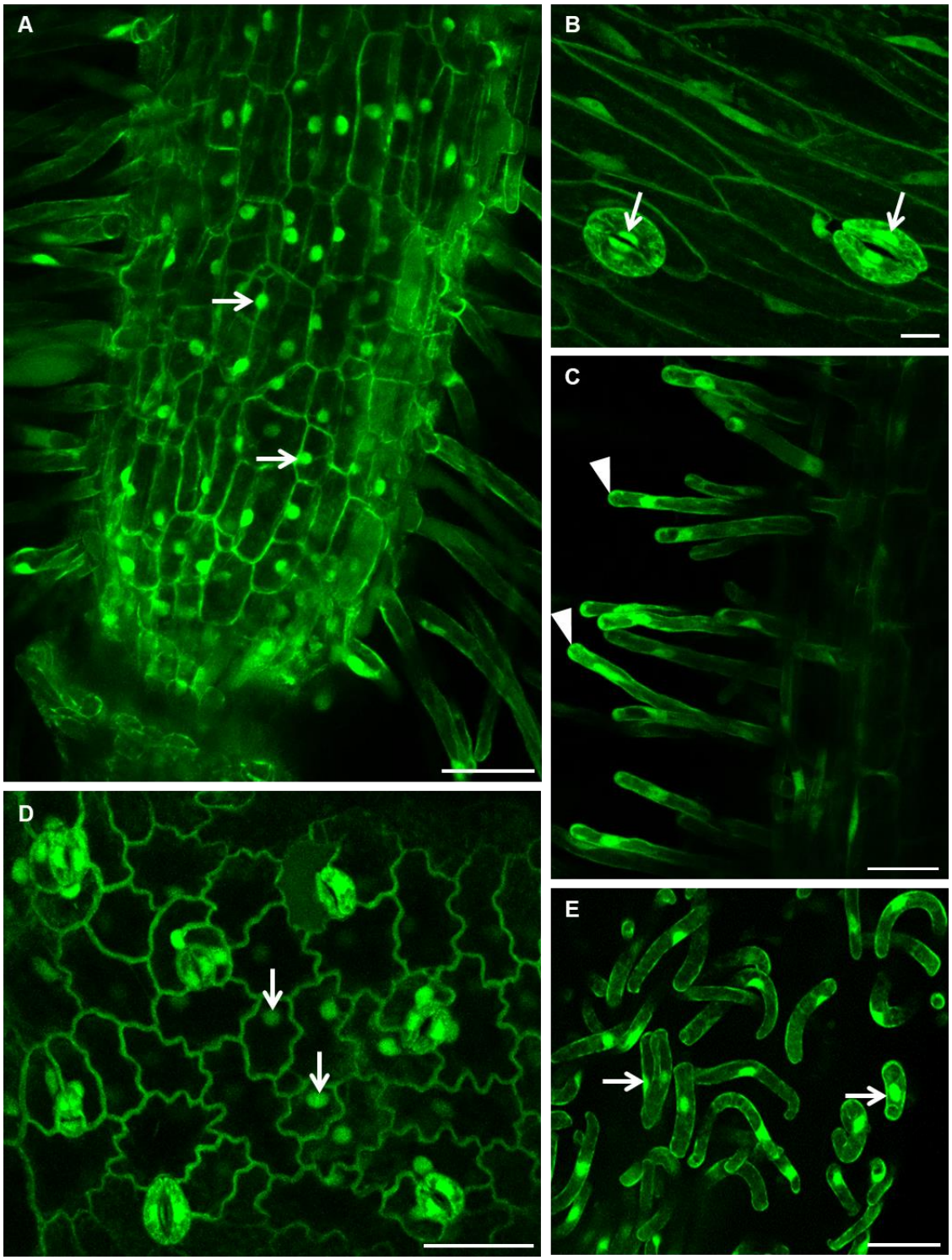

Figure 6 Subcellular localization of GFP-SIMK in stable transformed alfalfa plants using CLSM and Airyscan CLSM. **(A)** Overview of GFP-SIMK distribution pattern in the root. **(B)** Subcellular localization of GFP-SIMK in hypocotyl epidermal cells and stomata, **(C)** root hairs, **(D)** leaf epidermal cells and stomata, and **(E)** in border cells liberated from lateral root cap. Note localization of GFP-SIMK in nuclei (arrows in **A, B, D** and **E**), root hair tips (arrowheads in **C**) and in the cytoplasm. Scale bar: 50 µm in **(A, B, D, E)** and 20 µm in **(C)**.

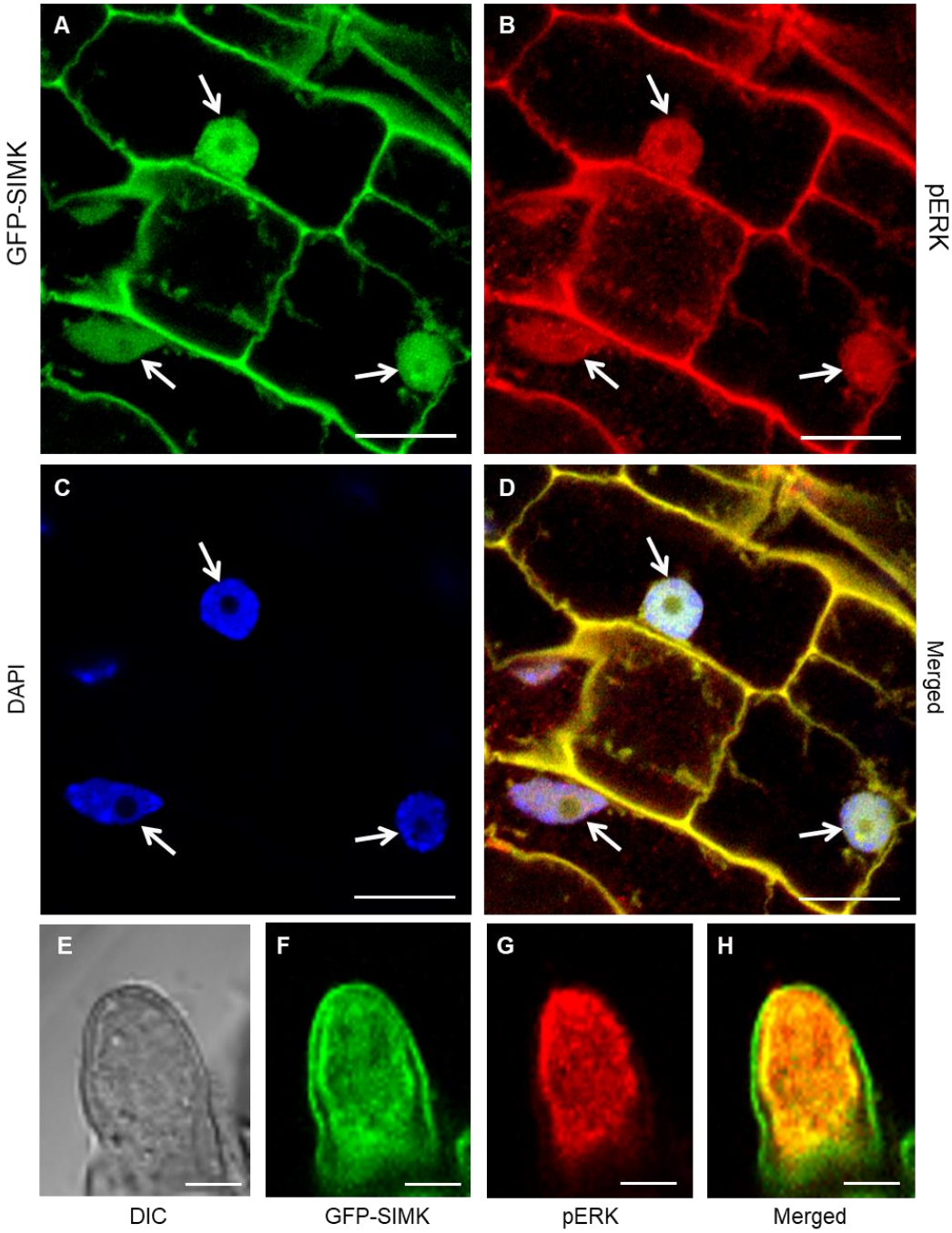

Figure 7 Whole-mount immunofluorescence localization of GFP-SIMK in epidermal cells and root hair tip of stable transformed alfalfa root using Airyscan CLSM. **(A-D)** Whole-mount immunofluorescence localization of GFP-SIMK in stable transformed root epidermal cells. **(A)** Immunolocalization of GFP-SIMK using anti-GFP antibody, **(B)** whole-mount immunolocalization of activated MAPKs (pERK) using anti-phospho-p44/42 antibody, **(C)** DAPI staining of DNA in nuclei, and **(D)** merged image of root epidermal cells. **(E-H)** Whole-mount immunofluorescence localization of GFP-SIMK in root hair tip of stable transformed alfalfa root with Airyscan CLSM. **(E)** Immunolocalization of root hair tip in DIC (differential interference contrast) microscopy, **(F)** immunolocalization of GFP-SIMK using anti-GFP antibody, **(G)** immunolocalization of activated MAPKs (pERK) using anti-phospho-p44/42 antibody, and **(H)** merged image of root hair tip. Note subcellular colocalization of SIMK and pERK in nuclei (arrows), in the cytoplasm **(D)** and in root hair tip **(H)**. Scale bar: 10 μm in **(A-D)**, 5 μm in **(E-H)**. Images provided by Olga Šamajová and Kateřina Hlaváčková. Adapted from Hrbáčková *et al.* (2021).

4.3 Discussion

The MAPK-mediated phosphorylation pathways are essential components of plant cellular signaling. They capture extracellular and developmental cues and translate them into intracellular signals. Typical MAPK cascade is composed of three functionally linked MAPKKK, MAPKK and MAPK (Šamajová *et al.,* 2013b). Recent studies using fluorescent protein tagging technology revealed subcellular localization of MAPKs in several model organisms, such as *A. thaliana* (Jia *et al.,* 2016), cotton (Zhang *et al.,* 2011), *Nicotiana* species (Ishihama *et al.,* 2011), or *Medicago* species (Hrbáčková *et al.,* 2021; Ovečka *et al.,* 2014; Šamaj *et al.,* 2002). MAPK-tagged with GFP fusions are usually driven by potent constitutive promoters, such as the *35S* promoter of cauliflower mosaic virus (Šamajová *et al.,* 2013) or by their own native promoter. In this work, alfalfa SIMKK and SIMK have been cloned with fluorescent marker proteins under *35S* promoter. CLSM have been applied to visualize different organelles and subcellular compartments in living plant organs.

Transient transformation is an efficient method for experiments aiming to visualize subcellular compartments such as nuclei, endoplasmic reticulum, endosome or cytoskeleton labeled by molecular markers containing GFP, YFP (yellow fluorescent protein) or RFP (Křenek *et al.,* 2015). In our laboratory we are routinely using *Agrobacterium*-mediated transient transformation of *N. benthamiana* leaves. First true leaves are suitable for agroinfiltration. Cloning of constructs for expression of N-terminal GFP-tagged SIMK protein and tagRFP-tagged SIMKK driven under *35S* promoter (*35S::GFP:SIMK*, *35S::tagRFP:SIMKK*) was performed in pB7m34GW,0 by MultiSite Gateway®Three-fragment vector construction kit using modified and optimized *att* sites to permit transfer of heterologous DNA sequences between vectors. Vectors with transgenes were transiently transformed into *N. benthamiana* epidermal leaf cells for testing. Under control conditions, both GFP-tagged SIMK and tagRFP-tagged SIMKK preferentially localized to the nuclei, but they were also dispersed throughout the cytoplasm. Chen *et al.* (2017) constructed plasmids that expressed GFP-tagged MtMAPKK4 (ortholog of SIMKK) and GFP-tagged MtMAPK6 (ortholog of SIMK) fusion proteins under *35S* promoter. The plasmids were delivered to *N. benthamiana* leaves. The subcellular localization of *MtMAPKK4* and *MtMAPK6* genes were checked by a confocal microscope. Fused proteins were localized to the plasma membrane, cytoplasm and nucleus (Chen *et al.* 2017). These results were consistent with our observed localization of SIMK and SIMKK in transient system.

MAPKKs can activate their downstream MAPKs through phosphorylation. For example, AtMPK3, AtMPK6, AtMPK4 and their orthologs in plant species showed that they can be activated by several stimuli (Chen *et al.*, 2017). LjSIP2 from *L. japonicus* is orthologue of SIMKK and is important in symbiosis. LjSIP2 represents a typical plant MAPKK and interacts with AtMPK6 (orthologue of SIMK), but not with AtMPK3 in yeast cells. Recombinant LjSIP2 protein could phosphorylate casein and AtMPK6 *in vitro* (Chen *et al.*, 2012). Moreover, MtMAPKK4 (representing another ortholog of SIMKK) physically interacts with MtMAPK6 (ortholog of SIMK), which was verified in yeast and plant cells (Chen *et al.*, 2017). In alfalfa, yeast two-hybrid screen and activation studies identified SIMKK as an upstream activator of SIMK (Kiegerl *et al.*, 2000; Cardinale *et al.*, 2002). To determine whether SIMKK may be involved in mediating the salt-induced activation of SIMK, Kiegerl *et al.* (2000) performed co-expression experiments with SIMKK and SIMK in the presence and absence of salt stress. The co-expression experiments were performed in protoplasts from suspension-cultured parsley cells. SIMK showed very little kinase activity and was only slightly activated by salt stress, but co-expression with SIMKK resulted in considerably stronger SIMK activation. In order to examine colocalization of GFP-SIMK and tagRFP-SIMKK fusion proteins, *N. benthamiana* leaves were infiltrated by co-cultivated bacteria solution of these two constructs. Ovečka *et al.* (2014) described salt-induced subcellular relocation of these two kinases. They have studied the activation and localization of the alfalfa SIMKK-SIMK module after NaCl treatment. Both SIMK and SIMKK were tagged with different fluorescent proteins, YFP and CFP, respectively. They were co-expressed in *A. thaliana* protoplasts to investigate their colocalization. Under control conditions in the inactivate state, both YFP-tagged SIMK and CFP-tagged SIMKK preferentially colocalized in the nuclei and were also dispersed throughout the cytoplasm. In contrast, SIMK-YFP and SIMKK-CFP relocated to cytoplasmic punctate compartments where the colocalized after 30 minutes of salt stress (Ovečka *et al.*, 2014). This was consistent with our results showing colocalization of GFP-SIMK and tagRFP-SIMKK fusion proteins. We showed first their colocalization in transiently transformed *N. benthamiana* epidermal leaf cells. After exposure to the salt stress, GFP-SIMK and tagRFP-SIMKK relocated to cytoplasmic compartments. Colocalization studies suggest that SIMKK functions as a scaffold protein for SIMK, or other unknown scaffold proteins can bind both SIMKK and SIMK (Ovečka *et al.*, 2014). The dynamic relocalization of the MAPK and MAPKK components from the nucleus to the cytoplasm seems to challenge the traditional view of MAPKs translocation from the cytoplasm to the nucleus after its activation. SIMK and SIMKK relocation into the cytoplasmic compartments could represent activation-dependent

mechanism regulated by salt stress. Supporting evidence that the process of activation is linked with MAPK relocation to membrane targets may be related to studies dealing with salt overly sensitive (SOS) pathway (Kim *et al.,* 2012; Ovečka *et al.,* 2008, 2014). In addition, salt stress can trigger different MAPK signaling pathways in *Arabidopsis* resulting in positive or negative plant tolerance. In this respect, it is well known that AtMPK6 and AtMPK3 are activated by salt stress and it seems to be important for salt tolerance of *A. thaliana* (Ichimura *et al.,* 2000). Another recent report showed the colocalization of *Arabidopsis* MPK6 (orthologue of SIMK) and clathrin at the plasma membrane and at trans-Golgi network (TGN) vesicles (Müller *et al.,* 2010). In this context, activated kinase modules have been identified in clathrin-coated vesicles and signaling endosomes in animals (Sorkin and Von Zastrow, 2002).

Expression efficiency of GFP-SIMK and tagRFP-SIMKK fusion proteins was checked using transient transformation before stable transformation of alfalfa. In this thesis, a highly efficient stable transformation approach using *A. tumefaciens* was selected for introducing foreign genes of interests into alfalfa. The stable transformation of plants mediated by *A. tumefaciens* is inheritable and persists in transformant progeny (Samac and Austin-Phillips, 2006; Sangra *et al.,* 2019). The modified protocol of Samac and Austin-Phillips (2006) for alfalfa transformation was used for this purpose. Normally, the protocol provides 80-100 % of successfully transformed plants regenerated from somatic embryos. The protocol described in this thesis utilizes highly regenerable genotype from the variety Regen-SY (Bingham, 1991) and allows a long-term maintenance of somatic embryogenesis through subculture cycles (Samac and Austin-Phillips, 2006; Sangra *et al.,* 2019). The original protocol works with *A. tumefaciens* strain LB4404, but we tried and optimized this stable transformation with *A. tumefaciens* strain GV3101. An appropriate compositions of culture media played a key role in alfalfa propagation protocol. To increase efficiency, various substances, such as vitamins, amino acids, growth regulators, or organic acids were added to the culture media (Amini *et al.,* 2016). Transgenic alfalfa plants can be regenerated within 9-14 weeks after the co-cultivation with agrobacteria. Wild type control plants and stably transformed alfalfa lines can be further regenerated and propagated *via* somatic embryogenesis.

Using stable transgenic line, we were able to describe subcellular localization of GFP-SIMK protein in planta. Our subcellular and developmental observations using live cell CLSM imaging revealed GFP-SIMK localization predominantly to the nucleus and cytoplasm in various cell types (e.g. root border cells, root hairs, epidermis, pavement cells and stomata) of diverse organs including roots, hypocotyls and leaves. Some previous studies showed *in situ*

hybridization and immunolocalization of both SIMK transcript and protein in alfalfa root hairs (Baluška *et al.*, 2000; Munnik *et al.*, 1999; Ovečka *et al.*, 2014; Šamaj *et al.*, 2002). Later on, Chen *et al.* (2017) cloned *MtMAPKK4* and *MtMAPK6* cDNAs from *M. truncatula* with *GFP* under the *35S* promoter. GFP-tagged fusion proteins were delivered to *N. benthamiana* leaves to check their expression. They also examined mRNA transcript levels in different tissues and nodulation stages by quantitative RT-PCR. The expression of *MtMAPKK4* and *MtMAPK6* genes was detected in almost all examined tissues, including roots, stems, leaves and nodules of *M. truncatula.* We stably transformed alfalfa to examine GFP-SIMK localization patterns using modern fluorescent microscopy methods. SIMK was strongly expressed under constitutive *35S* promoter in alfalfa root cells and it accumulated within nuclei of both meristematic and postmitotic cells of the transition zone, consistently with previous *in situ* localization study (Baluška *et al.*, 2000a). The situation changed dramatically in growing root hairs. GFP-tagged SIMK redistributed from nuclei into the cytoplasm and accumulated at the tips of emerging and growing root hairs. This was in agreement with previously published SIMK localization pattern using immunofluorescence microscopy (Šamaj *et al.*, 2002). Concerning subnuclear localization, SIMK was less abundant in nucleoli. The pattern of subcellular localization of SIMK protein in root cells was confirmed in GFP-SIMK transgenic lines by using whole-mount immunofluorescence co-labeling with GFP-specific and phospho-specific (anti-phospho-p44/42) antibodies. Moreover, activated state of SIMK was confirmed with whole-mount immunolabeling in the root hair tip using phospho-specific antibodies. This whole-mount immunolabeling protocol is relatively fast and enables high-resolution microscopy imaging without the need to control proper physiological and environmental conditions during imaging, which are crucial for live cell imaging (Tichá *et al.*, 2020a). Nevertheless, it is mostly restricted only to root apices, and it is not suitable for dynamic live cell imaging provided by recombinant GFP technology used in this study.

5 Part III - Overexpression of SIMK promotes root hair growth, IT and nodule clustering and shoot biomass production in *M. sativa*

5.1 Material and methods

5.1.1 Plant and bacterial material and growth conditions

Somatic embryos of wild type plants of alfalfa RSY (two independent lines L1 and L2), transgenic lines with *SIMKK RNAi* (SIMKKi, two independent lines L3 and L4) and transgenic lines carrying *35S::GFP:SIMK* construct (GFP-SIMK, two independent lines L5 and L6) with well-developed root poles were separated, individually transferred and inserted into root and plant development medium (MMS) or Fåhreus medium without nitrogen (FAH-N_2). Regenerated plants were inoculated with *S. meliloti* Sm2011 strain. Plants were grown in an environmental chamber at 21 °C and 70% humidity in a 16-h light/8-h dark cycle. Illumination intensity was 150 μmol $m^{-2}s^{-1}$.

5.1.2 Root hair phenotypic analysis and *M. sativa* plant inoculation with *S. meliloti*

Wild type plants of alfalfa RSY (lines L1 and L2), transgenic plants with *SIMKK RNAi* (SIMKKi, lines L3 and L4) and transgenic plants carrying *35S::GFP:SIMK* construct (GFP-SIMK, lines L5 and L6) were used for phenotypic analysis of root hairs. Regenerated plants (18-day-old, originating from somatic embryos) were transferred to Petri dishes with FAH-N_2 medium containing 13 g/L micro agar. These plants were used for root hair imaging with Axio Zoom.V16 (Carl Zeiss, Germany). Statistics was calculated in SigmaPlot11.0 using Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's Method) and was based on N=529-1924. Different lower case letters indicated statistical significance between treatments ($p < 0.05$). Plants were inoculated with bacteria *S. meliloti* strain Sm2011 labeled with mRFP with $OD_{600} = 0.5$ (Boivin *et al.*, 1990). After 10 days post inoculation (10 dpi) infection threads were counted using Axio Zoom.V16 (Carl Zeiss, Germany) with excitation laser line 590 nm and emission filter 558 – 583 nm for mRFP and plants were scanned 5, 10, 15 and 20 dpi for evaluation of nodule numbers and arrangements.

5.1.3 Shoot biomass phenotypic analysis

Images of above ground parts of RSY line L1, SIMKKi line L4 and GFP-SIMK line L5 regrown in pots 60 days after shoot cutting were acquired by digital camera (Nikon D5000, Japan). Individual shoots were detached from the plants and shoot length (in cm), shoot weight (in g), number of shoots per plant, and biomass weight per plant (in g) were recorded.

Quantitative analysis was performed in SigmaPlot11.0 using Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's Method) or using One Way Analysis of Variance (Holm-Sidak method) and was based on N=94-196 shoots (for shoot length and weight) and N=4-13 plants (for number of shoots per plant and biomass weight). Ivan Luptovčiak performed statistical analysis. Images of above ground parts were taken with help of Dominik Novák.

5.1.4 Immunoblotting analysis

Immunoblotting analysis was performed as described in Takáč *et al.* (2017). Plants of 20-day-old alfalfa RSY L1, transgenic line with *SIMKK RNAi* construct (SIMKKi L4) and transgenic line carrying *35S::GFP:SIMK* construct (GFP-SIMK L5) were used for immunoblotting analysis. Roots from 20-day-old alfalfa plants were homogenized using liquid nitrogen to fine powder and the proteins were extracted in E-buffer [50 mM HEPES (pH 7.5), 75 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM NaF, 10 % (v/v) glycerol, Complete™ EDTA-free protease inhibitor and PhosSTOP™ phosphatase inhibitor cocktails (both from Roche, Basel, Switzerland)]. After centrifugation, supernatants were mixed with Laemmli buffer [final concentration 62.5 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 10 % (v/v) glycerol, 300 mM 2-mercaptoethanol]. After protein concentration measurement using Bradford assay equal protein amounts (10 ng) were separated on 12% TGX Stain-Free™ (Bio-Rad) gels (Biorad). Overnight proteins were transferred to polyvinylidene difluoride (PVDF) membranes in a wet tank unit (Bio-Rad) at 24 V and 4 °C using the Tris-glycin-methanol transfer buffer. Membranes were blocked in 4% (w/v) bovine serum albumin in Tris-buffered-saline (TBS, 100 mM Tris-HCl; 150 mM NaCl; pH 7.4) at 4 °C overnight. Following washing step with TBS-T (TBS, 0.1 % Tween 20) membranes were incubated with polyclonal anti-AtMPK6 antibody (Sigma, Life Science, USA), highly specific for SIMK detection (Bekešová *et al.*, 2015), diluted 1:15000 in TBST-T containing 1% (w/v) BSA or with anti-phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204) antibody (Cell Signaling, Netherlands) diluted 1:1000 in TBS-T containing 1% (w/v) BSA at 4 °C overnight. After five washing steps in TBS-T blots were incubated with a horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody (diluted 1:5000) in the case of both anti-AtMPK6 and anti-phospho-p44/42 primary antibodies. The signals were developed using Clarity Western ECL substrate (Biorad, Hercules, CA) and detected on Chemidoc MP documentation system (Biorad). In total nine immunoblots were performed from three biological samples representing different lines. Arbitrary units measured from immunoblotting using software ImageLab (Biorad) were normalized according to stain-free gels for corrections of imbalanced loading. After normalization, relative protein levels were

calculated as a ratio to control RSY L1, thus RSY level is one (zero in $\log_2$ graphs) without dispersion of variation. Statistics was calculated in Microsoft Excel using t-Test and was based on N=3-8. Error bars represent SD. Asterisks indicated statistical significance between treatments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n. s. means no statistical significance. Ivan Luptovčiak performed statistical analysis.

5.1.5 Statistical analysis

All statistical parameters of the performed experiments are included in the figures or figure legends, number of samples (N), type of statistical tests and methods used, statistical significance denoted by lowercase letters or stars. Statistics was calculated in SigmaPlot11.0 using Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's method) if normality and/or equal variance failed or using One Way Analysis of Variance (Duncan's method) or Two Way Analysis of Variance (Holm-Sidak method) if normality and equal variance passed. Different lowercase letters indicate statistical significance between treatments ($p < 0.05$). Statistical analysis using t-Test was done in Microsoft Excel and statistical significance between treatments is indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

5.2 Results

5.2.1 Root hair phenotypes in stable transformed *M. sativa* plants

Root hair phenotypes were examined in stable transformed alfalfa lines with downregulated or upregulated *SIMK*, using *SIMKK RNAi* or overexpression (both under constitutive *35S* promoter) approaches, respectively. The appropriate parameter of root hair length in mature parts of the root was selected and measured in these lines for evaluation of root hair growth efficiency. In control wild type lines (RSY, lines L1 and L2) root hair length median value in both of them was 471 μm (**Figure 8A,B,G**). In transgenic lines carrying *SIMKK RNAi* construct (annotated as SIMKKi, lines L3 and L4), root hair length median decreased to 345 μm and 311 μm, respectively (**Figure 8C,D,G**). In contrast, overexpressor transgenic lines carrying *35S::GFP:SIMK* construct in wild type RSY background (annotated as GFP-SIMK, lines L5 and L6) showed an increase of root hair length median to 527 μm and 504 μm, respectively (**Figure 8E,F,G**). Root hair phenotypes of alfalfa lines were depicted in the form of contingency graph with 25 μm intervals (**Figure 9**). Graph showed a relative root hair number (%) found within each root hair length interval. In SIMKKi lines (L3, L4), the root hair distribution pattern was shifted to the left (**Figure 9**) in comparison to RSY (L1, L2), which means an earlier cessation of root hair tip growth. In contrast, the distribution of root hairs in

GFP-SIMK lines (L5, L6) was shifted to the right while distribution curves showed higher values in the range of longer root hairs (**Figure 9**), which means later cessation of the tip growth and higher proportion of longer root hairs.

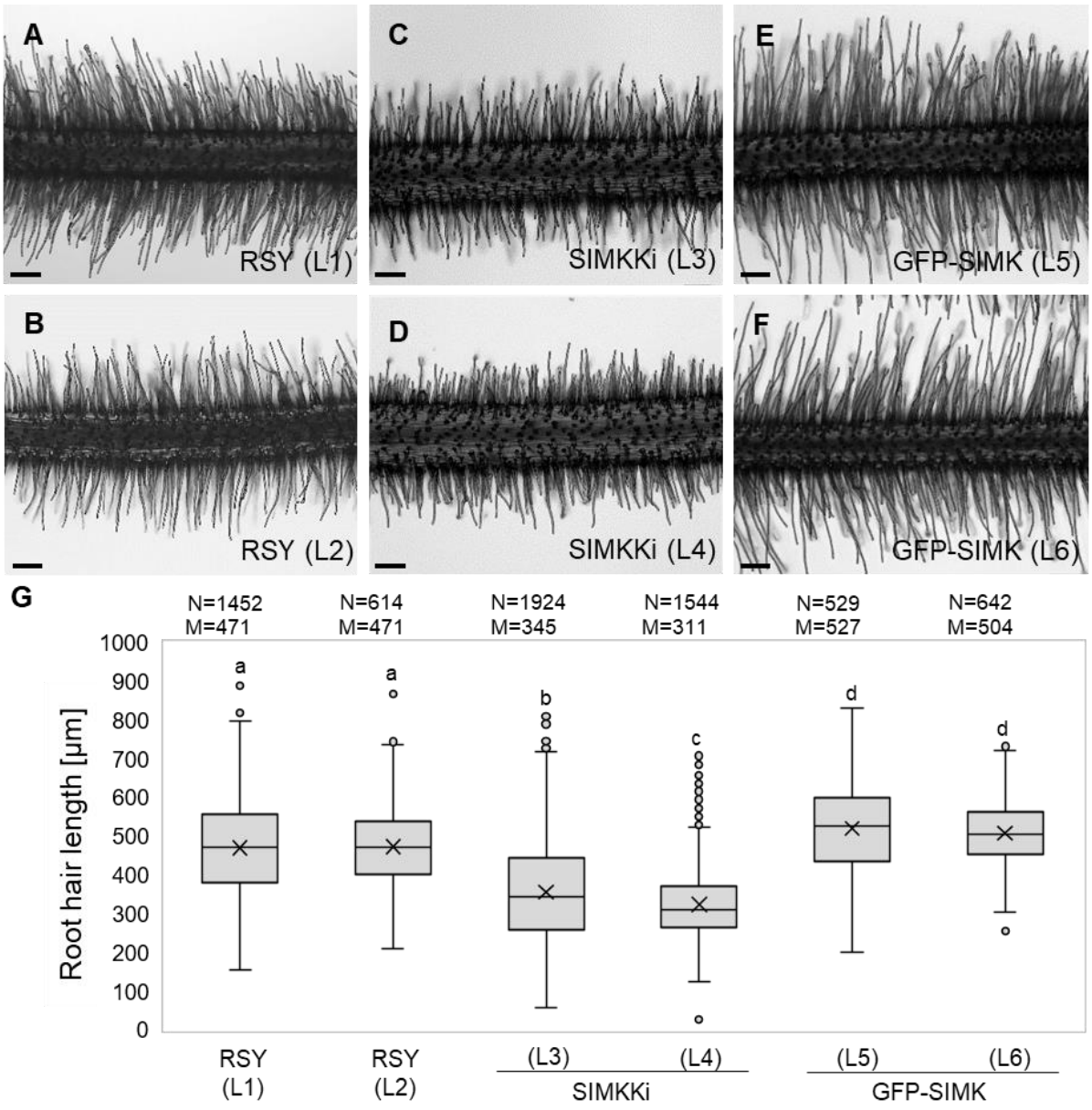

Figure 8 Root hair phenotypes in alfalfa RSY, SIMKK RNAi (SIMKKi) lines and lines overexpressing GFP-SIMK. **(A,B)** Representative images of root hair phenotypes of plants from two independent lines (L1, L2) of control wild type RSY, **(C,D)** two independent transgenic lines with SIMKK RNAi construct (SIMKKi L3, L4), and **(E,F)** two independent transgenic lines expressing *35S::GFP:SIMK* in wild type RSY background (GFP-SIMK L5, L6). **(G)** Box plot graph depicting comparison of root hair lengths of indicated lines, number of observations N and median value M. Statistics was calculated in SigmaPlot11.0 using Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's Method) and is based on N=529-1924. The numbers of root hairs observed were 1452 (RSY L1), 614 (RSY L2), 1924 (SIMKKi L3), 1544 (SIMKKi L4), 529 (GFP-SIMK L5), 642 (GFP-SIMK L6). Different lower case letters indicate statistical significance between treatments ($p < 0.05$). Scale bar: 200 µm in **(A-F)**. Adapted from Hrbáčková *et al.* (2021).

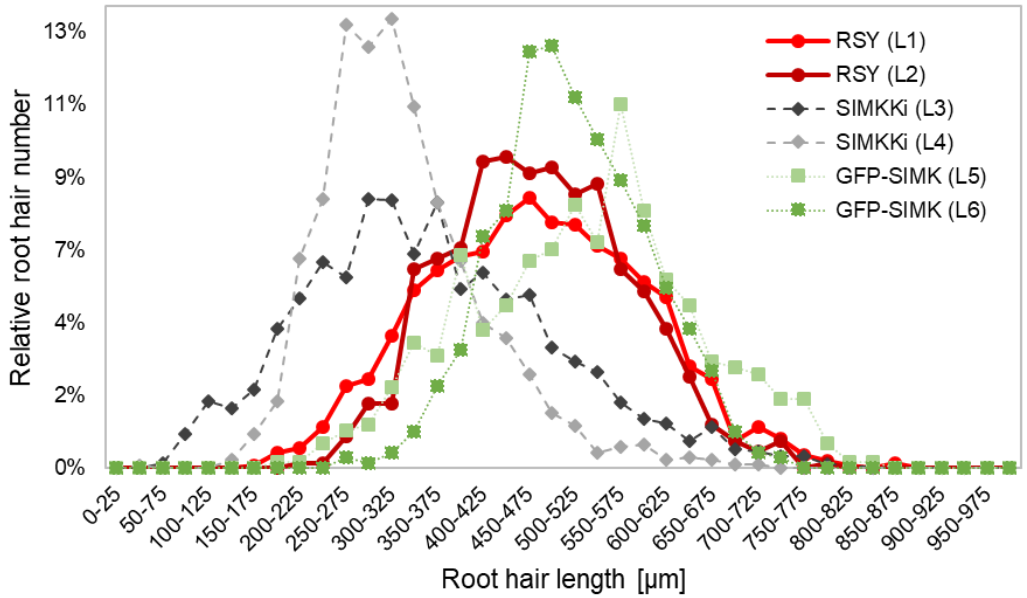

Figure 9 Relative distribution of root hair lengths in indicated alfalfa lines. Normalized root hair number was evaluated using 25 µm intervals distribution. Transgenic lines show different distribution pattern of root hair lengths as compared to RSY wild type lines.

Immunoblot analysis for semiquantive evaluation of SIMK protein level (**Figure 10A,C**) and phosphorylated SIMK (pSIMK) protein level (**Figure 10B,D**) was performed in order to explain previously obtained phenotypical results at the level of protein abundance and activity. Endogenous SIMK protein with molecular mass around 46 kDa and recombinant GFP-SIMK protein with molecular mass around 72 kDa (**Figure 10A**) were quantified (**Figure 10C**). Relative SIMK abundance was strongly decreased in SIMKKi line L4 to approximately 1 % (**Figure 10A,C**). Relative GFP-SIMK abundance was strongly increased in *35S::GFP:SIMK* line L5 to approximately 6.48 times (**Figure 10A,C**), while relative abundance of endogenous SIMK showed a decrease to approximately 49 % (**Figure 10A,C**). These results are consistent with the root hair length phenotypes and indicate that relative SIMK abundance in above-mentioned lines correlates with effectiveness of the root hair tip growth. Phospho-specific pERK antibody was used to check out activity status of respective proteins. Endogenous phosphorylated pSIMK protein with molecular mass around 46 kDa and also phosphorylated GFP-pSIMK with molecular mass around 72 kDa (**Figure 10B**) were quantified (**Figure 10D**). Relative level of pSIMK was considerably decreased in SIMKKi line L4 to approximately 12 % (**Figure 10B,D**) while relative level of GFP-pSIMK was strongly increased in *35S::GFP:SIMK* line L5 to approximately 8.21 times and relative level of endogenous pSIMK level showed non-significant change compared to RSY line L1 (**Figure 10B,D**). These results are also consistent with root hair length phenotypic results.

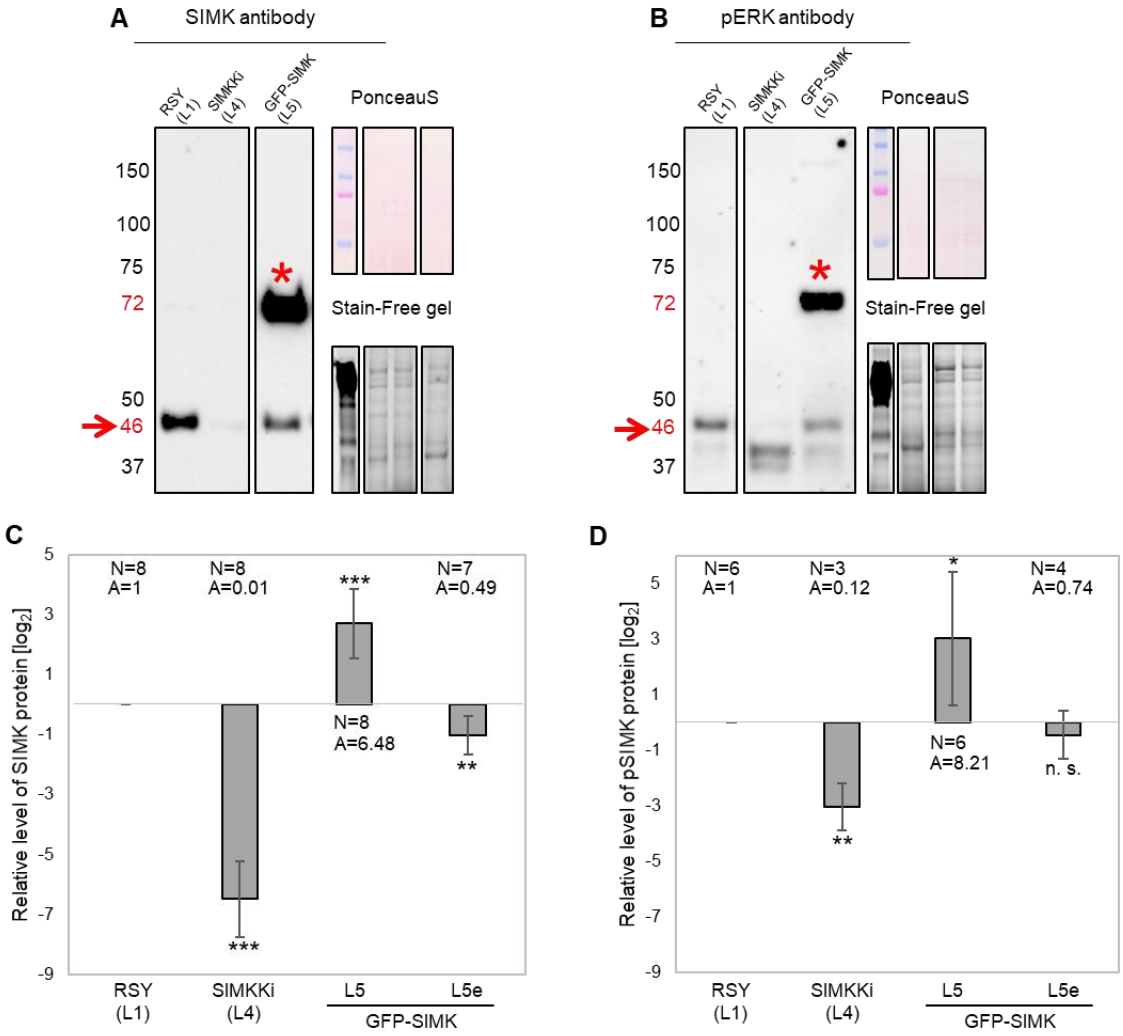

Figure 10 Immunoblotting analysis of total endogenous SIMK, active endogenous SIMK and both total and active GFP-SIMK. **(A)** Western blot detection of SIMK and GFP-SIMK bands using SIMK antibody and **(B)** detection of phosphorylated proteins pSIMK and GFP-pSIMK bands using pERK antibody in root tissue of control and transgenic alfalfa plants of SIMKKi (L4) and expressing *35S::GFP:SIMK* (L5). Arrows point to the 46 kDa which corresponds to **(A)** endogenous SIMK and **(B)** endogenous pSIMK, while asterisks show bands around 72 kDa which corresponds to **(A)** GFP-SIMK and **(B)** GFP-pSIMK. **(C,D)** Log_2 graphs depicting comparison of protein levels in respective lines (SIMKKi L4, GFP-SIMK L5) relative to RSY L1, number of observations N and average value A (presented as inversed log_2 values). GFP-SIMK L5e refer to endogenous level of protein, while GFP-SIMK L5 refer to GFP-SIMK level. **(C)** Relative SIMK protein level in roots of control and transgenic plants (RSY1 L1, SIMKKi L4, GFP-SIMK L5). **(D)** Relative pSIMK protein level in roots of control and transgenic plants (RSY1 L1, SIMKKi L4, GFP-SIMK L5). **(C,D)** Statistics was calculated in Microsoft Excel using t-Test and is based on N=3-8. Error bars show ± SD. Asterisks indicate statistical significance between treatments ($p < 0.05$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n. s. indicates no statistical significance. Adapted from Hrbáčková *et al.* (2021).

5.2.2 Impact of overexpressed GFP-SIMK on infection thread formation

Possible function of GFP-SIMK in infection thread (IT) formation was examined after inoculation with *S. meliloti* (Sm2011 strain) labelled with monomeric red fluorescent protein (mRFP). Evaluation of ITs was performed 10 day-post-inoculation (10 dpi) per whole root

system in alfalfa RSY plants L1 (**Figure 11A**), transgenic SIMKKi plants L4 (**Figure 11B**) and GFP-SIMK plants L5 (**Figure 11C**). Transgenic lines were compared to RSY and between each other. GFP-SIMK line L5 showed IT clustering (**Figure 11C-E**). ITs were also longer, which was consistent with longer root hairs. Quantitative analysis showed that most of ITs in RSY L1 (76.34 %) and SIMKKi L4 (83.61 %) developed individually, while only 45.74 % of ITs was spatially separated in GFP-SIMK L5 (**Figure 11D**). The rest, 54.26 % of ITs in GFP-SIMK L5 line was present in clusters. Portion of ITs in clusters was only 23.66 % in RSY L1 and 16.39 % in SIMKKi L4 (**Figure 11D**). In RSY L1 and SIMKKi L4 most of the clusters contained two or three ITs, while in GFP-SIMK L5 there was a significant amount of clusters possessing also four or five ITs. In 5.7 % of clusters in GFP-SIMK L5 we found five and more ITs; it occurred in only 4.5 % of clusters in RSY L1, while it was absent completely in SIMKKi L4 (**Figure 11E**).

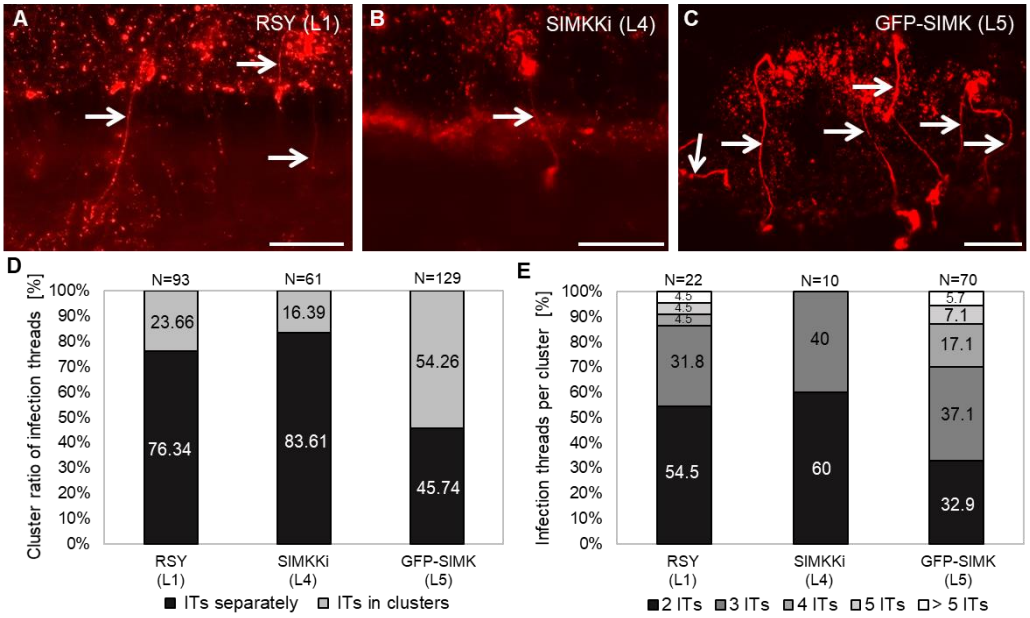

Figure 11 Infection thread and nodule formation in alfalfa roots inoculated with *Sinorhizobium meliloti* labelled with mRFP. **(A-C)** Overview of the infection threads containing *S. meliloti* labelled with mRFP (white arrows) in roots of **(A)** wild type RSY line L1, **(B)** in transgenic SIMKKi line L4 and **(C)** in transgenic GFP-SIMK line L5 at 10 dpi. **(D)** Ratio of individual/clustered infection threads (in %) at 10 dpi. **(E)** Number of infection threads per cluster (in %) at 10 dpi. N = number of observations. Scale bar: 100 μm in (**A-C**). Adapted from Hrbáčková *et al.* (2021).

Live cell microscopic observation of GFP-SIMK in root nodules of L5 line (**Figure 12**) inoculated with mRFP-marked *S. meliloti*. Nodules were harvested 10 dpi (**Figure 12A**) and 20 dpi (**Figure 12B**) and analyzed by CLSM live-cell imaging with the appropriate settings of lasers for GFP and mRFP channels. GFP-SIMK was expressed in young nodules (**Figure 12A**) as well as in mature nodules, including meristematic (I), infection (II), and symbiotic (III) zones (**Figure 12B**).

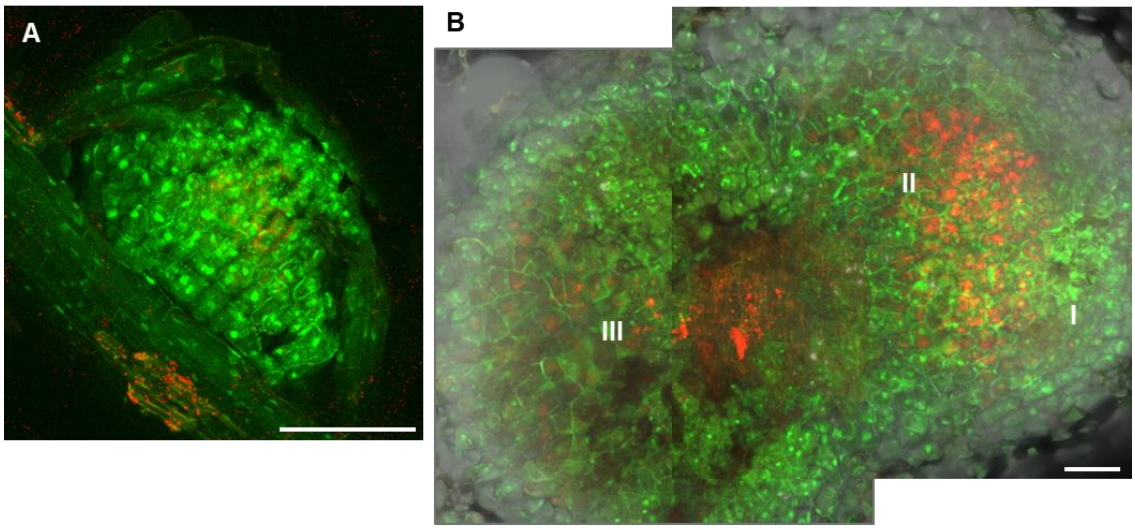

Figure 12 Localization of GFP-SIMK in alfalfa root nodules. Examples of nodule **(A)** at the early stage of development and **(B)** at the late stage of development observed by CLSM. Localization of GFP-SIMK fusion protein (green) in root nodules developed after inoculation of GFP-SIMK line L5 with *S. meliloti* marked with mRFP (red) at 10 dpi **(A)** and 20 dpi **(B)**. Tissue organization of the late nodule: I, meristematic zone; II, infection and differentiation zone; III, symbiotic zone. Scale bar: 100 μm in **(A)**; 200 μm in **(B)**. Adapted from Hrbáčková *et al.* (2021).

5.2.3 Impact of overexpressed GFP-SIMK on nodule formation

When ITs reach the nodule primordium, rhizobia are released into host cells by an endocytosis, which allows to form functional nitrogen-fixing bacteroids within infected plant cells of the root nodule. Possible function of GFP-SIMK in nodule formation was examined after alfalfa inoculation with *S. meliloti* (Sm2011 strain) labelled with monomeric red fluorescent protein (mRFP). Evaluation of nodules was performed 15 days-post-inoculation (15 dpi) per whole root system in alfalfa RSY plants L1 (**Figure 13A**), transgenic SIMKKi plants L4 (**Figure 13B**) and GFP-SIMK plants L5 (**Figure 13C**). GFP-SIMK line L5 often produced nodules in clusters (**Figure 13C**), which was consistent with previous results in infection thread formation. This was less frequent in RSY line L1 and in SIMKKi line L4 (**Figure 13B**). Analysis of nodule clustering showed that 89.8 % of nodules in RSY L1, 95 % in SIMKKi L4 and 87.4 % in GFP-SIMK L5 developed individually (**Figure 13D**). However, clusters in transgenic GFP-SIMK L5 line contained much higher number of nodules in comparison to RSY L1 and SIMKKi L4 (**Figure 13E**). Detailed analysis revealed that 27.3 % of clusters in GFP-SIMK line L5 possessed five and more nodules while in RSY L1 it was only in 2.9 % of clusters (**Figure 13E**) and SIMKKi line L4 did not form clusters with five or more nodules (**Figure 13E**). On the contrary, RSY line L1 and SIMKKi line L4 had 62.9 % and 61.5 % of clusters formed from two nodules only, respectively, as compared to 18.2 % of such clusters in GFP-

SIMK line L5 (**Figure 13E**). It is resembling IT clustering where the ratio of clusters with two ITs represented 54.5 % and 60 % in RSY line L1 and SIMKKi line L4, respectively, but it was only 32.9 % in GFP-SIMK line L5 (**Figure 11E**). GFP-SIMK line L5 was able to very effectively produce ITs and nodules spatially organized in bigger clusters (**Figure 11C, 13C**).

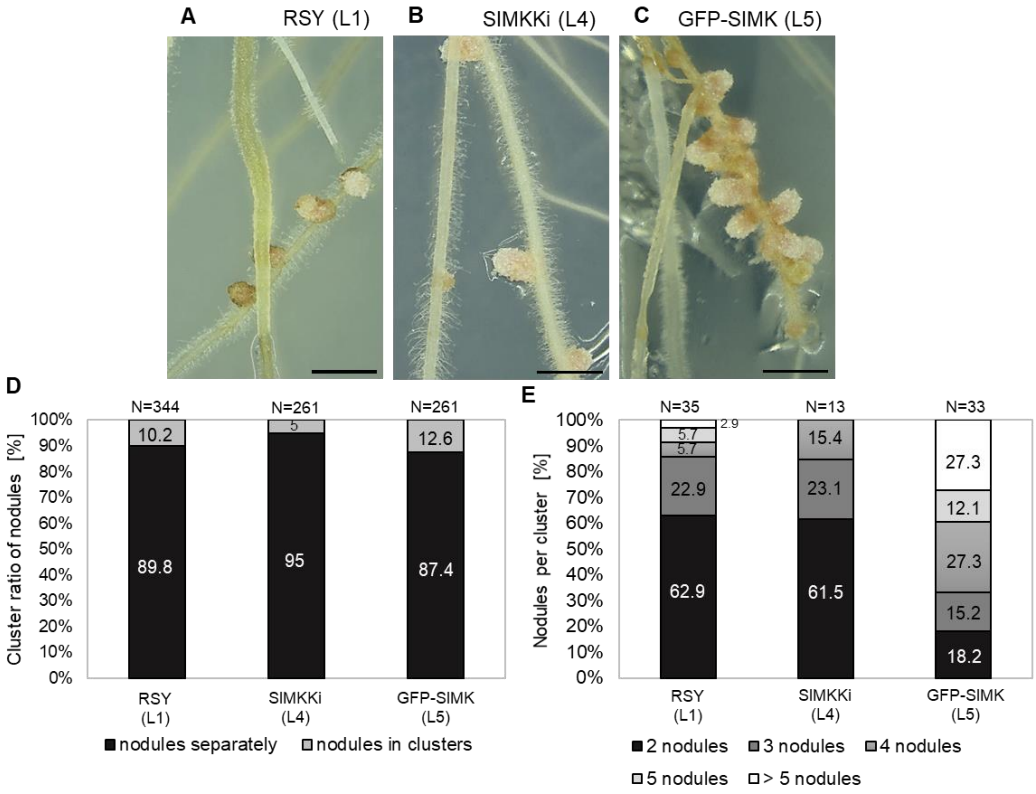

Figure 13 Nodule formation in alfalfa roots inoculated with *Sinorhizobium meliloti*. **(A-C)** Representative images of root nodules formed in respective alfalfa lines, **(A)** control RSY line L1, **(B)** SIMKKi line L4 and **(C)** GFP-SIMK line L5 inoculated with *S. meliloti* labelled with mRFP on Fåhreus medium at 15 dpi. **(D)** Ratio of individual/clustered nodules (in %) at 15 dpi. **(E)** Number of nodules per cluster (in %) at 15 dpi. N = number of observations. Scale bar: 1 cm in **(A-C)**. Adapted from Hrbáčková *et al.* (2021).

5.2.4 Impact of overexpressed GFP-SIMK on shoot biomass formation

In addition to root hair phenotypes and symbiotic interaction with *S. meliloti*, the role of overexpressed GFP-SIMK was also examined in above ground biomass production (**Figure 14**). Formation and regrowth of new individual alfalfa shoots was induced and synchronized by cutting off the green part. Documentation was taken after 60 days plant growth in pots with soil. SIMKKi line L4 showed smaller and thinner habitus of shoots (**Figure 14B**) in comparison to RSY line L1 (**Figure 14A**). Above ground parts in GFP-SIMK line L5 showed more robust and bushy habitus (**Figure 14C**). In quantitative terms, GFP-SIMK line L5 produced significantly longer shoots (**Figure 14D**) with significantly higher weight (**Figure 14E**) in comparison to both RSY line L1 and SIMKKi L4 plants. SIMKKi line L4 developed significantly lower number of shoots per plant in comparison to both RSY L1 and GFP-SIMK

L5 (**Figure 14F**). Consequently, the biomass weight per plant was significantly decreased in SIMKKi L4 plants, but significantly increased in GFP-SIMK L5 plants in comparison to control RSY L1 (**Figure 14G**).

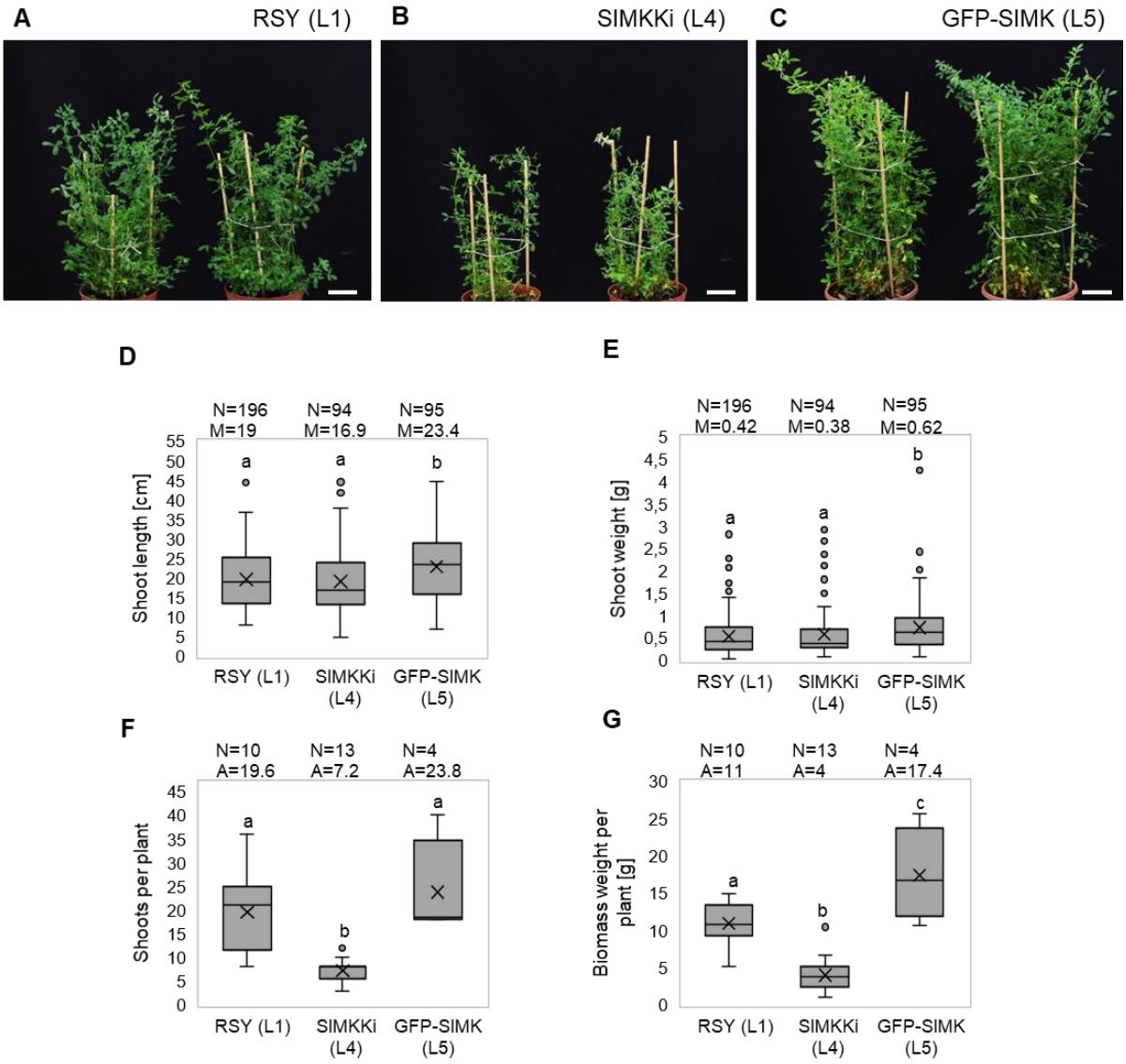

Figure 14 Shoot biomass production in transgenic alfalfa plants grown *in vivo*. **(A-C)** Representative images of above ground parts of mature plants grown in pots in control RSY L1 **(A)**, SIMKKi L4 **(B)** and GFP-SIMK L5 **(C).** Regrown plants were documented 60 days after cutting the shoots. **(D)** Box plot graph depicting comparison of shoot length of indicated lines, number of observations N and median value M. **(E)** Box plot graph depicting comparison of shoot weight of indicated lines, number of observations N and median value M. **(F)** Box blot graph depicting comparison of shoots number per plant of indicated lines, number of observations N and average value A. **(G)** Box plot graph depicting comparison of biomass weight per plant of indicated lines, number of observations N and average value A. Statistics was calculated in SigmaPlot11.0 using Kruskal-Wallis One Way Analysis of Variance on Ranks (Dunn's method) **(D,E)** or using One Way Analysis of Variance (Holm-Sidak method) **(F,G)** and is based on **(D,E)** N=94-196 and **(F,G)** N=4-13. Different lower case letters indicate statistical significance between treatments ($p < 0.05$). Scale bar: 4 cm in **(A-C)**. Images were provided by Dominik Novák. Statistics was provided by Ivan Luptovčiak. Adapted from Hrbáčková *et al.* (2021).

5.3 Discussion

Leguminous plant species are important members of the agricultural ecosystems and are widely utilized also in nutritional production industry. They are able to grow in soils deficient for nitrogen due to the symbiotic interaction with rhizobia in root nodules, specialized organs for atmospheric nitrogen fixation. Both initial and later interactions between legumes and rhizobia require exchange of different signals and activation of signal transduction pathways. MAPK-dependent signal transduction cascades regulate many developmental and cellular processes in plants (Šamajová *et al.*, 2013). Systemic approaches indicated that legume-rhizobia interactions and subsequent root nodule development involve activity of various protein kinases (Roy *et al.*, 2020). Main effort in research of symbiotic nitrogen fixation is conducted in legumes important for food production (Oldroyd, 2013). However, the regulation of symbiotic interactions, nodule development and nitrogen fixation, including possible involvement of MAPK signaling, is much less clear in alfalfa.

In this chapter, we studied effects of SIMK downregulation and overexpression in alfalfa using genetically modified transgenic lines. We characterized parameters like length of root hairs, phenotype of above ground plant parts, but also addressed possible involvement of SIMK in the efficiency of root nodulation, through determination of clustering of ITs and nodules. In order to decrease SIMK functions, we prepared two independent RNAi transgenic lines downregulating SIMKK, an upstream activator of SIMK (SIMKKi lines). We confirmed that SIMK expression was strongly downregulated in these lines.

Alfalfa nodulation requires attachment of *S. meliloti* to root hairs. In general, root hairs provide easier water and nutrient uptake from the soil into the plant and help to keep the plant body stable in the soil (Šamaj *et al.*, 2004). To gain further insight into the importance of SIMK in root hair development, independent transgenic lines of alfalfa were compared. The question remained whether genetically-based downregulation or overexpression of SIMK might have an effect on the root hair growth in homologous alfalfa species. We addressed this question and report here about earlier cessation of root hair tip growth leading to short root hairs in SIMKKi lines, while GFP-SIMK overexpressing lines showed an opposite phenotype manifested by later cessation of the tip growth and longer root hairs. Crucial components of the tip-growth machinery are: a tip-focused cytoplasmic Ca^{2+} gradient, polarly targeted vesicular traffic and the actin cytoskeleton (Šamaj *et al.*, 2002). From the results showed in previous chapter of this thesis, the SIMK kinase is strongly expressed in growing root hairs. SIMK is strongly expressed in root cells and the SIMK protein is accumulated in nuclei. The situation start to change during

root hairs formation. SIMK redistributes from nuclei into the cytoplasm and into the tips of growing root hairs (Šamaj *et al.,* 2002, 2004). During this redistribution, SIMK is active (Šamaj *et al.,* 2002). This selective enrichment of active SIMK in tips of growing root hairs coincides with dynamic F-actin meshworks (Baluška *et al.,* 2000). Depolymerization and stabilization of F-actin activates SIMK, indicating that MAPK activity is directly affected by F-actin dynamics (Šamaj *et al.,* 2002). After treatment with actin disruptors such as LB or cytochalasin D, tip-focused localization of SIMK disappeared and resulted in nuclear accumulation of SIMK. On the other side, an inducer of actin polymerization, jasplakinolide, also activated SIMK (Šamaj *et al.,* 2002). Moreover, Šamaj *et al.* (2002) by exchanging the homologous amino acid in SIMK observed higher MAPK activity in transformed protoplasts and plants of tobacco line SR1. This gain-of-function construct of SIMK induced longer root hairs, which is consistent with results in the case of overexpressed GFP-SIMK in alfalfa. On the other hand, loss-of-function construct of SIMK in tobacco showed no visible root hair phenotype. Participation of SIMK and other MAPKs in the regulation of root hair tip growth in general is part of complex signal transduction pathways. MAPKs can participate in the transcriptional regulation of genes involved in root hair development. Previously, Bekešová *et al.* (2015) showed decreased accumulation of phosphorylated SIMK in SIMKKi lines. SIMKKi transgenic line exhibited strong downregulation of SIMK protein, and showed shorter root hairs. Such decreases in root hair growth in the SIMKKi line and increase growth in the overexpressed transgenic line support a positive role of SIMK in the root hair formation.

Effectivity in the root hair tip growth is an important aspect affecting early stages of plant-rhizobia interaction. MAPK cascades play central roles in various intracellular signal transduction processes through sequential phosphorylation of three-linked kinases (MAPKKK, MAPKK, MAPK) (Šamajová *et al.,* 2013). Publications reporting involvement of MAPK signaling cascades in nodule development are rather scarce. A comparative study confirmed that MAPK signaling cascade and stress-related responses are activated early upon plant infection with symbiotic rhizobia (Lopez-Gomez *et al.,* 2012).

Most of the advances in the area originate from the studies of two model legumes, *M. truncatula* and *L. japonicus* (Clúa *et al.,* 2018). It has been shown that the legume LjSIP2 is a MAPKK in *L. japonicus* directly interacting with receptor-like kinase SymRK and have an essential role in the early symbiosis signaling and nodule organogenesis (Chen *et al.,* 2012). Recent study demonstrated that phosphorylation target of LjSIP2 is LjMPK6 (orthologue of SIMK) (Yin *et al.,* 2019). LjSIP2/SymRK interaction inhibits the kinase activity of LjSIP2 on

the LjMPK6 substrate. Thus, signaling module SymRK-SIP2-MPK6 is required for nodulation, playing a positive role in nodule formation and organogenesis in *L. japonicus* (Yan *et al.*, 2020). Moreover, Yan *et al.* (2020) also showed that LjPP2C, a PP2C-type phosphatase, specifically interacts with LjMPK6 *in vitro* and dephosphorylates LjMPK6. Phosphatase assay demonstrated that LjPP2C is a genuine protein phosphorylating the LjMPK6 in cellular signal transduction as reported for other plant PP2C (Yan *et al.*, 2020). Meskiene *et al.* (2003) showed, that MP2C, a wound-induced alfalfa PP2C, negatively regulate and also directly inactivates SIMK.

In alfalfa, SIMKK is specific upstream activator of SIMK under salt stress (Kiegerl *et al.*, 2000). Interestingly, SIMKK shares 88% amino acid similarity with LjSIP2 (Chen *et al.*, 2012). The orthologue of SIP2 has been identified in *M. truncatula* as MtMAPKK4 (Chen *et al.*, 2017). It is involved in the regulation of different plant developmental processes and also mediates root nodule formation. Chen *et al.* (2012) prepared *SIP2 RNAi* line and after inoculation with *Mesorhizobium loti*, average nodule number was lower in comparison to control line. In another study, Chen *et al.* (2017) prepared heterozygous mutant *mapkk4/+*. The *mapkk4/+* heterozygotes showed also reduced numbers of ITs and nodules which is consistent with our SIMKKi transgenic line exhibited with less numbers of ITs and nodules. (Chen *et al.*, 2017). Downstream interacting partners of MtMKK4 are MtMPK3 and MtMPK6. Moreover, another *M. truncatula* MAPKK, namely MtMKK5, also interacts with MtMPK3 and MtMPK6 in alternative signaling pathway, having a negative role in the symbiotic nodule formation (Chen *et al.*, 2017; Ryu *et al.*, 2017). On the other side, overexpressed GFP-SIMK transgenic line showed higher numbers of ITs and nodules. Yin *et al.* (2019) prepared overexpressed *LjMPK6-ox* transgenic lines, which showed significantly higher numbers of ITs and nodules compared to control plants after inoculation with *M. loti* bacteria strain. Such decreases in formation of ITs and nodules in the SIMKKi line but enhanced amounts of ITs and nodules in overexpression GFP-SIMK line support a positive role of SIMK in the alfalfa nodulation.

Clustering of ITs after inoculation with *S. meliloti* in overexpressor GFP-SIMK line is another interesting finding. Moreover, clustered development of ITs correlated well with clustered formation of fully developed and equally growing root nodules. This may represent important aspect of root nodule formation, since appropriate number of nodules developed in whole root system is tightly regulated by the plant and depends on overall physiological conditions. It has been observed that legumes tend to maintain development of the minimal number of nodules that are required for optimal growth at given growth conditions (Mortier *et*

al., 2012). This mechanism is regulated by local and systemic endogenous signals. Locally, the number of developing nodules is controlled through ethylene signaling pathway, restricting the initiation of nodule primordia to cortical cells close to xylem poles (Heidstra *et al.*, 1997), and through nitrate-induced signaling peptides of the CLAVATA family (Mortier *et al.*, 2010). A particular class of these small signaling CLE peptides, induced by rhizobia infection, controls also systemic regulation of nodulation (Mortier *et al.*, 2010; Concha and Doerner, 2020). CLE peptides move as a long-distance signals from roots to shoots where specifically interact with shoot receptors, like leucine-rich-repeat receptor SUNN in *M. truncatula* (Schnabel *et al.*, 2005), and negatively autoregulate the nodule number. On the other hand, root competence for nodulation is controlled also by the microRNAs like miR2111, which is produced upon activity of the receptor CRA2 in shoots, and affecting positively root nodulation as systemic regulation signal (Gautrat *et al.*, 2020). This feedback mechanism is controlled by the number, the activity and the age of early-developed nodules (Caetano-Anollés *et al.*, 1991). Formation of IT and nodule clusters in GFP-SIMK lines may indicate a new SIMK role in spatial control of nodule formation on the root system. Nodules developing close to each other and forming clusters might require SIMK involvement in IT formation and viability, as less infection events might abort in early stages of development. This unique aspect of nodulation process and mode of its regulation within the whole root system certainly deserves further detailed study.

Another aspect interesting from the biotechnological point of view, is the development and production of above ground plant parts. Particularly in alfalfa, an important forage crop, shoot biomass production is one of the agronomical parameters of interest. In this respect, genetic manipulation of SIMK brought interesting and potentially promising results. Downregulation of *SIMKK* and *SIMK* genes led to decreased root hair growth, numbers of ITs, or nodules. Moreover, downregulation of *SIMKK* and *SIMK* genes in SIMKKi lines influenced negatively the development of above ground plant parts, leading to production of less shoot biomass per plant. SIMK overexpression in GFP-SIMK lines, on the other hand, resulted in higher shoot biomass per plant. This result may support a general effort of alfalfa biotechnological improvement as a forage crop. Nowadays, genetic, genomic and recombinant DNA technology approaches are widely utilized in alfalfa improvements, including leaf production parameters and biomass yield (Lei *et al.*, 2017). Compared to other crops, alfalfa shows relatively high drought tolerance (Arshad *et al.*, 2017; Lei *et al.*, 2017). Also salinity is a big threat for alfalfa production (Arshad *et al.*, 2017). Previous studies provided some interesting information. For example, overexpression of *WXP1*, a gene encoding AP2 domain-

containing putative transcription factor from *M. truncatula* under the control of the *35S* promoter in alfalfa resulted in excessive formation of cuticular wax layer on leaves. Such leaves were more resistant to water loss making these plants more resistant against drought stress (Zhang *et al.,* 2005). A number of microRNAs have been used to improve various crop species via genetic engineering. For example, miR156 is a plant microRNA characterized in many plants (Aung *et al.,* 2015). Overexpression of miR156 in alfalfa caused silencing of seven *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes, and thereby enhanced biomass production and shoot branching (Aung *et al.,* 2017). In another study, genetic modification of the *MsSPL8* gene in alfalfa significantly altered shoot architecture. Knockdown of *MsSPL8* significantly increased shoot branching and biomass yield, however, shoot branching was suppressed and biomass yield was reduced by *MsSPL8* overexpression (Gou *et al.,* 2018). We observed increase in total leaf surface area and shoot biomass production in SIMK-overexpressing lines.

6 Conclusions

This Ph.D. thesis describes preparation and characterization of SIMK and SIMKK transgenic alfalfa lines expressed under the *35S* promoter. We have studied in detail the role of overexpressed SIMK in root hair growth, IT and nodule clustering and shoot biomass production.

The thesis consists of three parts. The first part summarizes current knowledge on biotechnological potential of alfalfa, including genetic transformation methods as well as genomic and transcriptomic perspectives focused on alfalfa responses to abiotic and biotic stress. This section also includes important information on legume-rhizobia interactions. The main functions of Nod factors and flavonoids as symbiotic signals as well as structural aspects of nodule development are described. The last subsection is dedicated to the large number of MAPKs identified in alfalfa and in the model plant organism *A. thaliana*.

In the second part, the process of cloning of fluorescently-tagged *SIMK* and *SIMKK* genes is described. Expression of GFP-tagged SIMK and tagRFP-tagged SIMKK was checked using transient transformation of *N. benthamiana* leaves before stable transformation of alfalfa. Our results supported localization of GFP-SIMK and tagRFP-SIMKK in nuclei and dispersed in cytoplasm. Moreover, we also studied colocalization of SIMK and SIMKK and showed formation of *SIMKK-SIMK* spot-like structures in the cytoplasm after salt stress. New transgenic alfalfa lines have been prepared by improved and optimized protocol for stable transformation by *Agrobacterium tumefaciens* with high efficiency. Up to now, localization experiments were performed solely on fixed plant material, such as root protoplasts or root tips. For the first time, we were able to perform live cell imaging of GFP-SIMK in the whole alfalfa plant including different cell types and organs. A systemic study of GFP-SIMK localization patterns at whole organ, tissue and cellular levels can help to better understand its role in alfalfa. Modern microscopic methods such as CLSM and Airyscan CLSM were used for subcellular localization of GFP-SIMK and for colocalization with activated MAPKs in alfalfa using live cell imaging or immunolabeling. The investigation of GFP-SIMK localization during development of alfalfa roots revealed its strong accumulation in the nuclei of root tip and epidermal cells but signal was weaker in nucleoli. GFP-SIMK regularly accumulated in tips of growing root hairs. It was also localized in root border cells detached from lateral root cap. In the above ground plant parts, GFP-SIMK accumulated in the nuclei and less in the cytoplasm

of hypocotyl cells. Similar subcellular localization was found also in leaf epidermal cells and stomata. Immunolabeling method confirmed the pattern of subcellular localization of GFP-SIMK in root cells by using whole-mount immunofluorescence co-immunolabeling with GFP-specific and phospho-specific (anti-phospho-p44/42) antibodies. Imaging of co-immunolabeled samples with Airyscan CLSM revealed that GFP-SIMK is localized in distinct spot-like structures in the nucleoplasm and in cytoplasmic structures, preferentially in activated form. Moreover, GFP-SIMK was localized in activated form in the root hair tip.

The last part of the thesis deals with genetic manipulation (overexpression and suppression) of *SIMK* and *SIMKK*. In more detail, we generated stable transgenic alfalfa lines overexpressing GFP-tagged SIMK as well as transgenic SIMKK RNAi (SIMKKi) line with downregulated *SIMKK* and *SIMK* genes. We confirmed that SIMK expression was strongly downregulated in these lines. Next, we quantitatively characterized parameters like root hair growth, possible involvement of SIMK in the efficiency of IT and nodule formation and shoot biomass production in these alfalfa transgenic lines. We showed that downregulation or overexpression of *SIMK* affected root hair formation and growth in alfalfa. GFP-SIMK overexpressor line revealed longer root hairs phenotype manifested also by later cessation of their tip growth as compared to the control and the SIMKKi lines. Clustering of ITs and nodules after inoculation with *S. meliloti* in overexpressor GFP-SIMK line is an interesting finding. It is possible that nodules developing close to each other and forming clusters might require SIMK involvement in IT formation and viability. Besides a new role of SIMK in alfalfa nodulation process, we also showed positive impacts of SIMK overexpression on some other important agronomical factors, such as shoot biomass production, petiole and leaf development, leading to the formation of bigger leaves and production of greener biomass. This shows a biotechnological potential of SIMK for genetic improvement of alfalfa as a forage crop.

7 References

Abdelrahman, M., Jogaiah, S., Burritt, D. J., Tran, L. S. P. (2018). Legume genetic resources and transcriptome dynamics under abiotic stress conditions. *Plant Cell Environ.* 41, 1972–1983. doi: 10.1111/pce.13123

Albrecht, K. A., Beauchemin, K. A. (2003). Alfalfa and other perennial legume silage. *Sil. Sci. Tech.*, 42, 633–664. doi: 10.2134/agronmonogr42.c14

Annicchiarico, P., Barrett, B., Brummer, E. C., Julier, B., Marshall A. H. (2015). Achievements and challenges in improving temperate perennial forage legumes. *Crit Rev Plant Sci.* 34, 327–380. doi: 10.1080/07352689.2014.898462

Ari, Ş., Arikan, M. (2016). "Next-generation sequencing: advantages, disadvantages, and future," in Plant Omics: Trends and Applications, eds. K. Hakeem, H. Tombuloglu, G. Tombuloglu (Cham: Springer). 109–135. doi: 10.1007/978-3-319-31703-8_5

Arshad, M., Feyissa, B. A., Amyot, L., Aung, B., Hannoufa, A. (2017). MicroRNA156 improves drought stress tolerance in alfalfa (*Medicago sativa*) by silencing SPL13. *Plant Sci.* 258, 122–136. doi: 10.1016/j.plantsci.2017.01.018

Baluška, F., Salaj, J., Mathur, J., Braun, M., Jasper, F., Šamaj, J., Volkmann, D. (2000). Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Develop. Biol.* 227, 618–632. doi: 10.1006/dbio.2000.9908

Beever, D.E., Thorp, C. (1996). "Advances in the understanding of factors influencing the nutritive value of legumes," in Legumes in sustainable agriculture, eds. D. Yonie. (Occasional Symp. 30. British Grassland Society, Reading, UK). 194–207

Bekešová, S., Komis, G., Křenek, P., Vyplelová, P., Ovečka, M., Luptovčiak, I., Šamaj, J. (2015). Monitoring protein phosphorylation by acrylamide pendant Phos-Tag™ in various plants. *Front. Plant Sci.* 6, 336. doi: 10.3389/fpls.2015.00336

Berger, A., Boscari, A., Frendo, P., Brouquisse, R. (2019). Nitric oxide signaling, metabolism and toxicity in nitrogen-fixing symbiosis. *J. Exp. Bot.* 70, 4505–4520. doi: 10.1093/jxb/erz159

Bevan, M. W., Uauy, C., Wulff, B. B., Zhou, J., Krasileva, K., Clark, M. D. (2017). Genomic innovation for crop improvement. *Nature* 543, 346–354. doi: 10.1038/nature22011

Bögre, L., Calderini, O., Binarova, P., Mattauch, M., Till, S., Kiegerl, S., et al. (1999). A MAP kinase is activated late in plant mitosis and becomes localized to the plane of cell division. Plant Cell 11, 101–113. doi: 10.1105/tpc.11.1.101

Bohnert, H. J., Jensen, R. G. (1996). Strategies for engineering water-stress tolerance in plants. *Trends Biotechnol.* 14, 89–97. doi: 10.1016/0167-7799(96)80929-2

Bonaldi, K., Gourion, B., Fardoux, J., Hannibal, L., Cartieaux, F., Boursot, M., et al. (2010). Large-scale transposon mutagenesis of photosynthetic Bradyrhizobium sp. strain ORS278 reveals new genetic loci putatively important for nod-independent symbiosis with Aeschynomene indica. *Mol Plant-Microbe Interact.* 23, 760–770. doi: 10.1094/MPMI-23-6-0760

Brencic, A., Winans, S. C. (2005). Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria detection of and response to signals involved in hostmicrobe interactions by plant-associated bacteria. *Microbiol. Mol. Biol. Rev.* 69,155–194. doi: 10.1128/MMBR.69.1.155

Caetano-Anollés, G., Paparozzi, E. T., Gresshoff, P. M. (1991). Mature nodules and root tips control nodulation in soybean. *J. Plant. Physiol.* 137, 389–396. 10.1016/S0176-1617(11)80306-8

Calderini, O., Bogre, L., Vicente, O., Binarová, P., Heberle-Bors, E., Wilson, C. (1998). A cell cycle regulated MAP kinase with a possible role in cytokinesis in tobacco cells. *J. Cell Sci.* 111, 3091–3100

Calvert, H. E., Pence, M. K., Pierce, M., Malik, N. S., Bauer, W. D. (1984). Anatomical analysis of the development and distribution of Rhizobium infections in soybean roots. *Can. J. Bot.* 62, 2375–2384. doi: 10.1139/b84-324

Cardinale, F., Meskiene, I., Ouaked, F., Hirt, H. (2002). Convergence and divergence of stress-induced mitogen-activated protein kinase signaling pathways at the level of two distinct mitogen-activated protein kinase kinases. *Plant Cell* 14, 703–711. doi: 10.1105/tpc.010256

Charron, D., Pingret, J. L., Chabaud, M., Journet, E. P., Barker, D. G. (2004). Pharmacological evidence that multiple phospholipid signaling pathways link Rhizobium nodulation factor perception in Medicago truncatula root hairs to intracellular responses, including Ca^{2+} spiking and specific ENOD gene expression. *Plant Physiol.* 136, 3582–3593. doi: 10.1104/pp.104.051110

Chen, T. H., Murata, N. (2002). Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol*. 5, 250–257. doi: 10.1016/s1369-5266(02)00255-8

Chen, T., Zhou, B., Duan, L., Zhu, H., Zhang, Z. (2017). MtMAPKK4 is an essential gene for growth and reproduction of *Medicago truncatula*. *Physiol. Plant.* 159, 492–503. doi: 10.1111/ppl.12533

Chen, T., Zhu, H., Ke, D., Cai, K., Wang, C., Gou, H., et al. (2012). A MAP kinase kinase interacts with SymRK and regulates nodule organogenesis in *Lotus japonicus*. *Plant Cell* 24, 823–838. doi: 10.1105/tpc.112.095984

Cheung, F., Haas, B. J., Goldberg, S. M. D., May, G. D., Xiao, Y., Town C. D. (2006). Sequencing Medicago truncatula expressed sequenced tags using 454 Life Sciences technology. BMC *Genom.* 7, 272. doi: 10.1186/1471-2164-7-272

Clúa, J., Roda, C., Zanetti, M. E., Blanco, F. A. (2018). Compatibility between Legumes and Rhizobia for the Establishment of a Successful Nitrogen-Fixing Symbiosis. *Genes* 9, 125. doi: 10.3390/genes9030125

Concha, C., Doerner, P. (2020). The impact of the rhizobia–legume symbiosis on host root system architecture. *J. Exp. Bot.* 71, 3902–3921. doi: 10.1093/jxb/eraa198

Cooper, J. E. (2007). Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *J. Appl. Microbiol.* 103, 1355–1365. doi: 10.1111/j.1365-2672.2007.03366.x

de Ruijter, N. C. A., Bisseling, T., Emons, A. M. C. (1999). Rhizobium Nod factors induce an increase in sub-apical fine bundles of actin filaments in Vicia sativa root hairs within minutes. *Mol. Plant-Microbe Interact.* 12, 829–832. doi: 10.1094/MPMI.1999.12.9.829

Dixon, R.A., Steele, C.L. (1999). Flavonoids and isoflavonoids – a gold mine for metabolic engineering. *Trends Plant Sci.* 4, 394–400. doi: 10.1016/S1360-1385(99)01471-5

Dong, L., Liu, H., Zhang, J., Yang, S., Kong, G., Chu, J. S., et al. (2015). Single-molecule real-time transcript sequencing facilitates common wheat genome annotation and grain transcriptome research. *BMC Genom.* 16, 1039. doi: 10.1186/s12864-015-2257-y

Doyle, J. J., Luckow, M. A. (2003). The rest of the iceberg. Legume diversity and evolution in a phylogenetic context. *Plant Physiol.* 131, 900–910. doi: 10.1104/pp.102.018150

Dupont, L., Hérouart, D., Alloing, G., Hopkins, J., Pierre, O., Frendo, P., et al. (2012). The legume root nodule: from symbiotic nitrogen fixation to senescence. London: INTECH Open Access Publisher. IntechOpen. doi: 10.5772/34438

Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., et al. (2009). Real-time DNA sequencing from single polymerase molecules. *Science* 323, 133–138. doi: 10.1126/science

Esseling, J. J., Lhuissier, F. G., Emons, A. M. (2003). Nod factor-induced root hair curling: continuous polar growth towards the point of nod factor application. *Plant Physiol.* 132, 1982–1988. doi: 10.1104/pp.103.021634

Ferguson, B. J., Mathesius, U. (2014). Phytohormone regulation of legume-rhizobia interactions. *J. Chem. Ecol.* 40, 770–790. doi: 10.1007/s10886-014-0472-7

Foucher, F., Kondorosi, E. (2000). Cell cycle regulation in the course of nodule organogenesis in *Medicago*. *Plant Mol. Biol.* 43, 773–786. doi: 10.1023/a:1006405029600

Franche, C., Lindström, K., Elmerich, C. (2009). Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant Soil* 321, 35–59. doi: 10.1007/s11104-008- 9833-8

Gage, D. J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol Biol Rev.* 68, 280–300. doi: 10.1128/MMBR.68.2.280-300.2004

García, A. N., Ayub, N. D., Fox, A. R., Gómez, M. C., Diéguez, M. J., Pagano, E. M., et al. (2014). Alfalfa snakin-1 prevents fungal colonization and probably coevolved with rhizobia. *BMC Plant Biol.* 14, 248. doi: 10.1186/s12870-014-0248-9

Gautrat, P., Laffont, C., Frugier, F. (2020). Compact root architecture 2 promotes root competence for nodulation through the miR2111 systemic effector. *Curr. Biol.* 30, 1339–1345. doi: 10.1016/j.cub.2020.01.084

Genre, A., Chabaud, M., Timmers, T., Bonfante, P., Barker, D. G. (2005). Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. *Plant Cell* 17, 3489–3499. doi: 10.1105/tpc.105.035410

Glazebrook, J., Walker, G. C. (1989). A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by Rhizobium meliloti. *Cell* 56, 661–672. doi: 10.1016/0092-8674(89)90588-6

Gou, J., Debnath, S., Sun, L., Flanagan, A., Tang, Y., Jiang, Q., et al. (2018). From model to crop: functional characterization of SPL 8 in *M. truncatula* led to genetic improvement of biomass yield and abiotic stress tolerance in alfalfa. *Plant Biotechnol. J.* 16, 951–962. doi: 10.1111/pbi.12841

Gourion, B., Berrabah, F., Ratet, P., Stacey, G. (2015). Rhizobium-legume symbioses: the crucial role of plant immunity. *Trends Plant Sci.* 20, 186–194. doi: 10.1016/j. tplants.2014.11.008

Griffin, G. D. (1990). "Diseases caused by nematodes," in: Compendium of Alfalfa Diseases, second edition, eds. D.L. Stuteville and D.C. Erwin (APS Press. St. Paul, MN). 46–51

Gull, A., Lone, A. A., Wani, N. U. I. (2019). Biotic and abiotic stresses in plants. *Ab. Biot. Stress Plants* 1–19. doi: 10.5772/intechopen.85832

Hafez, S. L., Sundararaj, P. (2009). "Nematodes of alfalfa and their management," in: Proceedings, Western Alfalfa & Forage Conference, Reno, Nevada, 2–4

Halbleib, C. M., Ludden, P. W. (2000). Regulation of biological nitrogen fixation. *J. Nutr.* 130, 1081–1084. doi: 10.1093/jn/130.5.1081

Hrbáčková, M., Dvořák, P., Takáč, T., Tichá, M., Luptovčiak, I., Šamajová, O., et al. (2020). Biotechnological Perspectives of Omics and Genetic Engineering Methods in Alfalfa. *Front. Plant Sci.* 11, 592. doi: 10.3389/fpls.2020.00592

Hrbáčková, M., Luptovčiak, I., Hlaváčková, K., Dvořák, P., Tichá, M., Šamajová, O., et al. (2021). Overexpression of alfalfa SIMK promotes root hair growth, nodule clustering and shoot biomass production. *Plant Biotechnol. J.* 1–18. doi: 10.1111/pbi.13503

Ichimura, K., Shinozaki, K., Tena, G., Sheen, J., Henry, Y., Champion, A., Heberle-Bors, E. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends Plant Sci.* 7, 301–308. doi: 10.1016/s1360-1385(02)02302-6

Janczarek, M., Rachwal, K., Marzec, A., Grzadziel, J., and Palusinska-Szysz, M. (2015). Signal molecules and cell-surface components involved in early stages of the legume-Rhizobium interactions. *Appl. Soil Ecol.* 85, 94–113. doi: 10.1016/j.apsoil.2014.08.010

Janczarek, M., Rachwał, K., Marzec, A., Grzadziel, J., Palusińska-Szysz, M. (2014). Signal molecules and cell-surface components involved in early stages of the legume-rhizobium interactions. *Appl. Soil Ecol.* 85, 94–113. doi: 10.1016/j.apsoil.2014.08.010

Jia, W., Li, B., Li, S., Liang, Y., Wu, X., Ma, M., Wang, Y. (2016). Mitogen-activated protein kinase cascade MKK7-MPK6 plays important roles in plant development and regulates shoot branching by phosphorylating PIN1 in Arabidopsis. *PLoS Biol.* 14, e1002550. doi: 10.1371/journal.pbio.1002550

Jonak, C., Kiegerl, S., Ligterink, W., Barker, P. J., Huskisson, N. S., Hirt, H. (1996). Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Natl. Acad. Sci. USA.* 93, 11274–11279. doi: 10.1073/pnas.93.20.11274

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., Walker, G. C. (2007). How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nature Rev. Microbiol.* 5, 619–633. doi: 10.1038/nrmicro1705

Kape, R., Parniske, M., Brandt, S., Werner, D. (1992). Isoliquiritigenin, a strong nod gene-inducing and glyceollin resistance-inducing flavonoid from soybean root exudate. *Appl. Environ. Microbiol*. 1992, 1705–1710. doi: 10.1128/AEM.58.5.1705-1710.1992

Karimi, M., Bleys, A., Vanderhaeghen, R., Hilson, P. (2007). Building blocks for plant gene assembly. *Plant Physiol.* 145, 1183–1191. doi: 10.1104/pp.107.110411

Kiegerl, S., Cardinale, F., Siligan, C., Gross, A., Baudouin, E., Liwosz, A., et al. (2000). SIMKK, a mitogen-activated protein kinase (MAPK) kinase, is a specific activator of the salt stress–induced MAPK, SIMK. *Plant Cell* 12, 2247–2258. doi: 10.1105/tpc.12.11.2247

Kineman, B.D., Brummer, E.C., Paiva, N.L., Birt, D.F. (2010): Resveratrol from transgenic alfalfa for prevention of aberrant crypt foci in mice. *Nutr. Canc.* 623, 351–361. doi: 10.1080/01635580903407213

Křenek, P., Šamajová, O., Luptovčiak, I., Doskočilová, A., Komis, G., Šamaj, J. (2015). Transient plant transformation mediated by Agrobacterium tumefaciens: Principles, methods and applications. *Biotechnol. Adv.* 33, 1024–1042. doi: 10.1016/j.biotechadv.2015.03.012

Kumar, T., Bao, A. K., Bao, Z., Wang, F., Gao, L., Wang, S. M. (2018). The progress of genetic improvement in alfalfa (Medicago sativa L.). *Czech J. Genet. Plant Breed.* 54, 41–51. doi: 10.17221/46/2017-CJGPB

Lardi, M., Pessi, G. (2018). Functional genomics approaches to studying symbioses between legumes and nitrogen-fixing rhizobia. *High Throughput.* 7, 15. doi: 10.3390/ht7020015

Lei, Y., Hannoufa, A., Yu, P. (2017). The use of gene modification and advanced molecular structure analyses towards improving alfalfa forage. *Int. J. Mol. Sci.* 18, 298. doi: 10.3390/ijms18020298

Lin, J., Frank, M., Reid, D. (2020). No home without hormones: how plant hormones control legume nodule organogenesis. *Plant Commun.* 1, 100104. doi: 10.1016/j.xplc.2020.100104

Liu, C. W., Murray, J. (2016). The role of flavonoids in nodulation host-range specificity: an update. *Plants* 5, 33. doi: 10.3390/plants5030033

Long, S. R. (2001). Genes and signals in the rhizobium-legume symbiosis. *Plant Physiol.* 125, 69–72. doi: 10.1104/pp.125.1.69

Lu, H., Giordano, F., Ning, Z. (2016). Oxford Nanopore MinION sequencing and genome assembly. *Genom. Proteom. Bioinf.* 14, 265–279. doi: 10.1016/j.gpb.2016.05.004

Luo, D., Zhou, Q., Wu, Y., Chai, X., Liu, W., Wang, Y., et al. (2019). Full-length transcript sequencing and comparative transcriptomic analysis to evaluate the contribution of osmotic and ionic stress components towards salinity tolerance in the roots of cultivated alfalfa (*Medicago sativa* L.). *BMC Plant Biol.* 19, 32. doi: 10.1186/s12870-019-1630-4

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437, 376–380. doi: 10.1038/nature03959

Masonbrink, R. E., Severin, A. J., Seetharam, A. S. (2017). "Comparative genomics of soybean and other legumes," in: The Soybean Genome, eds. H. Nguyen, M. Bhattacharyya (Cham: Springer). 83–93. doi: 10.1007/978-3-319-64198-0_6

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., et al. (2006). Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium–legume symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5230–5235. doi: 10.1073/pnas.0600912103

Meskiene, I., Baudouin, E., Schweighofer, A., Liwosz, A., Jonak, C., Rodriguez, P. L., et al. (2003). Stress-induced protein phosphatase 2C is a negative regulator of a mitogen-activated protein kinase. *J. Biol. Chem.* 278, 18945–18952. doi: 10.1074/jbc.M300878200

Mittler, R., Blumwald, E. (2015). The roles of ROS and ABA in systemic acquired acclimation. *Plant Cell* 27, 64–70. doi: 10.1105/tpc.114.133090

Mortier, V., Den Herder, G., Whitford, R., Van de Velde, W., Rombauts, S., D'Haeseleer, K., et al. (2010). CLE peptides control *Medicago truncatula* nodulation locally and systemically. *Plant Physiol.* 153, 222–237. doi: 10.1104/pp.110.153718

Mortier, V., Holsters, M., Goormachtig, S. (2012). Never too many? How legumes control nodule numbers. *Plant Cell Environ.* 35, 245–258. doi: 10.1111/j.1365-3040.2011.02406.x

Müller, J., Beck, M., Mettbach, U., Komis, G., Hause, G., Menzel, D., et al. (2010). Arabidopsis MPK6 is involved in cell division plane control during early root development, and localizes to the pre-prophase band, phragmoplast, trans-Golgi network and plasma membrane. *Plant J.* 61, 234–248. doi: 10.1111/j.1365-313X.2009.04046.x

Munnik, T., Ligterink, W., Meskiene, I., Calderini, O., Beyerly, J., Musgrave, A. (1999). Distinct osmo-sensing protein kinase pathways are involved in signalling moderate and severe hyper-osmotic stress. *Plant J.* 20, 381–388. doi: 10.1046/j.1365-313x.1999.00610.x

Nakano, K., Shiroma, A., Shimoji, M., Tamotsu, H., Ashimine, N., Ohki, S., et al. (2017). Advantages of genome sequencing by long-read sequencer using SMRT technology in medical area. Hum. *Cell* 30, 149–161. doi: 10.1007/s13577-017-0168-8

Oldroyd, G. E. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Rev. Microbiol.* 11, 252–264. doi: 10.1038/nrmicro2990

Oldroyd, G. E., Downie, J. A. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annu. Rev. Plant Biol.* 59, 519–546. doi: 10.1146/annurev.arplant.59.032607.092839

Oldroyd, G. E., Murray, J. D., Poole, P. S., Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Ann. Rev. Ann.* 45, 119–144. doi: 10.1146/annurev-genet-110410-132549

Ovečka, M., Lichtscheidl, I. K., Baluška, F., Šamaj, J., Volkmann, D., Hirt, H. (2008). "Regulation Of Root Hair Tip Growth: Can Mitogen-Activated Protein Kinases Be Taken Into Account?," in: The Plant Cytoskeleton: a Key Tool for Agro-Biotechnology, eds. Y. B. Blume, W. V. Baird, A. I. Yemets, D. Breviario (Springer, Dordrecht), 91–128. doi: 10.1007/978-1-4020-8843-8

Ovečka, M., Takáč, T., Komis, G., Vadovič, P., Bekešová, S., Doskočilová, A., et al. (2014). Salt-induced subcellular kinase relocation and seedling susceptibility caused by overexpression of Medicago SIMKK in Arabidopsis. *J. Exp. Bot.* 65, 2335–2350. doi: 10.1093/jxb/eru115

Peck, M. C., Fisher, R. F., Long, S. R. (2006). Diverse flavonoids stimulate NodD1 binding to nod gene promoters in *Sinorhizobium meliloti*. *J. Bacteriol.* 188, 5417–5427. doi: 10.1128/JB.00376-06

Pellock, B. J., Cheng, H. P., Walker, G. C. (2000). Alfalfa Root Nodule Invasion Efficiency Is Dependent on *Sinorhizobium meliloti*Polysaccharides. *J. Bacteriol.* 182, 4310–4318. doi: 10.1128/JB.182.15.4310-4318.2000

Perret, X., Staehelin, C., Broughton, W. J. (2000). Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* 64, 180–201. doi: 10.1128/mmbr.64.1.180-201.2000

Playne, M. J., McDonald, P. (1966). The buffering constituents of herbage and silage. *J. Sci. Food Agric.* 17, 264–268. doi: 10.1002/jsfa.2740170609

Postnikova, O. A., Hult, M., Shao, J., Skantar, A., Nemchinov, L. G. (2015). Transcriptome analysis of resistant and susceptible alfalfa cultivars infected with root-knot nematode *Meloidogyne incognita*. *PLoS One* 10, e0123157. doi: 10.1371/journal.pone.0118269

Pratt, R. G., Rowe D. E. (2002). Enhanced resistance to *Sclerotium rolfsii* in populations of alfalfa selected for quantitative resistance to *Sclerotinia trifoliorum. J. Phytopathol.* 92, 204–209. doi: 10.1094/PHYTO.2002.92.2.204

Putnam, D., Russelle, M., Orloff, S., Kuhn, J., Fitzhugh, L., Godfrey, L., et al. (2001) "Alfalfa, wildlife and the environment," in: The importance and benefits of alfalfa in the 21st century (California Alfalfa and Forage Association), 1–24.

Radović, J., Sokolović, D., Marković, J. (2009). Alfalfa-most important perennial forage legume in animal husbandry. *Biotechnol. Anim. Husb.* 25, 465–475. doi: 10.2298/BAH0906465R

Raja, V., Majeed, U., Kang, H., Andrabi, K. I., John, R. (2017). Abiotic stress: interplay between ROS, hormones and MAPKs. *Environ. Exp. Bot.* 137, 142–157. doi: 10.1016/j.envexpbot.2017.02.010

Rashmi, R., Sarkar, M., Vikramaditya, T. (1997). Cultivaton of alfalfa (*Medicago sativa L.). Anc. Sci. Life* 17, 117–119.

Redmond, J. W., Batley, M., Djordjevic, M. A., Innes, R. W., Kuempel, P. L., Rolfe, B. G. (1986). Flavones induce expression of nodulation genes in *Rhizobium. Nature*, 323, 632-635.

Ringli, C., Baumberger, N., Diet, A., Frey, B., & Keller, B. (2002). ACTIN2 is essential for bulge site selection and tip growth during root hair development of Arabidopsis. *Plant Physiol.* 129, 1464–72. doi: 10.1104/pp.005777

Rothberg, J. M., Hinz, W., Rearick, T. M., Schultz, J., Mileski, W., Davey, M., et al. (2011). An integrated semiconductor device enabling non-optical genome sequencing. *Nature* 475, 348–352. doi: 10.1038/nature10242

Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., et al. (2020). Celebrating 20 years of genetic discoveries in legume nodulation and symbiotic nitrogen fixation. *Plant Cell* 32, 15–41. doi: 10.1105/tpc.19.00279

Rubiales D., Fondevilla S., Chen W., Gentzbittel L., Higgins T.J.V., Castillejo M.A., et al. (2015). Achievements and challenges in legume breeding for pest and disease resistance. *Crit. Rev. Plant Sci.* 34, 195–236. doi: 10.1080/07352689.2014.898445

Samac D. A., Temple S. J. (2004). "Development and utilization of transformation in Medicago species," in: Genetically Modified Crops, Their Development, Uses and Risks, eds. G. H. Liang, D. Z. Skinner (New York, NY: The Haworth Press), 165–202.

Samac, D. A., Austin-Phillips, S. (2006). Alfalfa (Medicago sativa L.). Agrobacterium protocols, 301-312. doi: 10.1385/1-59745-130-4:301

Šamaj, J., Ovečka, M., Hlavacka, A., Lecourieux, F., Meskiene, I., Lichtscheidl, I., Hirt, H. (2002). Involvement of the mitogen-activated protein kinase SIMK in regulation of root hair tip growth. *EMBO J.* 21, 3296–3306. doi: 10.1093/emboj/cdf349

Šamajová, O., Komis, G., Šamaj, J. (2013). Emerging topics in the cell biology of mitogen-1190 activated protein kinases. *T. Plant Sci.* 18, 140–148. doi.org/10.1016/j.tplants.2012.11.004. 1191

Sangra, A., Shahin, L., Dhir, S. K. (2019). Long-term maintainable somatic embryogenesis system in alfalfa (*Medicago sativa*) using leaf explants: embryogenic sustainability approach. *Plants 8*, 278. doi: 10.3390/plants8080278

Schena, M., Shalon, D., Davis, R. W., Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467–470. doi: 10.1126/science.270.5235.467

Segura, A., Moreno, M., Madueno, F., Molina, A., Garcia-Olmedo, F. (1999). Snakin-1, a peptide from potato that is active against plant pathogens. *Mol. Plant Microbe Interact.* 12, 16–23. doi: 10.1094/MPMI.1999.12.1.16

Severin, A. J., Cannon, S. B., Graham, M. M., Grant, D., Shoemaker, R. C. (2011). Changes in twelve homoeologous genomic regions in soybean following three rounds of polyploidy. *Plant Cell* 23, 3129–3136. doi: 10.1105/tpc.111.089573

Sieberer, B. J., Timmers, A. C., Emons, A. M. C. (2005). Nod factors alter the microtubule cytoskeleton in *Medicago truncatula* root hairs to allow root hair reorientation. *Mol. Plant Microbe Interact.* 18, 1195–1204. doi: 10.1094/MPMI-18-1195

Singer, S. D., Hannoufa, A., Acharya, S. (2018). Molecular improvement of alfalfa for enhanced productivity and adaptability in a changing environment. *Plant Cell Environ.* 41, 1955–1971. doi: 10.1111/pce.13090

Sorkin, A., Von Zastrow, M. (2002). Signal transduction and endocytosis: close encounters of many kinds. *Nat. Rev. Mol. Cell Biol.* 3, 600–614. doi: 10.1038/nrm883

Stambulska, U. Y., Bayliak, M. M. (2020). "Legume-rhizobium symbiosis: Secondary metabolites, free radical processes, and effects of heavy metals," in: Co-Evolution of Secondary Metabolites, eds. J.-M. Mérillon and K. G. Ramawat (Springer International Publishing). 291–322. doi: 10.1007/978-3-319-96397-6

Subramanian, S., Stacey, G., Yu, O. (2007). Distinct, crucial roles of flavonoids during legume nodulation. *Trends Plant Sci* 12, 282–285. doi: 10.1016/j.tplants.2007.06.006

Takáč, T., Šamajová, O., Luptovčiak, I., Pechan, T., Šamaj, J. (2017). Feedback microtubule control and microtubule-actin cross-talk in Arabidopsis revealed by integrative proteomic and cell biology analysis of KATANIN 1 mutants. *Mol. Cel. Prot.* 16, 1591–1609. doi: 10.1074/mcp.M117.068015

Tang, H., Krishnakumar, V., Bidwell, S., Rosen, B., Chan, A., Zhou, S., et al. (2014). An improved genome release (version Mt4.0) for the model legume *Medicago truncatula. BMC genom.* 15, 312. doi: 10.1186/1471-2164-15-312

Tichá, M., Hlaváčková, K., Hrbáčková, M., Ovečka, M., Šamajová, O., Šamaj, J. (2020a). Super-resolution imaging of microtubules in Medicago sativa. *Met. Cell Biol.* 160, 237–251. doi: 10.3390/ijms22041903

Tichá, M., Illésová, P., Hrbáčková, M., Basheer, J., Novák, D., Hlaváčková, K., et al. (2020b). Tissue culture, genetic transformation, interaction with beneficial microbes, and modern bio-imaging techniques in alfalfa research. *Crit. Rev. Biotechnol.* 40,1265–1280. doi: 10.1080/07388551.2020.1814689

Timmers, A. C., Auriac, M. C., Truchet, G. (1999). Refined analysis of early symbiotic steps of the *Rhizobium–Medicago* interaction in relationship with microtubular cytoskeleton rearrangements. *Develop.* 126, 3617–3628.

Toth, E., Bakheit, B. R. (1983). Results of resistance breeding in alfalfa. II. Resistance to *Verticillium wilt*. *Acta Biol. Hung.* 32, 78–85

Tu, X., Zhao, H., Zhang, Z. (2018). Transcriptome approach to understand the potential mechanisms of resistant and susceptible alfalfa (*Medicago sativa* L.) cultivars in response to aphid feeding. *J. Integr. Agric.* 17, 2518–2527. doi: 10.1016/S2095-3119(17)61843-4

Volkov, V., Wang, B., Dominy, P. J., Fricke, W., Amtmann, A. (2004). Thellungiella halophila, a salt-tolerant relative of Arabidopsis thaliana, possesses effective mechanisms to discriminate between potassium and sodium. *Plant Cell Environ.* 27, 1–14. doi: 10.1046/j.0016-8025.2003.01116.x

Wang, K., Wang, Z., Li, F., Ye, W., Wang, J., et al. (2012). The draft genome of a diploid cotton Gossypium raimondii. *Nature Gen.* 44, 1098–1103. doi: 10.1038/ng.2371

Wang, Q., Liu, J., Zhu, H. (2018). Genetic and molecular mechanisms underlying symbiotic specificity in legume-rhizobium interactions. *Front. Plant Sci.* 9, 1–8. doi: 10.3389/ fpls.2018.00313

Wang, Z., Gerstein, M., Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10, 57–63. doi: 10.1038/nrg2484

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant Physiol.* 126, 485–493. doi: 10.1104/pp.126.2.485

Xiao, T. T., Schilderink, S., Moling, S., Deinum, E. E., Kondorosi, E., Franssen, H., et al. (2014). Fate map of *Medicago truncatula* root nodules. *Develop.* 141, 3517–3528. doi: 10.1242/dev.110775

Xu, C., Liu, R., Zhang, Q., Chen, X., Qian, Y., Fang, W. (2017). The diversification of evolutionarily conserved MAPK cascades correlates with the evolution of fungal species and development of lifestyles. *Genome Biol. Evol.* 9, 311–322. doi: 10.1093/gbe/evw051

Yan, Z., Cao, J., Fan, Q., Chao, H., Guan, X., Zhang, Z., et al. (2020). Dephosphorylation of LjMPK6 by Phosphatase LjPP2C is Involved in Regulating Nodule Organogenesis in *Lotus japonicus. Int. J. Mol. Sci.* 21, 5565. doi: 10.3390/ijms21155565

Yang, S. S., Xu, W. W., Tesfaye, M., Lamb, J. F., Jung, H. J. G., VandenBosch, K. A., et al. (2010). Transcript profiling of two alfalfa genotypes with contrasting cell wall composition in stems using a cross-species platform: optimizing analysis by masking biased probes. *BMC genom.* 11, 323. doi: 10.1186/1471-2164-11-323

Yin, J., Guan, X., Zhang, H., Wang, L., Li, H., Zhang, Q., et al. (2019). An MAP kinase interacts with LHK1 and regulates nodule organogenesis in *Lotus japonicus*. *Sci. Chin. Life Sci.* 62, 1203–1217. doi: 10.1007/s11427-018-9444-9

Zanke, B. W., Boudreau, K., Rubie, E., Winnett, E., Tibbles, L. A., Zon, L., et al. (1996). The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. *Curr. Biol.* 6, 606–613. doi: 10.1016/S0960-9822(02)00547-X

Zemenchik, R. A., Albrecht, K. A., Wollenhaupt, N. C. (1997). Soil erosion and forage production from established alfalfa and smooth bromegrass in rotation with corn. *In Proc.* 6, 251–255.

Zhang, J. Y., Broeckling, C. D., Blancaflor, E. B., Sledge, M. K., Sumner, L. W., Wang, Z.Y. (2005). Overexpression of WXP1, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). *Plant J.* 42, 689–707. doi: 10.1111/j.1365-313X.2005.02405.x

Zhang, J., Subramanian, S., Stacey, G., Yu, O. (2009). Flavones and flavonols play distinct critical roles during nodulation of *Medicago truncatula* by *Sinorhizobium meliloti*. *Plant J.* 57, 171–183. doi: 10.1111/j.1365-313X.2008.03676.x

Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. *Ann. Rev. Plant Biol.* 53, 247–273. doi: 10.1146/annurev.arplant.53.091401.143329

8 List of publications

- **Hrbáčková, M.**, Dvořák, P., Takáč, T., Tichá, M., Luptovčiak, I., Šamajová, O., Ovečka, M., Šamaj, J. (2020). Biotechnological Perspectives of Omics and Genetic Engineering Methods in Alfalfa. *Frontiers in Plant Science*, 11. doi: 10.3389/fpls.2020.00592.
- **Hrbáčková, M.,** Luptovčiak, I., Hlaváčková, K., Dvořák, P., Tichá, M., Šamajová, O., Novák, D., Bednarz, H., Niehaus, K., Ovečka, M., Šamaj, J. (2021). Overexpression of alfalfa SIMK promotes root hair growth, nodule clustering and shoot biomass production. *Plant Biotechnology Journal*, doi: 10.1111/pbi.13503.
- Tichá, M., Richter, H., Ovečka, M., Maghelli, N., **Hrbáčková, M.,** Dvořák, P., Šamaj, J., Šamajová, O. (2020). Advanced microscopy reveals complex developmental and subcellular localization patterns of ANNEXIN 1 in Arabidopsis. *Frontiers in Plant Science 11,* 1153. doi: 1153.10.3389/fpls.2020.01153.
- Tichá, M., Illésová, P., **Hrbáčková, M.,** Basheer, J., Novák, D., Hlaváčková, K., Šamajová, O., Niehaus, K., Ovečka, M., Šamaj, J. (2020). Tissue culture, genetic transformation and modern bio-imaging techniques in alfalfa research. *Critical Reviews in Biotechnology*, published online Sept 17. doi: 10.1080/07388551.2020.1814689.
- Tichá, M., Hlaváčková, K., **Hrbáčková, M.**, Ovečka, M., Šamajová, O., Šamaj, J. (2020). Super-resolution imaging of microtubules in Medicago sativa. *Methods in Cell Biology*. Published online April 2020, doi: 10.1016/bs.mcb.2020.03.004.

9 Supplements

9.1 Abstrakt v slovenskom jazyku

Strukoviny sú schopné vytvárať symbiotické interakcie s rhizobaktériami, ktoré sú schopné premieňať atmosférický dusík na amoniak, ktorý je následne asimilovaný hostiteľskou rastlinou. Signalizácia pomocou mitogen-aktivovaných protein kináz (MAPK) môže byť do tejto symbiózy zapojená. MAPK kaskády sú jedny z najviac konzervovaných a najlepšie charakterizovaných proteín kinázových signalizačných dráh. U lucerny siatej (*Medicago sativa*) bola identifikovaná SIMK ako MAPK indukovaná soľným stresom a elicitormi. SIMKK je nadradeným aktivátorom SIMK a bolo dokázané, že aktivuje SIMK predovšetkým pri soľnom strese. Jedným z hlavných cieľov tejto dizertačnej práce bola príprava a transformácia konštruktov pre fluorescenčne značené SIMK a SIMKK do rastlinných buniek. Boli využité pokročilé mikroskopické metódy na vizualizáciu a imunolokalizáciu SIMK v živých a fixovaných pletivách a bunkách *M. sativa*. Ďalej bola študovaná nadexpresia SIMK a jej úloha pri raste koreňových vláskov, vytváraní infekčných vlákien, klastrovaní koreňových hľúzok a tvorbe zelenej biomasy.

Prvá časť dizertačnej práce je zameraná na plodinu *M. sativa*. Sumarizuje základné poznatky a popisuje jej biotechnologický potenciál. Táto kapitola sa tiež zaoberá interakciami strukovín s pôdnymi rhizobaktériami a MAPK identifikovanými v *M. sativa* a modelovej rastline *Arabidopsis thaliana.*

Nasledujúca kapitola je venovaná príprave a testovaniu fúznych proteínov GFP-SIMK a tagRFP-SIMKK. Expresia fluorescenčne značených SIMK a SIMKK bola overená pomocou tranzientnej transformácie listov *Nicotiana benthamiana*. Na prípravu nových stabilne transformovaných línií lucerny bol použitý vylepšený a účinný transformačný protokol pomocou *Agrobacterium tumefacins* a somatickej embrogenézy.

Posledná časť dizertačnej práce je zameraná na charakterizáciu a parametre produkcie u transgénnych línií lucerny s geneticky upravenou SIMK po inokulácii *Sinorhizobium meliloti*. U transgénnych línií *SIMKK RNAi* bolo zazanmenané výrazné potlačenie expresie *SIMKK* a *SIMK* génov. Tieto línie sa vyznačujú zníženým rastom koreňových vláskov a nižšou schopnosťou tvoriť infekčné vlákna, a následne tiež koreňové hľúzky. Naopak, konštitutívna nadexpresia GFP-značeného SIMK vyvolala u transgénnej línie rast dlhších koreňových vláskov a vytváranie klastrov infekčných vlákien a hľúzok. Zníženie hladiny *SIMK* a *SIMKK*

transkriptov viedlo k redukcii, zatiaľ čo nadexpresia GFP-SIMK znamenala zvýšenie produkcie zelenej biomasy u stabilne transformovaných rastlín *M. sativa*. Tieto výsledky poukazujú na to, že génové úpravy *SIMK* ovplyvňujú rast koreňových vláskov, klastrovanie nodulov a produkciu zelenej biomasy. To poukazuje na významný biotechnologický potenciál tejto proteín kinázy.

Klíčová slova: *Medicago sativa,* lucerna siata, SIMK, SIMKK, koreňový vlások, *Sinorhizobium meliloti,* infekčné vlákno, nodul, CLSM, imunoflourescenčné značenie

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