Palacký University in Olomouc

Master thesis

Olomouc 2016 **Bc. Martina Janyšková**

Palacký University in Olomouc

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Genetic and hormonal control of crown-root initiation and development in barley (*Hordeum vulgare* L.)

Master thesis

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Study program: Biology

Study branch: Molecular and cell biology

Study mode: Full-time

Olomouc 2016

Advisor: Véronique Bergougnoux-Fojtík, Ph.D.

Univerzita Palackého v Olomouci

Přírodovědecká fakulta

Katedra buněčné biologie a genetiky

Genetická a hormonální kontrola iniciace a vývoje nodálních kořenů u ječmene (*Hordeum vulgare* L.)

Diplomová práce

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Studijní program: Biologie

Studijní obor: Molekulární a buněčná biologie

Forma studia: Prezenční

Olomouc 2016 Vedoucí práce: Véronique Bergougnoux-Fojtík, Ph.D.

Declaration

I hereby declare that this thesis has been prepared by myself during my Master degree under the leadership of my supervisor Véronique Bergougnoux-Fojtík, Ph. D. All sources used in this thesis are cited and included in the „References“ part.

In Olomouc …….. ……...

Acknowledgements

I wish to express my sincere gratitude and indebtedness to my supervisor Véronique Bergougnoux-Fojtík, Ph. D. for her continuous guidance and help throughout the study. I also thank my supervisor for her constructive criticism and suggestions during the preparation of my master thesis. My thanks belong to Mgr. Aleš Pěnčík Ph.D. for auxin quantification. Thanks also belong to all members of Department of Molecular Biology for the pleasant working atmosphere and help that I received.

Summary

I worked out a master thesis on the topic „Genetic and hormonal control of crown-root initiaton and development in barley (*Hordeum vulgare* L.)“. Barley is the 4th most important cereal crops worldwide. Environmental changes, characterized by extreme climates including drought periods, induce important losses in term of yield which strongly impacts economie. Delivery of crops which are able to withstand drought is of great interest. One strategy would be to increase and improve the root system of crops. The aims of the work were i) to identify and characterize candidate genes involved in the initiation and development of CRs in barley, and ii) study the role of phytohormones in the process.

In the theoretical part of my master thesis I dealt with the description of root system architecture (RSA) of plants. I mostly focused on the root system of monocots which is mainly composed of crown-roots (CRs). CRs are post-embryonically formed and developed from the junction between primary roots and stem. A part of the introduction is focused on plant hormones auxins, cytokinins and strigolactones, their general properties and their involvement in the RSA. In the last chapter of the theoretical part, I compiled informations concerning the genetic aspects of RSA. Among other information, I presented genes known to be involved in RSA in other plant species and with potential orthologs in barley genome.

In the practical part of my master thesis I identified candidate genes and studied their expression profiles during crown-root development. How auxins and cytokinins influence the process was also investigated, as well as their effect on the expression of genes potentially involved in the CR initiation and development. Six genes were identified and found to be potentially related during CR initiation and development. The role of auxin, cytokinins and strigolactones was investigated.

Souhrn

Vypracovala jsem diplomovou práci na téma "Genetická a hormonální kontrola iniciace a vývoje nodálních kořenů u ječmene (*Hordeum vulgare* L.)". Ječmen představuje celosvětově 4. nejdůležitější obilninu. Změny životního prostředí, zahrnující periody sucha, způsobují významné ztráty ve výnosech zemědělských plodin a výrazně tak ovlivňují ekonomiku. Zemědělské plodiny schopné odolat tomuto suchu jsou dnes předmětem výzkumu. Jednou ze strategií jak tohoto cíle dosáhnout je zvětšení a zlepšení architektury kořenového systému zemědělských plodin. Předmětem této práce bylo i) identifikovat a charakterizovat kandidátní geny zapojené v iniciaci a vývoji nodálních kořenů u ječmene a ii) studovat roli fytohormonů zapojených v těchto procesech.

V teoretické části své diplomové práce jsem se zabývala popisem architektury kořenového systému rostlin. Podrobněji jsem se zabývala kořenovým systémem jednoděložných rostlin, který je tvořen hlavně nodálními kořeny. Nodální kořeny jsou vytvářeny post-embryonálně a vyrůstají z báze stébla. Dále jsem se věnovala rostlinným hormonům, především auxinům, cytokininům a strigolaktonům, jejich obecným vlastnostem a vlivu na architekturu a vývoj kořenového systému. Poslední oddíl teoretické části jsem věnovala genetickým aspektům architektury kořenového systému jednoděložných, především již známým genům, které by potenciálně mohly mít své ortology v genomu ječmene.

V praktické části diplomové práce jsem se věnovala identifikaci a studiu profilu exprese kandidátních genů. Zabývala jsem se rolí auxinu a cytokininů v tomto procesu a studovala jsem také jejich vliv na expresi kandidátních genů. Celkem jsem nalezla 6 genů, které by potenciálně mohly hrát roli při iniciaci a vývoji nodálních kořenů u ječmene. Role auxinu, cytokininů a strigolaktonů během tohoto procesu byla také předmětem této práce.

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1. Aims of the thesis

1. Identify in barley orthologues of genes involved in the initiation and development of crown-roots.
2. Study by qRT-PCR their expression during crown-root development.
3. Investigate the role of different hormones (namely auxin, cytokinins and strigolactones) during the same process and their influence on the studied genes.

2. Introduction

Plants are an essential part of life on Earth, providing humans not only oxygen but also food, fuel, fibers, medicines. Cereals (wheat, maize, barley) due to their nutritional value are one of the most important food sources for human life. Except of human food supply barley is used in distilling and brewery industry and for animal feeding. The Food and Agriculture Organization (FAO) estimated a worldwide production of 143 megatons of barley for 2013. The area of barley production is very large covering extreme environments, thus making this crop particularly tolerant to cold, drought, salinity and alkali.

Because of increasing world population, modification of environmental conditions, or demand for biofuels, improvement of crops is becoming an increasingly pressing issue for breeders and scientists. Roots, the hidden organ of the plant, are important for a wide variety of processes, including nutrient and water uptake, anchoring, and involved in respiration and photosynthesis (Flores *et al*., 1993). Roots serve as the major interface between the plant and biotic/abiotic factors in the soil. Also, plants can dramatically alter their root architecture to optimize their growth in response to environmental pressure. Differences in root architecture can affect many physiological functions, such as water and nutrients aquisition, carbon allocation and adaptability to environmental stress (Coudert *et al*., 2010). Therefore understanding the development and architecture of roots is of importance to exploit and manipulate root characteristics to increase plant yield.

The root system of monocots differs from those of dicots by its fibrous architecture (Figure 1) characterized by embryonic primary and seminal roots and post-embryonic shoot-borne (crown or nodal) and lateral roots. In maize the embryonic roots are important for the early vigor of the seedling, whereas the root system is later dominated by the crown-roots. Proliferation of crown-roots may have a significant influence on the grain yield in water-limited condition (Hochholdinger and Tuberosa, 2009). Crown-root initiation and development is mainly studied in the rice model plant, a non-european cereal. Few studies were conducted on maize (Jenkins 1930, Hetz *et al*., 1996, Hochholdinger and Feix 1998) but interestingly, whereas crown-roots are very important organs for the development of the plant and the yield, very few information is available. In this study, we propose to understand the basic mechanisms of crown-roots initiation and development in barley, one of the most important European crops, linking gene expression and hormonal regulation.

3. Theoretical part

3.1. Barley (*Hordeum vulgare* L.)

Cultivated barley (*Hordeum vulgare* ssp. *vulgare*) is a monocot of the Poaceae family. *Hordeum vulgare* spp. *spontaneum* is described as its wild ancestor. It was one of the first domesticated crops, grown in the "Fertile Crescent" of the near East 10 000 years ago (Bard *et al*., 2000). Today barley represents the fourth most important cereal crop species worldwide (FAO statistics: http://faostat.fao.org/). Barley is adapted to diverse environmental conditions including extreme regions. It is more stress tolerant than its close relative wheat and remains a major source of food in poor countries (Nevo *et al*., 2012, Mayer *et al*., 2012). Barley is a diploid species with 7 pairs of chromosomes representing a genome of 5.1 Gb. The sequence of the whole barley genome was recently released and indicates the presence of more than 37 000 genes (Mayer *et al*., 2012).

3.2. Root system architecture

Roots are important to plants for a wide variety of processes, their key functions are nutrient and water uptake, mechanical support and anchoring plant in the soil. Many plants use roots for storage function. Roots constitute the interface between plant and biotic/abiotic factors in the soil environment. Many factors in soil lead to temporally and spatially heterogenous conditions, including erosion, mineral nutrient and water content, and biotic factors (microbial population, symbiotic organisms). Plants can dramatically alter their root system architecture (RSA) to optimize growth in response to environmental stresses. This developmental plasticity aims with maximal yield, especially under stress, and yield stability (Jung and McCouch, 2013). The relevance of the root system for food production has often been overlooked and was not a major selection criterion as part of crop development programmes of the 1960s' green revolution (Waines and Ehdaie, 2007). Improved access to deep soil water, reducing the need for irrigation, is one potential benefit that could be achieved by exploitation of RSA (Smith and De Smet, 2012).

The term root system architecture (RSA) refers to the spatial configuration of a plant's root system. On a macroscale, it describes the organization of the primary, lateral and accessory roots (crown, brace roots found in cereals). On a microscale, it describes the organization of root hairs that increase the surface area, aiding with water and nutrients uptake (Gilroy and Jones, 2000). As proposed by Fitter (1991) there are five main components to

classify the architecture of a root system: (1) branch magnitude – the number of exterior or interior links, (2) topology – the pattern of branching (harringbone, dichotomous or radial), (3) link/internode lengths – the distance between branches, (4) root angles – the radial angle of a lateral root´s base around the parent root´s circumference, (5) link radius – the diameter of a root.

3.3. Cereal root types

Most knowledge of root system has been accumulated in the dicot plant model *Arabidopsis thaliana*. Cereal root system differs from those of dicots (Figure 1) and is mostly studied on rice which represents the plant model for monocots (Rebouillat *et al*., 2009) and maize. Rice belongs to the grass family together with barley, wheat, sorgum and maize. They share a monophyletic origin indicating that information obtained from rice might be transferred to other cereal crops (Itoh *et al*., 2005).

Figure 1. Comparison of a dicot taproot (A, dandelion) and the fibrous root system of a monocot (B, grass) (from Raven *et al.*, 1986).

Cereal plants are characterized by a dense fibrous root system (Fig.1). In adult cereal plants, the root system is mainly formed by post-embryonic roots (Fig.2; Coudert *et al*., 2010). Nevertheless, this root system is complex, comprising embryonic and post-embryonic roots. Embryonic roots are radicle and seminal roots, whereas post-embryonic roots emerge from the nodes of the stem or from the crown (junction between embryonic roots and stem) (Fig.3C).

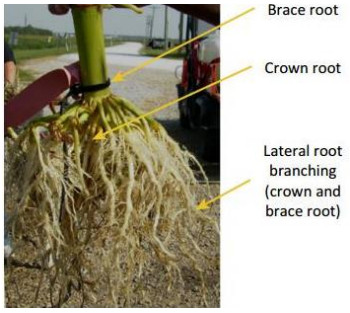

Figure 2. The root system of adult maize plant (from Meister *et al*., 2014).

Plant meristems (Fig.3A) are established during embryogenesis and provide most of the post-embryonic cells that constitute the organs of plants throughout their life cycle. The root apical meristem (RAM) is located underground at the root apex. The RAM contains a self-renewal stem-cell niche (SCN) with a central organizer quiescent centre (QC; rarely dividing cells which signal to the surrounding cells to organize and maintain the population of initial stem cells). The QC may vary in size from 4 in *Arabidopsis* to 800-1200 cells in maize (Jiang *et al*., 2003). The activity of the root apical meristem generates the radicle (primary root). The primary root becomes visible 2 or 3 days after germination (Coudert *et al*., 2010). The root itself broadly consists of xylem and phloem within a central vascular column and pericycle to constitute the stele. The stele is surrounded by concentric layers of epidermal, cortical and endodermal tissues (Fig.3B). The primary root of rice can have 10 to 15 cortical layers compared with the single layer found in *Arabidopsis thaliana* (Hochholdinger and Zimmermann, 2008). Cereal roots tissues tend to be larger and more complex.

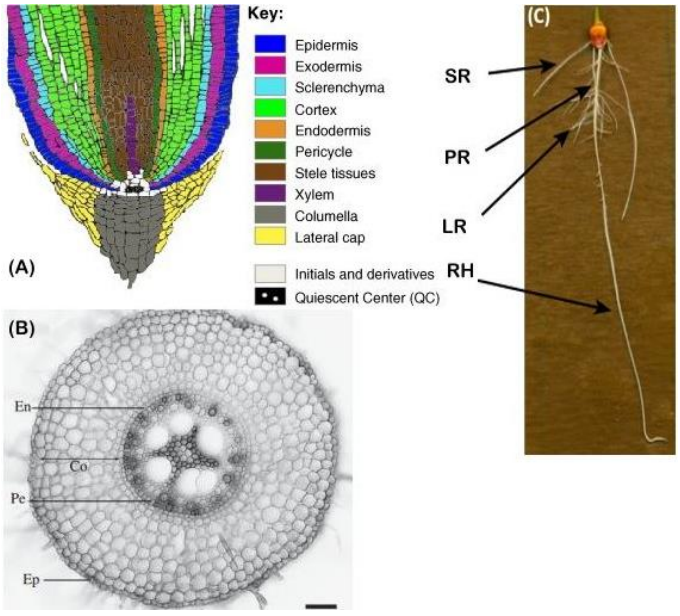

Figure 3. (A) Organization of monocot root tissues in rice; cell types are color coded as indicated in the key. **(B)** Transvere section through maize root. The epidermis (Ep), cortex (Co), endodermis (En) and pericycle (Pe) are indicated. Scale bar 100 µm. **(C)** Photograph of a young maize seedling illustrating the embryonic root system dominated by the primary (PR) and seminal (SR) roots. LR: lateral roots; RH: root hairs (adpated from Coudert *et al*., 2010, Meister *et al*., 2014, Smith and De Smet, 2012).

In rice, two or three days after germination 5 embryonic crown roots (seminal roots) emerge from the node of the coleoptile by breaking the sheath. Postembryonic crown roots differentiate during germination and throughout the life of plant from the nodes of the main stem and tillers (Coudert *et al*., 2010). Crown roots primordia initial cells of rice are produced from one or two periclinal divisions of few layers of parenchyma cells, which are adjacent to the peripheral cylinder of vascular bundles in the stem (Fig.4A). Those initial cells give rise to various tissues (epidermis, endodermis, cortex, stele and root cap) of the apical meristem of the new forming crown-root. Cells in the inner layer of the initial divide anticlinally and periclinally to form an epidermis-endodermis initial and a central cylinder initial. The outer

layer of the initials divides anticlinally to form the root cap initials (Fig.4B). The epidermis-endodermis initial undergo periclinal division to form epidermis and endodermis (Fig.4C). The root cap and central cylinder initial cells divide anticlinally and periclinally and increase their size (Fig.4C). The endodermal cells undergo numerous divisions to produce several cortical cell layers (Fig.4D). The root cap initial cells form columella and central cylinder initials continue divisions to become dome-shaped; at the same time various tissues of the vascular bundle are differentiated (Fig.4E). In the basal region of the primordium, cells of all tissues become vacuolated and elongated concurrently with the emergence of the crown-root from the stem (Fig.4F and 4G). Crown-root emergence coincides with the connection of the vascular bundle of the newly formed root with vascular system of the stem (Itoh *et al*., 2005).

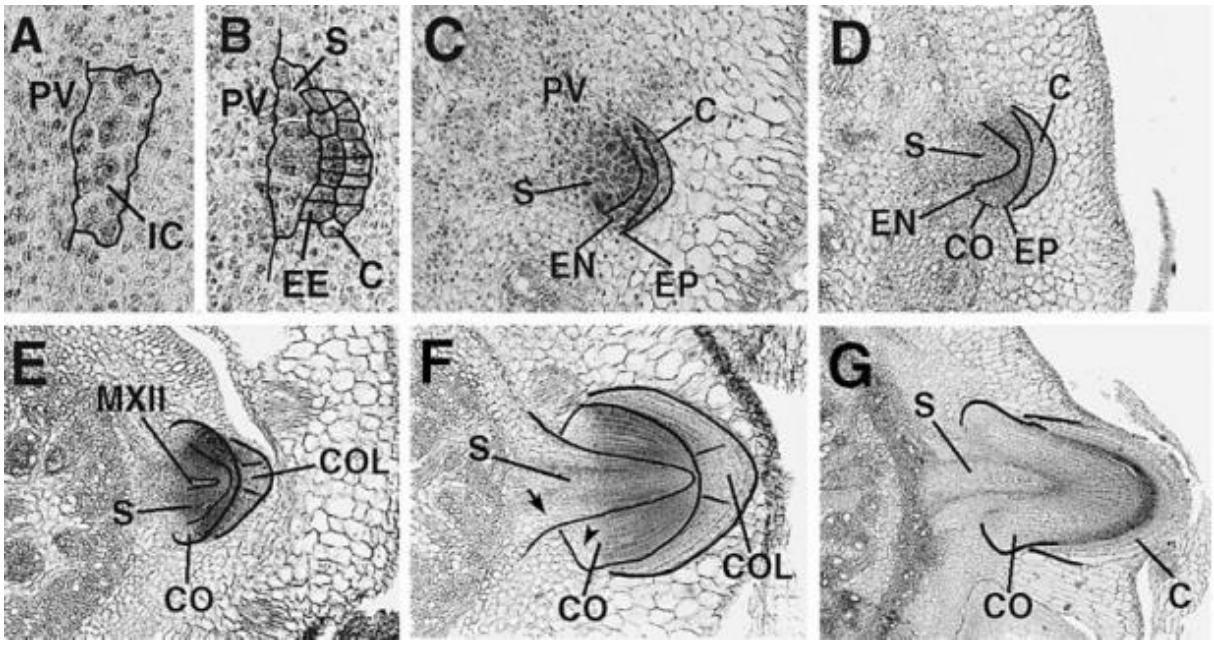

Figure 4. Crown root development in rice. **(A)** Establishment of initial cells. **(B)** Establishment of epidermis-endodermis and root cap initials. **(C)** Differentiation of epidermis-endodermis initial into epidermis and endodermis. **(D)** Cortex differentiation. **(E)** Establishment of fundamental organization of root primordium. **(F)** Onset of cell vacuolation (arrow head) in cortex and elongation (arrow) in stele. **(G)** Crown-root emergence. IC initial cells, PV peripheral cylinder of vascular bundle, C root cap or its initials, EE epidermis-endodermis initials, S stele, EP epidermis, EN endodermis, CO cortex, COL columella, MXII late meta-xylem vessel (from Itoh *et al*., 2005).

The radicle and crown-roots can branch, bearing two types of secondary post-embryonic roots: (i) large lateral roots characterized by a positive gravitropism and an undetermined growth, and (ii) small lateral roots ageotropic, short and showing determinate growth (Coudert *et al*., 2010). Root branching is essential and important for plant because it increases the area of the root system and subsequently the surface for absorption of water and nutrients. More extensive root system can reach more distant reserves of water and nutrient and improve soil anchorage (Smith and De Smet, 2012). Lateral roots play key role in soil

exploration and their production is generally developmental. Nevertheless they can also develop in adaptive response to environment pressure. Lateral roots are smaller in diameter than their parent root, due to a reduced number of cortical cell layers, and phloem and xylem poles (Jung and McCouch, 2013). A deep, thick and branched root system is correlated with better survival under adverse conditions (Rebouillat *et al*., 2009). Lateral root formation, in cereals, follows a succession of developmental steps including initiation, growth through the cortex and emergence through the epidermis. In dicots a specific subset of pericycle cells located opposite to protoxylem poles undergo an auxin-dependent cell cycle progression to form the founder cells. Lateral root initiation in cereals differs from dicots because founder cells originate from pericycle and endodermis cells located opposite to phloem poles. In monocots, roots have up to 10 or more phloem poles, resulting in a much more radial branched pattern around the parental root than observed in *Arabidopsis thaliana* (Smith and De Smet, 2012). Two longitudinal pericycle cells undergo asymmetric transverse division. In barley four to six such paired divisions occur at one time. Two short cells undergo periclinal divisions giving rise to two inner cells and two outer cells. The outer cells undergo numerous anticlinal and periclinal divisions. Endodermal cells adjacent to the dividing pericycle cells divide anticlinally to generate an additional cell layer. Thereafter, few cells at the forefront of the primordium divide synchronously and periclinaly to give rise to the inner layer of root cap initials and to the outer layer of root cap cells. During lateral root formation, the cortical and epidermal layers of parent root are reprogrammed to facilitate the emergence of the new root organ. This step is preceded by divisions of the cortical cells directly overlying lateral root primordia. Many of the resulting small cells enter H_2O_2-mediated cell death following the penetration of the lateral root primordium (Orman-Ligeza *et al*., 2013).

An important component of the root system architecture is presented by root hairs. They increase the surface and are one of the sites for plant/microbe interaction (Meister *et al*., 2014). The root-soil communication is facilitated by a structure called rhizosheath which contains tightly bound soil particles associated with root-hair-bearing roots and rhizobacteria. Rhizosheaths are only rarely seen in dicots (Hochholdinger *et al*., 2004).

3.4. Plant hormones

Plant hormones are small naturally occurring substances with very diverse chemical structures. Hormones regulate every aspects of plant life and can affect plant development and growth in small concentrations (Garay-Arryoy *et al*., 2012). Nine different plant hormones

have been discovered and isolated, so far: auxins, gibberellins, cytokinins, ethylene, abscisic acid, brassinosteroids, strigolactones, jasmonic acid, and salicylic acid. Plant hormones are important endogenous mediators that allow plants to rapidly adjust their development and growth to external cues. The activity of a given hormone depends on its biosynthesis, transport, accumulation in the vacuole, and degradation (Santner *et al*., 2009). All hormones regulate several processes independently; also a complex crosstalk between hormones regulates their biosynthesis, transport, and/or signaling. Then, the activity of one hormone is modulated by other contributing hormonal pathways (Benkova and Hejatko, 2009).

3.4.1. Auxin in root initiation and development

Auxins are known to be involved in the regulation of basic growth processes such as cell division and cell elongation. At the level tissues, organs and whole plant, they exhibit pleiotropic physiological effects such as curvature of stems toward a light source (Darwin, 1880), inhibition of root elongation, increase of lateral root production, induction of adventitious roots and root gravitropism (Zimmerman and Hitchcock, 1942; Wolverton *et al*., 2002). Auxins are involved in spatial and temporal coordination of plant morphogenesis and in plant responses to their environment. The most bioactive form of auxin is indole-3-acetic acid (IAA) (Zazimalova *et al*., 2007). Similar to other hormones, auxin can form inactive conjugates that may function in the storage of IAA, as intermediates in degradative processes or as a protection against oxidative degradation (Garay-Arryoy *et al*., 2012). Auxin concentration varies among different plant tissues and organs, and such graded distribution is correlated with different cellular behaviors (Friml *et al*., 2002). Several developmental processes seem to be dependent on the local asymmetric distribution of auxin molecules (Tanaka *et al*., 2009).

Auxin is mainly synthesized in young leaves and in the shoot apical meristem, and transported to the roots via the phloem. Auxin moves using two types of transport mechanisms. One of these mechanisms functions over long distances (long-range transport) dependents on the phloem, and moves auxin mainly from the aerial part of the plant to the roots. The second mechanism functions over short distances and is responsible for transport through plasma membranes via import-export mechanisms such as membrane diffusion, secretion and receptor- or transporter-mediated systems (Friml, 2003). The cell to cell auxin transport is used mainly to load and unload substances from the phloem and to distribute short-range signals within tissues. When this short-range transport involves influx and efflux carriers that are distributed asymmetrically in the plasma membrane, it is referred to as polar

auxin transport and gives directionality to auxin distribution. AUXIN RESISTANCE 1 (AUX1) and LIKE AUX (LAX) family members are auxin influx carriers whereas PIN FORMED (PIN) and ATP-BINDING CASETTE GROUP B (ABCB/MDRPGP) family members are auxin efflux transporters (Friml, 2003). In *Arabidopsis*, the intercellular trafficking of PIN1 is regulated by GNOM1, a membrane-associated guanine nucleotide exchange factor of the ADP-ribosylation factor G protein (ARF-GEF) family (Steinmann *et al*., 1999). GNOM1 is required for the first asymmetrical division of pairs of pericycle cells, which triggers lateral root initiation in *Arabidopsis* (Geldner *et al*., 2004). In rice, the identification and study of *crown rootless 4* and *Osgnom1* mutants demonstrated the importance of GNOM1 in the initiation of crown-roots and lateral roots (Kitomi *et al*., 2008 and Liu *et al*., 2009). In addition, in the *Osgnom1* mutant the expression of *OsPIN2*, *OsPIN5b* and *OsPIN9* was altered, indicating that polar auxin transport involving OsPINs and regulated by CRL4/OsGNOM1 is required for crown-root and lateral root initiation in rice. Several genes encoding auxin transporters have been characterized in dicotyledonous *Arabidopsis*, but most are unknown in monocotyledons. Nevertheless, a rice OsPIN1 gene which is expressed in the vascular tissues and root primordia was cloned and found to function in auxin-transport, crown-root emergence and tillering (Xu *et al*., 2005). The phylogenetic analysis of 12 *OsPIN*s revealed rice-genome specific (*OsPIN10a*, *OsPIN10b*) and monocot specific (*OsPIN9*) genes. The tissue-specific expression analysis of these genes showed that OsPIN9 protein was highly expressed in crown-root primordia and pericycle cells on stem-base. OsPIN10a and OsPIN10b were expressed in pericycle cells on stem-base. Authors concluded that these PIN proteins might have a particular role in crown-root development (Wang *et al*., 2009).

Auxin perception in plant cells begins when auxin binds to one of its multiple nuclear receptors including TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and closely related AUXIN SIGNALING F-BOX (AFB) proteins (Calderon Villalobos *et al*., 2012). TIR1 and AFB proteins are subunits of the ubiquitin ligase complex $SCF^{TIR/AFB}$. In the presence of auxin, $SCF^{TIR/AFB}$ complex degraded AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins. Aux/IAA are key regulators of the nuclear auxin response pathway, which functions as transcriptional repressors of the AUXIN RESPONSE FACTORS (ARF) transcription factor family (Garay-Arroyo *et al*., 2012, Orman-Ligeza *et al*., 2013).

In rice the *Oscand1* mutant is defective in crown-root emergence. OsCAND1 is the rice homolog of AtCAND1 which is required for $SCF^{TIR/AFB}$ activity. Loss-of-function of *OsCAND1* resulted in cessation of the G2/M transition in the meristem of crown-roots, and consequently in the failure of crown-root emergence (Wang *et al*., 2011). Auxin signaling is

also regulated by microRNAs (miRNA). OsMir393a and OsMir393b are negative regulators of the messenger RNAs *OsTIR1* and *OsAFB2*. Overexpression of 35S::Mir393 leads to a reduced number of crown-roots and a strong auxin-resistant phenotype (Bian *et al*., 2012).

The Aux/IAA degradation effectively releases ARF proteins, thereby activating a set of downstream target genes including LATERAL ORGAN BOUNDARIES DOMAIN (LBD) proteins. In rice OsARF16 specifically interact with the auxin-response element (AuxRE) in the promotor of *CROWN ROOTLESS1* (*CRL1*) gene which encodes an ASYMETRIC LEAVES2 (AS2)/LATERAL ORGAN BOUNDRAIES (LOB) domain transcription factor resulting in root initiation. The *crl1* mutant is defective in crown-root formation and exhibits other auxin-related phenotypic traits in the roots such as decreased lateral root number, auxin insensitivity in lateral root formation and impaired root gravitropism (Inukai *et al*., 2005). The maize *rtcs* (*ROOTLESS CONCERNING CROWN AND SEMINAL ROOTS*) mutant is defective in initiation of crown- and seminal roots. *RTCS* gene is orthologous to the rice *CRL1* gene and is a major regulator of crown-root formation in maize (Majer *et al*., 2012). Genetic pathway leading to crown-root initiation in rice is showed in Figure 5.

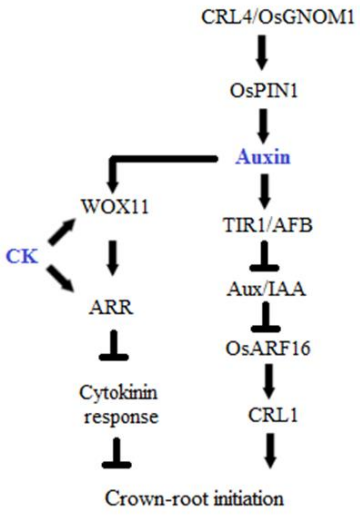

Figure 5. Gene regulatory network controlling crown-root initiation in rice: influence of auxin and cytoninin. Arrows represent the positive regulatory action of one element of the network on another one; a line ending with a trait represents the negative regulatory action of one element of the network on another one. Genes are denoted in black and hormons in blue (adapted from Coudert *et al.* 2010)

3.4.2. Cytokinins in root initiation and development

Cytokinins (CKs) are signaling hormonal molecules that play roles in many aspects of plant growth and development including apical dominance, repression of leaf senescence, root cell differentiation, vascular tissue development, pathogene responses, nutrient mobilization, seed germination and shoot apical meristem maintenance (Garay-Arroyo *et al*., 2012). The role of CKs in roots and shoots is opposite: in young shoot organs, CKs positively regulate development and promote shoot growth, whereas in roots they are negative regulators of growth and development (Howell *et al*., 2003, Werner *et al*., 2001). CKs are adenine

derivatives that are abundant in proliferating tissues such as shoot apical meristem, young leaves and immature seeds. The synthesis of CKs is initiated in a rate-limiting step by the ATP/ADP-isopentenyl-transferase, which transfer an isopentenyl group to an adenine nucleotide (Garay-Arroyo *et al*., 2012). Inactive cytokinin nucleotides can by activated by LONELY GUY proteins that directly converted them to the bioactive freebase (Kyozuka 2007). CKs inactivation depends on the activity of the CYTOKININ OXIDASE/DEHYDROGENASE (CKX) protein family (Werner *et al*., 2001). One of the main site in which CK biosynthesis occurs is the root tip, specifically the root cap cells (Aloni *et al*., 2004). CK can act within the region where they are synthesized or they can move. From the root cap, CKs are transported upward through plasmodesmata, which provide symplastic continuity in the meristematic and elongation zones, and from the differentiation zone through vessels of the xylem by the transpiration stream, mainly toward the developing shoot organs with high transpiration rates (Aloni *et al*., 2006).

In *Arabidopsis*, CKs are perceived by a two-component system that involves a histidine kinase receptor located in the plasma membrane that induces a phosphorylation cascade and subsequently activates transcription factors in the nucleus (Muller and Sheen, 2007). Three independent histidine kinase receptors (AHK2, AHK3 and CRE1/WOL/AHK4) bind to CKs, autophosphorylate and subsequently transfer the phosphoryl group to a histidine phosphotransfer protein that translocates to the nucleus and phosphorylates ARABIDOPSIS RESPONSE REGULATORS (ARR). Type-B ARRs are positive regulators that initiate the transcription of CK-responsive genes. Type-A ARRs are repressors that lack a DNA-binding domain and predominantly localize to the nucleus, where they act in conjunction with other transcription factors to regulate gene expression (Garay-Aroyo *et al*., 2012).

Many of processes controlled by CKs are controlled in coordination with other hormones, particularly auxin. CK and auxin have antagonistic effect on root development. Indeed, whereas auxin promotes the formation of lateral roots, adventitious roots and crown roots in cereals (Woodward and Bartel 2005; Sorin *et al*., 2005; Inukai *et al*., 2005), CK application at physiological concentrations inhibited root formation and reverses the auxin effect (Aloni *et al*., 2006). A low CK content in CK-deficient transgenic plants, which overexpress the *CKX* genes, resulted in an enlarged root meristem, formation of lateral roots closer to the root apical meristem, increased root branching and promotion of adventitious root formation (Werner *et al*., 2001; 2003). Similar morphological alterations were observed also in barley CK-deficient plants (Mrizova *et al*., 2013). Laplaze *et al*. (2007) reveals that CK acts directly on lateral root founder cells to inhibit root initiation in *Arabidopsis.* They

observed that CKs perturb the expression of *PIN* genes in lateral root founder cells and prevent the formation of an auxin gradient that is required to pattern lateral root primordia. CKs are also involved in crown-root initiation and development (Fig.5). In rice, the *WUSCHEL-Related Homeobox* (*WOX11*) gene is involved in the control of crown-root initiation and development, and interferes with CK signaling elements. The *wox11* mutant had a reduced number of crown-roots and showed a reduced development of the primary root. Plants overexpressing *WOX11* harboured an overproduction of crown-roots. *WOX11* is expressed in early crown-root primordia and its expression is maintained in the cell division zone of the root meristem. The expression of *WOX11* is induced by both auxin and CK. WOX11 suppresses the expression of the type-A RESPONSE REGULATOR2 (RR2) by direct binding to the promoter, suppressing thus the CK signaling. In rice, WOX11 integrates auxin and CK signals to regulate cell proliferation via RR2 during crown-root initiation and development (Zhao *et al*., 2009).

3.4.3. Strigolactones in root initiation and development

Strigolactones (SLs) are a novel class of phytohormones, which are proposed to be synthesized from carotenoids in the root tissues (Gomez-Roland *et al*., 2008). It is known that SLs or their derivatives suppress lateral shoot branching and are also involved in interactions with root parasitic plants and symbiotic arbuscular mycorrhizal fungi (Xie *et al*., 2008). A new role in the regulation of root development was attributed to SLs. Indeed, in roots, they increase the cell numbers in the primary root meristem, regulate lateral root formation, and enhance root hair elongation in the primary root (Ruyter-Spira *et al*., 2011, Kapulnik *et al*., 2011). SLs are a group of terpenoid lactones, consisting of a tricyclic lactone and a methylbutenolide coupled with an enol ether bond (Koltai, 2011). About 17 natural SLs have been isolated so far from different plant species (Koltai, 2015). The biosynthetic pathway of strigolactones is partially known. They are produced in a wide variety of plant species, including dicots, monocots and primitive plants mainly from the roots. They result from the cleavage of a carotenoid precursor by the activity of several enzymes. These include two carotenoid cleavage dioxygenases (CCDs), a carotenoid isomerase (DWARF27) and a class-III cytochrome P450 monooxygenase (Koltai, 2015). SLs are sensed by plants via a specific reception system which includes a D14-like/MAX2-like/SCF complex that, upon perception of strigolactone signaling leads to certain degradation of receptors and to the release of downstream targets. Degradation of the protein leads to the expression of SLs-responsive genes and lateral root or adventitious root formation (Koltai, 2014). The action of SLs on root

phenotypes is thought to be due to modulation of auxin flux in the root, via SL-regulated membrane cycling of PIN auxin efflux carrier proteins (Ruyter-Spira *et al*., 2011). SLs play a role in positively regulating crown-root elongation through promoting root meristematic cell division. Rice *dwarf* mutants for genes involved in SL biosynthesis (SL-deficient rice mutants *max3/rms5/d17, max4/rms1/d10,* and *d27*) or SL signaling (SL-insensitive rice mutants *max2/rms4/d3* and *d14*) were found to have a short CR phenotype due to an apparent decrease in cell division, leading to a narrower meristematic zone (Arite *et al*., 2012).

3.5. Genes involved in root system formation in cereals

The role and importance of root system has been already mentioned. Despite this fact, little is known about genetic basis of root system formation and architecture in major crop species. A great progress in understanding the molecular processes underlying root development has been achieved only in *Arabidopsis thaliana* (Scheres *et al*., 2002, Casimiro *et al*., 2003, Casson and Lindsay 2003, Ueda *et al*., 2005, Zhang *et al*., 2007, Busov *et al*., 2008). Several root mutant have been reported in three cereal species rice (Ma *et al*., 2001, Zimmer *et al*., 2003, Liu *et al*., 2005, Inukai *et al*., 2005, Jiang *et al*., 2005, Li *et al*., 2006a, Kim *et al*., 2007), maize (Lim *et al*., 2005, Woll *et al*., 2005, Wen *et al*., 2005, Hochholdinger *et al*., 2008) and wheat (Wang *et al*., 2006). Some of them have become the subject of studies that have led to the identification of homologous and novel genes controlling root system formation in monocotyledons (Morita and Kyozuka 2007). There is, however, a lack of similar knowledge in barley. In a recent *in silico* analysis, Orman and co-workers (2011) search for potential orthologs between *Arabidopsis* and barley using rice genes for confirmation and between barley and already reported genes in other monocotyledons. From this analysis ten genes (Table 1) were selected for their study in barley during crown-root initiation and development.

Table 1. Selection of candidate genes for identification of barley orthologues with potential role in crown-root initiation and development.

Gene name	Gene Acc. No.	Description
TaRAN1	AF488730	The wheat *TaRAN1*, from the family of Ran GTPases, regulates cell division and is involved in auxin signaling pathway (Wang *et al*., 2006)
TaRHD3	AY557340	The *root hair defective 3* gene encodes a protein with GTP-binding motifs, necessary to regulate cell expansion both during root epidermis development and root tip growth (Shan *et al*., 2005)
ZmRTH1	AY265854	The *root-hairless 1* encodes a homolog of the SEC3 exocyst subunit, playing a role in root elongation but not in root initiation (Wen *et al*., 2005)
ZmRTH3	AY265855	The *root-hairless 3* encodes a COBRA-like protein unique to monocots and required for root hair elongation and normal grain yield (Hochholdinger *et al*., 2008)
OsCRL1	Os03g05510	The *crown rootless 1* encodes a member of the plant-specific ASYMETRIC LEAVES2/LATERAL ORGAN BOUNDARIES protein family. It is expressed in tissue where crown and lateral roots are initiated and its expression is directly regulated by the auxin signaling pathway (Inukai *et al*., 2005)
OsCKI1	Os02g0622100	The *casein kinase 1* encodes a putative 463-aa protein. It is involved in auxin metabolism and its localization in the nucleus hypothesized a role in regulating gene expression (Liu *et al*., 2003)
OsCSLD1	Os10g0578200	The *cellulose synthase-like D1* is involved in the elongation of root hairs (Kim *et al*., 2007)
OsRAA1	Os01g0257300	The *root architecture associated 1* functions in the auxin-mediated development of rice roots (Ge *et al*., 2004)
OsPIN1	Os02g0743400	*OsPIN1* is a member of the *PIN1* family gene and is involved in the auxin-dependent adventitious root emergence and tillering in rice (Xu *et al*., 2005)
OsSLR1-1	XM_469478	The rice slender mutant *slr1-1* results in a constitutive gibberellin response phenotype. The mutant has a reduced number of roots which are also shorter than those from the wild-type (Ikeda *et al*., 2001)

4. Practical part

4.1. Equipment

Chemicals were weighted by analytical balance 5034EX (Nahita, China). Laboratory glassware was autoclaved by autoclave HST 5.6.8 (Zirbus technology, Germany). Samples were mixed by using vortex Combi-spin FVL-2400N (BioSan) and centrifuged by centrifuge ScanSpeed 1730R (LaboGene, Denmark). A concentration of nucleic acids was measured by spectrophotometer NanoDrop Lite (Thermo Scientific, USA). Samples were heated by Thermocell cooling and heating block (BIOER, China) and by thermocycler T-Personal (Biometra, Germany). Gels were visualised by UV transluminator (East Scientific Port, Czech Republic). The transcript level of genes was quantified by StepOnePlus™Real-Time PCR System (Applied Biosystems, USA).

4.2. Enzymes and kits

For RNA isolation was used ZR Plant RNA MiniPrep ™ kit (Zymo Research, USA). TURBO DNA-*free*™ Kit and RevertAid H Minus reverse transcriptase and buffers were from Thermo Scientific, USA. For real-time PCR was used gb SG Master Mix from GENERI BIOTECH, Czech Republic.

4.3. Media and buffers

Minimal nutritive solution – A complete nutrient solution containing macroelements and microelements described by Hackett (1986) was used for the experiments. The composition of the solution is given in Table 2.

To investigate the effects of auxin on number of crown-roots or gene expression a various concentrations of 1-naphtalenacetic acid (NAA) were added into the nutrient solution. A 1 mM stock solution of NAA was prepared as followed: 0.0093 g of NAA were dissolved with 1 ml of 1M NaOH and the volume was subsequently fill up to 50 ml with ultrapure water. For this study 5 different concentrations of NAA (1 nM, 10 nM, 100 nM, 1 μM and 10 μM) were used.

Table 2. Composition of the nutritive solution as described by Hackett (1968).

Major elements			
	mg/L	MW (g/mol)	concentration (mM)
$Ca(NO_3)_2.4H_2O$	218.00	236.15	0.92
KH_2PO_4	20.30	136.09	0.15
KNO_3	32.00	101.10	0.32
$MgSO_4.7H_2O$	61.60	246.47	0.25
Minor elements			
	mg/L	MW (g/mol)	concentration (mM)
$CuSO_4.5H_2O$	0.04	246.69	0.16
H_3BO_3	0.57	61.83	9.22
KCl	1.05	74.55	14.08
$MnSO_4.4H_2O$	0.81	223.06	3.63
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.02	1235.86	0.02
$ZnSO_4.7H_2O$	0.22	287.56	0.77

4.4. Sequences of primers used in practical part

The sequences and efficiency of the primers used by real-time PCR to identify the most stable reference gene and to investigate the expression of candidate genes are given in table 3. The specificity of all primers was verified by evaluating the real-time PCR products on 2 % agarose gel electrophoresis stained with ethidium bromide and the melt curve analysis.

Table 3. Sequence of primers used for real-time RT-PCR

Gene	**Sequence of primers**	**Primer efficiency (%)**
12395	Fw: 5'-GCCTCTGCCGCTACAAATAA -3'	219.0
	Rv: 5'-TGGTGGTGGACTGGAGAA-3'	
20934	Fw: 5'-AGTCACAACCCAACTGGTAAA -3'	97.4
	Rv: 5'-CAGGACAAGCGGTCTATCTATG -3'	
5439	Fw: 5'-GATTGAGGTGGAGAGGGTATTG -3'	88.7
	Rv: 5'-CTCCTGGTCTGTTAGCAGTTT -3'	
Act	Fw: 5'-TTGACCTCCAAAGGAAGCTATTCT -3'	116.1
	Rv: 5'-GGTGCAAGACCTGCTGTTGA -3'	
EIF52A	Fw: 5'-AGGGTGTATGCGGATGTGA -3'	85.4
	Rv: 5'-AATAGCATTCTCGGCTTCCA -3'	
EF2-α	Fw: 5'-CCGCACTGTCATGAGCAAGT-3'	127.0
	Rv: 5'-GGGCGAGCTTCCATGTAAAG-3'	
UPL	Fw: 5'-CTGAAGAGTTAGGCGGGAAA-3'	99.4
	Rv: 5'-ATCGCATGAACGTAGTGCA-3'	
CCD7	Fw: 5'-TGAAGACCAAGGAGTTGCTG -3'	82.6
	Rv: 5'-TGGCATAGCTCGGATTTATCG -3'	

Table 3. Continuation.

CKI1	Fw: 5'-TGTTATCGACTATGGCCTTGC -3'	109.8
	Rv: 5'-CTCTCCTGCTTTGTTCTACTCC -3'	
CRL1	Fw: 5'-CACCCCAACCATCCTCAG -3'	109.8
	Rv: 5'-TCAGATCCCCTACGCCTC -3'	
CSLD1	Fw: 5'-CACTCGGAAGCTCAAGATCC -3'	108.9
	Rv: 5'-ACAGCCAATCGGATGAGAAC -3'	
D27	Fw: 5'-GATGCCACCTTCAAGATTTTCC -3'	86.6
	Rv: 5'-TTCCATCGACTTCAGATTCCC -3'	
RAN1	Fw: 5'-TCGATTACCCCAGCTTCAAG -3'	104.4
	Rv: 5'-GGTGAAATCCAATGGGTGAAC -3'	
RHD3	Fw: 5'-CAACTACCTGGGACGAGATTC -3'	103.3
	Rv: 5'-ACTGCTTCCACACTGACTTG -3'	
RTH1	Fw: 5'-AGAGCTTTGTCCAGATGGTGACG -3'	103.3
	Rv: 5'-TCTCGGCAACCGGCATTATAGG -3'	
RTH3	Fw: 5'-TTTCTCCCAGGTGCTCAAC -3'	102.6
	Rv: 5'-AATGCTCGATCTTCCCCATC -3'	
slr 1.1	Fw: 5'-GCTCCAATGCCTACAAGCAG -3'	84.5
	Rv: 5'-AGGCACCCTTCCTTCTCTTC -3'	

4.5. Methods

4.5.1. Biological material

Experiments were conducted on *Hordeum vulgare* (L.) cultivar Golden Promise and on the mutant genotype overexpressing *CKX1* gene from *Arabidopsis thaliana* under the control of mild root specific β-glucosidase promotor from maize. The sequence of *AtCKX1* gene was slightly modified to ensure a cytosolic localization of the enzyme, as the natural enzyme bears a signal peptide for targeting into vacuole (Pospisilova *et al*., 2016).

4.5.2. Seed sterilization and sowing

Before each manipulation barley seeds were sterilized. Sterilization was done as followed: 5 min in 70% (v/v) ethanol, 3 rinses in sterile distilled water, 5 min in 5% sodium hypochlorite, 5 rinses in sterile distilled water. Prepared seeds were lied on wet filter paper in sterile petri dish and placed in a fridge for 2 days to ensure homogenous germination. Seeds in petri dishes were then transferred at room temperature to initiate germination.

4.5.3. Seedling growth and growing conditions

Seedlings were grown on filter paper-rolls as described by Bovina *et al*. (2011). Briefly, a filter paper sheet was fold in a half and wet with water. Germinated seeds were

placed 3 cm from the edge and finally the paper was rolled. Paper-rolls were placed in a baker with a minimal nutritive solution and placed in phytotron under long-day photoperiod (16h day/ 8h night) and controlled temperature (22 °C day/ 20 °C night) regimes.

To study the effect of auxin on the initiation of crown-root, a system of "mini-hydroponics" was developed. After the beginning of seed germination, 10 seedlings were fixed with piece of filter paper in 200 µl pipette tip box filled with 250 ml of minimal nutritive solution supplemented with appropriate concentration of NAA as described above (Fig. 6).

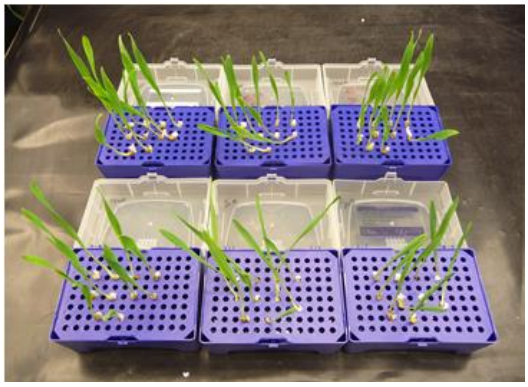

Figure 6. Photograph of the „mini-hydroponics“ system showing the pipette tip boxes with barley seedlings

4.5.4. Harvesting

After germination and growth on paper-roll or in pipette tip boxes, plants were harvested and used for experiments. Each day during five-day long experiment, 10 plants were harvested for RNA isolation. For this purpose only the junction between roots and aerial part, namely the crown zone, was harvested, immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

4.5.5. RNA isolation

All the RNA isolation procedure was performed in an RNase-free environment to achieve efficient isolation. The frozen plant material was ground in liquid nitrogen in fine powder using mortar and pestle. The plant powder was transferred into 2 ml Eppendorf tubes. Then the total RNA was extracted according instructions of the ZR Plant RNA MiniPrep kit. The RNA was eluted two times with 25 µl of DNase/RNase-free water. The DNase digestion procedure was performed with TURBO DNA-*free*™ Kit. To the RNA sample, 10 µl of 10X TURBO DNase Buffer, 2 µl of TURBO DNase and 38 µl of DNase/RNase-free water were added and mix gently. The mixture was incubated 45 min at 37 °C. Then 2 µl of enzyme were added again and incubation was continued for 45 min at the same temperature. Finally 50 µl

of 8M LiCl were added to start the precipitation of RNA. The mixture was incubated at -20 °C for at least 30 min, then centrifuged at 13 000 rpm at 4 °C for 30 min. The supernatant was discarded and pellet was washed with 500 µl of 70% ethanol followed by centrifugation at 13 000 rpm for 10 min. The supernatant was discarded and pellet was washed with 96% ethanol followed by centrifugation at 13 000 rpm for 10 min. The supernatant was discarded and pellet was air-dried and dissolved in 20 µl of DNase/RNase-free water. The concentration and quality of isolated RNA was determined with spectrophotometer.

4.5.6. Reverse transcription

Two micrograms of total RNA were used in reverse transcription reaction to obtain cDNA. First, 2 µg of RNA (in 13 µl) of, 1 µl of 100 µM oligo-(dT) primer and RNase-free water were mixed and incubated at 65 °C for 5 min. Then tubes were immediately transferred on ice. Four µl of 5X reaction buffer, 2 µl of 10 mM dNTPs each and 1 µl of revertAid H minus reverse transcriptase were added and mixture was incubated at 42 °C for 60 min. Reaction was stopped by heat deactivation at 70 °C for 10 min. Prepared cDNA were used immediately or stored at -20 °C.

4.5.7. Real-time PCR

For quantitative real-time PCR, cDNA samples were diluted by 5-fold. For each 10 µl PCR reaction, 1 µl of template and 9 µl of PCR mix were used. PCR mix was prepared from 5 µl of 2X gb SG PCR Master Mix, 1 µl of 5 µM Passive ROX Reference Dye and 0.3 µl of 10 µM primer each. Three technical repeats were run for each sample on the StepOnePlus real-time PCR system in a two-step amplification program. The initial denaturation at 94 °C for 10 s was followed by 40 cycles of 94 °C for 5 s and 60 °C for 20 s. A dissociation curve was obtained for each sample. Three independent biological repeats were analyzed for each sample. Cycle threshold values were normalized in respect to reference genes. After determination of primer efficiency, the Pfaffl method was used to determine the fold change in gene expression (Pfaffl, 2001). The relative quantification was made compared to the control. The results are expressed in term of fold change and represent the average ± standard error of mean (SEM) of 3 independent biological replicates.

4.5.8. Extraction and quantification of auxin content

Seeds of wild-type barley and barley genotype overexpressing cyt*AtCKX1* were sterilized. Germinated seedlings were grown in "mini-hydroponics". Shoots, roots of 8 day-

old plant, roots and nodal zones were harvested and immediately frozen in liquid nitrogen. The frozen plant material was ground in liquid nitrogen in fine powder using mortar and pestle. The powder was aliquoted to 15 mg per eppendorf tube. The auxin purification and quantification was performed by Mgr. Aleš Pěnčík Ph.D. (Laboratory of growth regulators, Palacký University, Olomouc) according a method described in Novak *et al*. (2012). Measurement of each sample consisted of at least three biological replicates.

4.5.9. Data analysis

Data were pooled for calculation of means and standard errors (SE) and assessed by non-parametric Kruskal-Wallis Anova test, followed by computation of least significant differences to compare means. All statistical analyses were performed using Statistica 12 (StatSoft CR, Czech Republic). In all analysis, the targeted level of statistical significance was $P< 0.0001$.

5. Results

5.1. Expression profiles and analysis of candidate reference genes

Eight genes were evaluated toward their ability to be used as housekeeping genes for the normalization of qRT-PCR data. Indeed, selection of housekeeping genes is a fundamental requirement for proper quantification of gene expression. Aside the well-known *Actine* and *EF2a* genes, 6 other genes were selected with Genevestigator based on the criteria of a specific expression in crown/roots: *EIF52A, UPL, TIP41*, 5439, 20934 and 12395. The specificity of the primer pairs was confirmed first by the presence of a single peak in the melting curve and second by the presence of a single band on the agarose gel (Fig.7). These results indicated that the primer pairs from these genes were highly specific. The efficiency of the primer pairs to recognize their target was assessed based on amplification of a standard curve taking into consideration sample diluted 5, 10, 50 and 100 times. In order to limit the „sample effect", the matrix used was a mixture of the different cDNA samples. The efficiency values of selected primers varied from 85.4 % for EIF52A to 219.0 % for „12359". The very high efficiency value (219 %) obtained for „12359" is surely connected with the fact that this gene was hardly detectable in our conditions (Ct values ≥ 34). Also it was removed from further analyses.

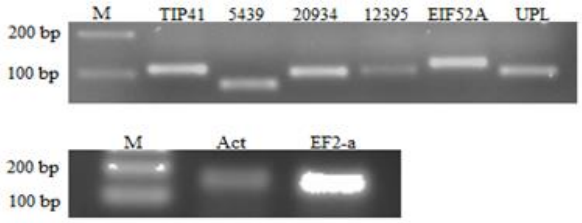

Figure 7. PCR products of housekeeping genes showed a single band. M – molecular weight.

The expression of candidate reference genes was detected by qRT-PCR in 5 samples collected from crown-zone of barley wild type plant at different time after germination. Their expression was plotted as a function of cycle threshold (C_t) values (Fig.8). The average C_t values of barley candidate genes ranged from 18 to 32 in all samples, mainly between 22 and 24. Among all these genes, *TIP 41* showed the highest expression with an average C_t value of 18.72, whereas „12395" showed the lowest expression level with an average value of 31.39. The C_t value variation indicated the difference in candidate gene expression stability among the different samples analyzed: smaller C_t variation of the gene across sample, more stable expression. Gene „12395" had the highest C_t variation 2.17 cycles and the most stable gene was „5439" with C_t variation 0.7 cycle.

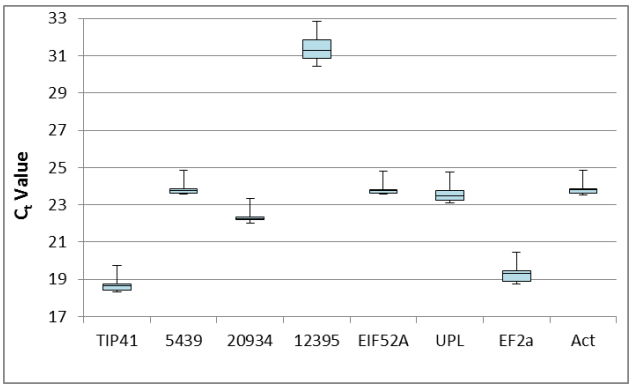

Figure 8. Expression levels of candidate reference genes across all samples. The line across the box depicts median.

5.1.1. geNorm analysis

The geNorm v3.5 software was used to analyze gene expression stability (Vandesompele *et al*., 2002). The reference genes were ranked according to the average *M* value expression (the larger the *M* value, the worse the gene's expression stability (Fig.9). In this study, all potential housekeeping genes presented an *M* value lower than the cutoff established by Vandesompele *et al*. 2002 (*M*<0.15). „5439*"* and „20934*"* had the lowest *M* values with the highest expression stability, while „12395" had the highest *M* value with the lowest expression stability.

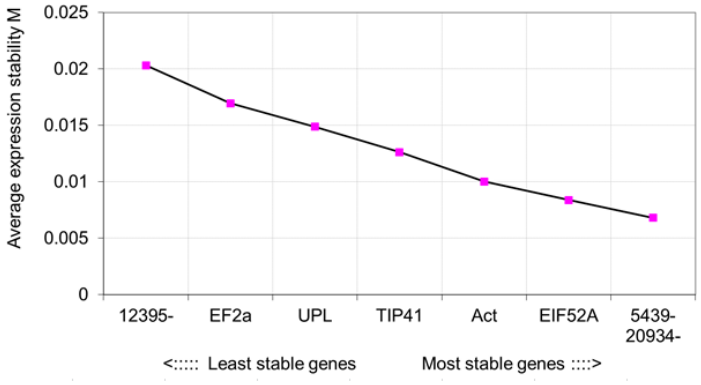

Figure 9. Gene expression stability and ranking of 8 candidate reference genes as calculated by geNorm (Vandesompele *et al*., 2002)

5.1.2. NormFinder analysis

Another program – NormFinder – was used to calculate the stability of the chosen potential housekeeping genes based on their intra- and inter-expression variation of expression (Andersen *et al*., 2004). Like geNorm software, lower values of the average expression stability indicated higher stability of a given gene. The stability values for all reference genes by NormFinder calculation are shown in Table 4. „20934*"* and *Act* were found to be the most stable, while „12395*"* was again determined at the least stable. Although the stability ranking of the 8 candidates with NormFinder was slightly different from the one

determined by geNorm, four most stable candidate genes (*20934*, *Act*, *EIF52A* and *5439*) were the same in NormFinder and geNorm.

Table 4. Expression stability of all the candidate reference genes as calculated by NormFinder (Andersen *et al*., 2004).

Rank	Gene name	Stability value
1	*20934*	0.002
2	*Act*	0.002
3	*EIF52A*	0.008
4	*5439*	0.010
5	*TIP41*	0.013
6	*UPL*	0.017
7	*EF2α*	0.023
8	*12395*	0.028

5.1.3. BestKeeper analysis

The BestKeeper program is another Excel-based software tool that analyzes the stability of the candidate reference gene by using the average C_t value of each sample (Pfaffl *et al*., 2004). The analysis is based on the CV (coefficient of variance) and SD (standard deviation) of the C_t. Data with SD [± C_t] < 1 were considered acceptable ranges of variation and the reference genes with lower coefficient of variance and standard deviation were identified as more stable genes. According the SD values all candidates were acceptable as stable genes (Table 5). According to the SD [± x-fold] the four most stable genes (*5439*, *EIF52A*, *20934* and *TIP41*) were almost identical as in geNorm and NormFinder. *TIP41* was the fifth most stable in geNorm and NormFinder.

Table 5. Expression stability of the candidate reference genes calculated by BestKeeper software (Pfaffl *et al*., 2004).

Gene name	CV [% CP]	SD [± CP]	SD [± x-fold]
5439	0.79	0.19	1.13
EIF52A	0.92	0.22	1.15
20934	1.02	0.23	1.15
Act	1.39	0.33	1.23
TIP41	1.60	0.30	1.21
EF2a	1.70	0.33	1.23
UPL	1.93	0.46	1.33
12395	2.10	0.66	1.51

Taking all together, our analysis of potential housekeeping genes revealed that *Actin* and *EF2α* genes, routinely used for normalization of qRT-PCR data, are not the most appropriate for our study. Instead 3 genes were identified as the most stable: „5439“, *EIF52A*

and „20934“. Also the normalization of our qRT-PCR data will be performed through these 3 genes.

5.2. Expression of candidate genes during the initiation and development of crown-roots in Golden Promise seedlings

The expression profile of ortholog genes identified by Orman *et al*. (2011) was investigated by real-time qRT-PCR to determine a possible role of these genes in crown-root initiation and development. Sequences of the corresponding genes were retrieved from the barley genome and specific pairs of primers were designed with Primer3Plus, specifically dedicated to the design of primers for qRT-PCR (Table 3). As for the potential housekeeping genes, the first step in our analysis was to determine their specificity and their efficiency. Except for *PIN1* and *RAA* genes, all other 8 genes produced a single peak in the melting curves obtained after amplification in qRT-PCR. This was confirmed by electrophoresis on 2% agarose gel of the amplified (Fig.10A).

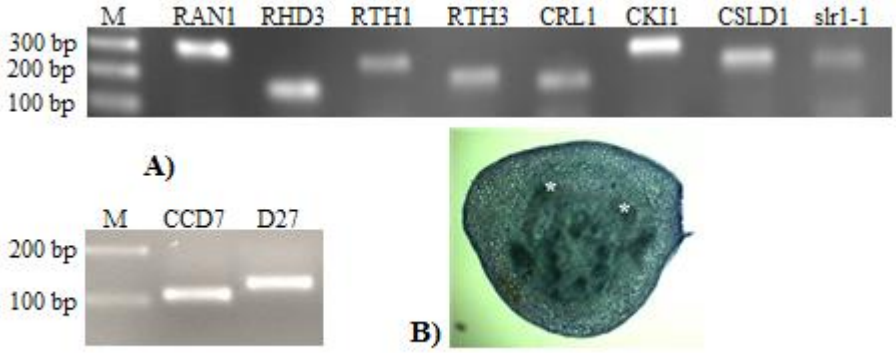

Figure 10. A) Electrophoresis on 2% agarose gel of the PCR products obtained after qRT-PCR amplification. All genes show a single band. M: molecular weight. **B)** Micrograph of hand-cutted sections of nodal zone of barley seedlings 5 DAG. Section was stained with toluidine blue. Stars indicate the crown-roots primordia.

The histological study of crown-root sections made from seedlings of different ages, demonstrated that crown-root primordia are already present 5 days after germination (DAG) (Fig.10B). Therefore, it was decided to study gene expression during the first five days after germination. The expression profile of 8 genes in the crown of young seedling was studied (Fig.11):

- *HvCRL1*-like encodes a LBD protein and might be close orthologue of OsCRL1/ZmRTCS, key component of the initiation of crown-root primordia.
- *HvRAN1* encodes a Ran GTPase, that regulates, in wheat, cell division and is involved in auxin signaling pathway (Wang *et al*., 2006).

- *HvRHD3* encodes a protein with GTP-binding motifs, necessary to regulate cell expansion both during root epidermis development and root tip growth (Shan *et al*., 2005).

- *HvCKI1* encodes a putative 463-aa protein. In rice, it is involved in auxin metabolism; its nuclear localization suggests a role in gene expression (Liu *et al*., 2003).

- *HvRTH3* encodes a COBRA-like protein unique to monocots, required for root hair elongation and normal grain yield (Hochholdinger *et al*., 2008).

- *HvCSLD1* whose orthologue in rice is involved in the elongation of root hairs (Kim *et al*., 2007)

- *HvSLR1.1* whose mutation in rice results in a constitutive gibberellin response phenotype with a reduced number of and shorter roots (Ikeda *et al*., 2001).

- *HvRTH1* encodes a homolog of the SEC3 exocyst subunit, playing a role in root elongation but not in root initiation (Wen *et al*., 2005).

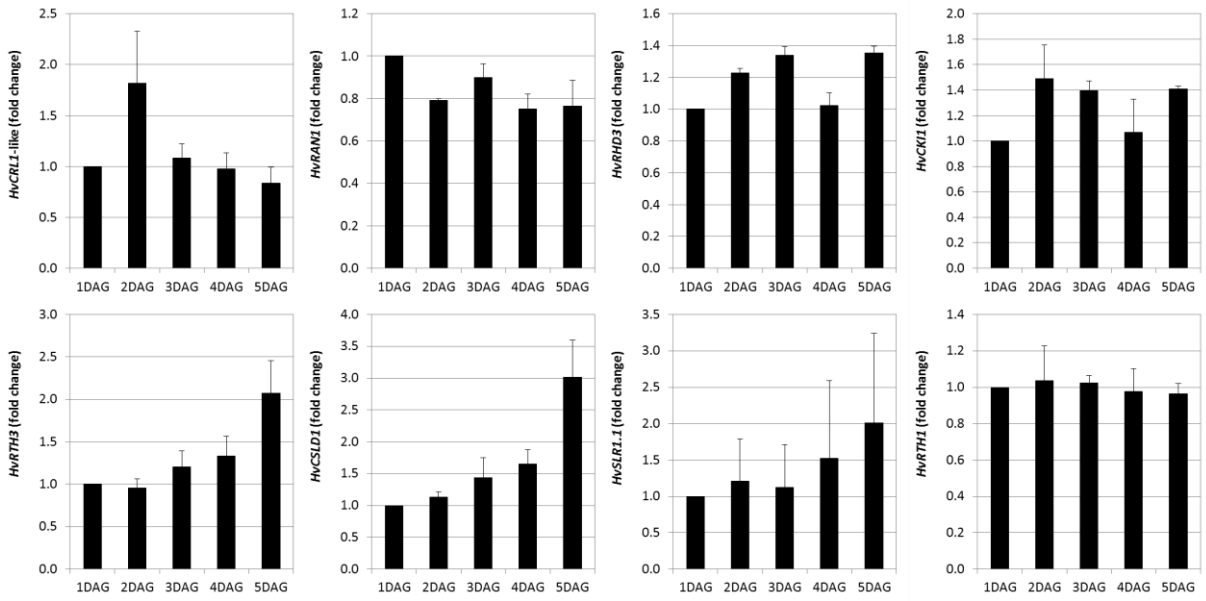

Figure 11. Expression profiles of 8 barley genes othologues to rice or maize genes with function in (crown-)roots development. Gene expression was monitored in the crown of Golden Promise seedlings harvested at different time after germination (DAG: day after germination). The results represent the geometrical average ± SEM of 3 independent biological replicates. Normalization was performed against 3 newly identified housekeeping genes („5439“, *EIF52A* and „20934“).

Two main expression profiles were observed: i) a „wavy” expression (*HvRAN1*, *HvRHD3* and *HvCKI1*) and ii) increasing expression along the time kinetic (*HvRTH3*, *HvCSLD1*). The expression of *HvCRL1*-like was stimulated in the 2nd day after germination, probably in connection with the signal required for the initiation of crown-root primordia. *HvSLR1.1* seemingly showed an increasing expression along the kinetic. However the large standard error bars represent a huge variation among the different replicates, without

consistency between independent biological replicates; therefore, it was exclude from the subsequent analyses. Finally, HvRTH1 gene did not show some variation in expression all long the time kinetic studied, excluding the possibility that it plays a role during the early development on seedling and the initiation/development of crown-root primordia.

5.3. Expression of candidate genes in the seedlings of a CK-deficient barley line

In order to investigate the role of CKs during initiation and development of crown-roots in barley, we used a line of barley overexpressing a cytosolic form of *AtCKX1* gene in a root-specific manner. Among other traits, this transgenic barley is characterized by lower endogenous CK content in roots and higher number of roots (Pospisilova *et al*., 2016). The figure 12 depicts the expression of all tested genes in the mutant during the first five days following germination. The relative quantification was made in relation to the expression observed in the transgenic line 1 DAG. Because the samples for the transgenic line were not collected in the same time as the samples of Golden Promise, it was not possible to compare the data between them, i.e. determine if the different genes are differently regulated in the transgenic lines compared to the wild-type Golden Promise.

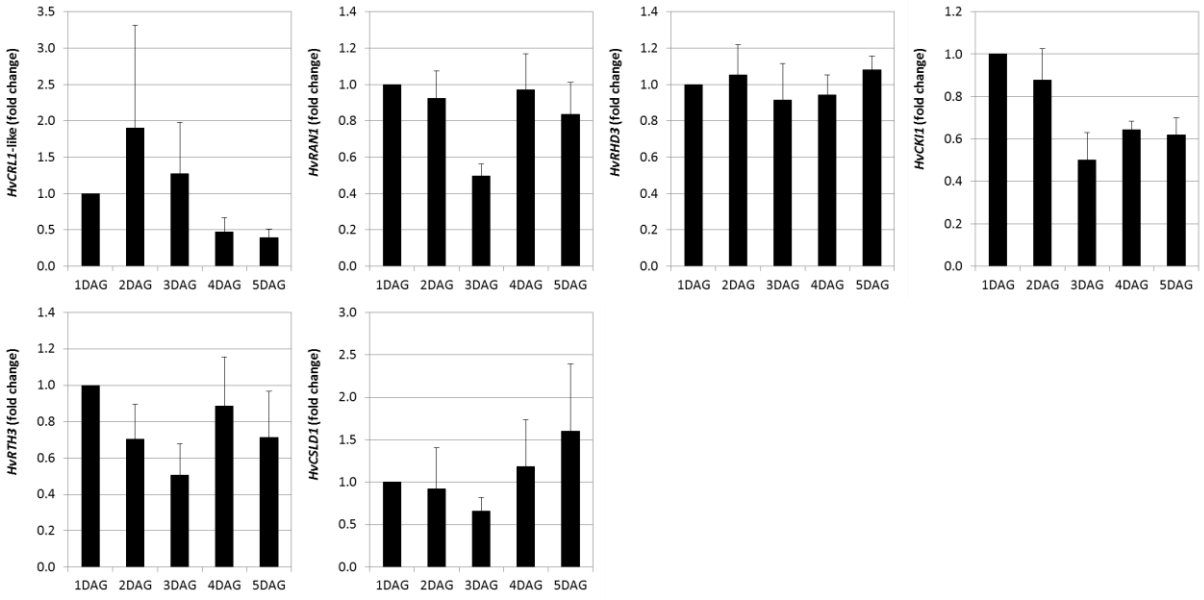

Figure 12. Expression profiles of 7 barley genes othologues to rice or maize genes with function in (crown-)roots development. Gene expression was monitored in the crown of transgenic barley overexpressing the cytosolic form of *AtCKX1* gene. Seedlings were harvested at different time after germination (DAG: day after germination). The results represent the geometrical average ± SEM of 3 independent biological replicates. Normalization was performed against 3 newly identified housekeeping genes („5439“, *EIF52A* and „20934“).

In general, standard error bars are very large, synonym of a large variability among the 3 independent biological replicates. Therefore, it will be of great interest to repeat this set of

experiments, also working in parallel with both genotypes in order to be able to estimate the effect of reduced endogenous CKs on the expression of the genes putatively involved in the initiation and development of crown-roots in barley.

Nevertheless, it appears that genes whose expression was regulated according a wave in the Golden Promise (*HvRAN1*, *HvRHD3* and *HvCKI1*) are also regulated in a rhythmic manner, but earlier in the kinetic analyzed, as shown by the shift from 2DAG in Golden Promise to 1DAG in the transgenic line. This is in agreement with the fact that this transgenic line harbors a faster/earlier development: indeed, it germinates earlier than the wild-type Golden Promise and consequently develops it first crown-roots earlier (data not shown).

5.4. Role of auxin during crown-root initiation and development

It is now accepted that high concentrations of auxin inhibits the growth of roots, low concentrations of auxin stimulates the initiation of crown-roots. In order to validate it in barley, 5 days-old seedling of Golden Promise barley were grown in „mini-hydroponics" in a nutritive solution containing different concentrations of auxin. The number of visible, emerged crown-roots was monitored after 10 days of culture in such conditions. As shown on the figure 13, the number of crown-roots increased in a dose-responsive manner up to 1 µM of IAA; higher concentration had no significant effect on the number of crown-roots. These results indicate the positive effect of auxin on crown-root initiation in barley. Nevertheless, we observed that exogenous auxin strongly inhibit the overall growth of embryonic roots (not shown).

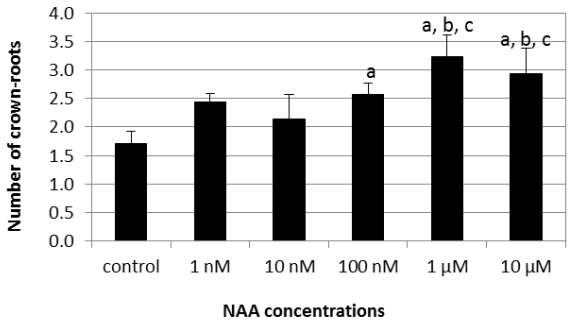

Figure 13. Effect of exogenous auxin treatment on number of crown-roots in barley. Data presents the averages of three biological repeats (10 plants per concentration). Analysis of variance (non-parametric Kruskal-Wallise ANOVA test) was used to compare plants at each auxin concentration. Statistically significant differences are indicated: (a) significantly different from control, (b) significantly different from 10 nM, (c) significantly different from 100 nM.

The pattern of expression of the genes under investigation was determined in relation to auxin treatment. For this purpose, crown of seedlings grown in presence of 100 nM of NAA were harvested immediately before or at 3h and 6h after start of the treatment.

Unfortunately, the experiment was performed only once and needs to be repeated. The figure 14 presents the data obtained. Again two groups of genes appeared: i) group of genes whose expression was very quickly stimulated by auxin (*HvCRL1*, *HvRAN1* and *HvCKI1*) and ii) group of genes whose expression was stimulated later, i.e. 6h after the application of auxin (*HvRHD3, HvRTH3, HvCSLD1* and *HvRTH1*). One might considers no effect of NAA treatment on the expression of *HvSLR1.1*.

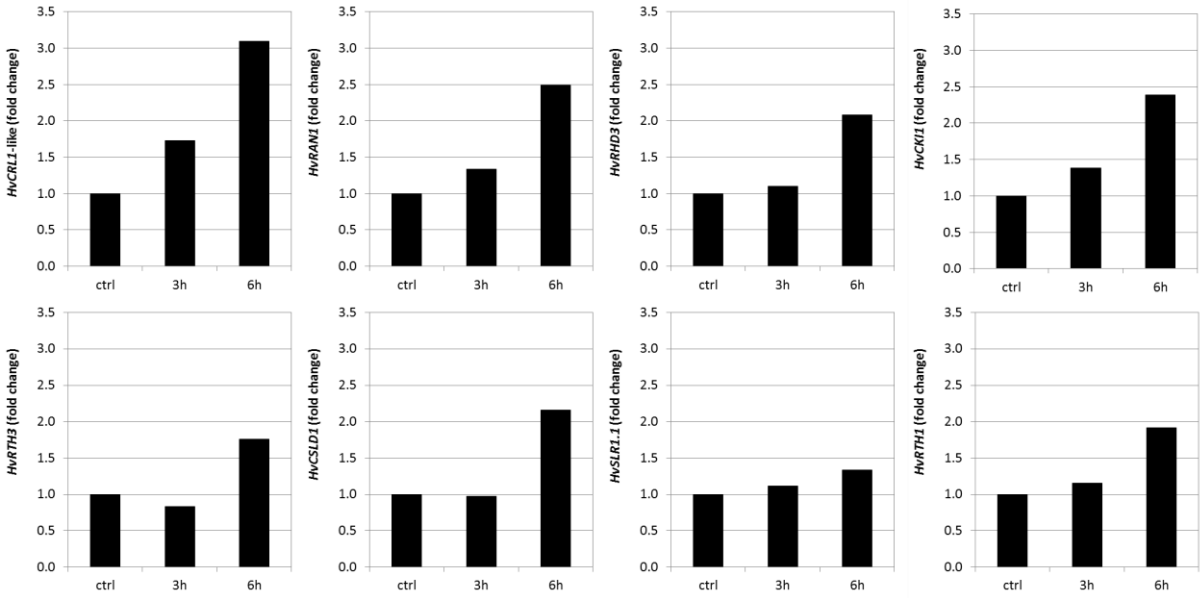

Figure 14. Effect of exogenous auxin (NAA) on the expression profiles of 8 barley genes orthologues to rice or maize genes with function in (crown-)roots development. Gene expression was monitored in the crown of 5-days old Golden promise seedlings grown for 3 and 6h in nutritive solution containing 100 nM NAA. Normalization was performed against 3 newly identified housekeeping genes („5439“, *EIF52A* and „20934“). No biological replicate.

Finally, the endogenous IAA content was analyzed in seedlings of the transgenic and in Golden Promise seedlings of the same age. IAA determination was performed from 3 different plants organs: shoots, roots and crowns. Several compounds involved in auxin metabolism (precursor, intermediates, products of degradation) were quantified. For better understanding and description of the table 6, we chose to include here the most recent scheme of IAA synthesis in plant (Fig.14; Ljung, 2013).

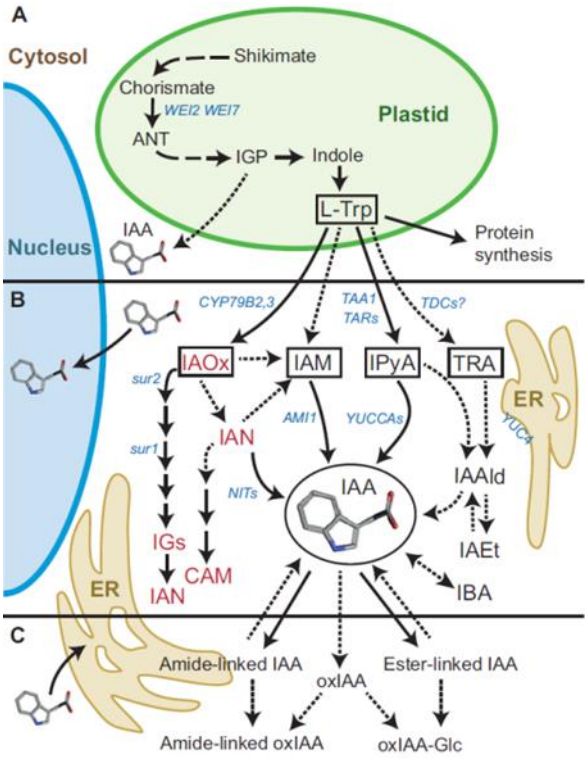

Figure 15. IAA metabolism in higher plants. (A) The biosynthesis L-Trp, the major IAA precursor via the shikimate pathway. (B) Four putative pathways for L-Trp-dependent IAA biosynthesis in higher plants are shown: the IAOx, IAM, IPyA and TRA pathways. The enzymes known to operate in each pathway are shown in blue. Intermediates that also act as precursors and degradation products of defense compounds (such as IGs and CAM) are in red. (C) Pathways for IAA degradation and conjugation. IAA can be conjugated to amino acids and sugars, or catabolized to form oxIAA. Some IAA conjugates can be regarded as storage forms that can be hydrolyzed to form free IAA. Solid arrows indicate pathways in which the enzymes, genes or intermediates are known, and dashed arrows indicate pathways that are not well defined. ANT, anthranilate; CAM, camalexin; Glu, glucose; IAAld, indole-3-acetaldehyde; IAEt, indole-3-ethanol; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; IBA, indole-3-butyric acid; IGP, indole-3-glycerol phosphate; IGs, indole glucosinolates; IPyA, indole-3-pyruvic acid; L-Trp, L-tryptophan; NITs, nitrilases; oxIAA, 2-oxoindole-3-acetic acid; TDCs, tryptophan decarboxylases; TRA, tryptamine. (from Ljung, 2013)

We were able to quantify free IAA from all types of tissue analyzed and in both genotypes. Interestingly, whereas in Golden Promise IAA content was higher in aerial parts (shoots and crowns) than in roots, the opposite could be observed in the transgenic line. Even more interesting is that whereas the transgenic line accumulates less IAA in its aerial part (shoots and crowns) than Golden Promise, the content of IAA in the roots of the transgenic line was higher than in the roots of Golden Promise. It is clear that the CK status modification in the transgenic line affects the IAA status of the plant. One might wonder which point of the IAA metabolism is affected by the modified CK status: synthesis or degradation/storage. L-tryptopan (TRP), the IAA precursor, was found to highly accumulate in all organs analyzed (roots, shoots and crowns) of both genotypes. Nevertheless, this accumulation was stronger in shoots compared to other organs. Interestingly, whereas the transgenic line accumulates less TRP in shoots and crowns compared to Golden Promise, it significantly accumulates more TRP in its roots than the wild-type. Because IAOx, IAN and IAM were not detected or in a very low amount in our material, we might assume that, in barley, IAA is not synthesized neither through the IAOx nor IAM pathway. Both indole-3-pyruvic acid (IPyA) and tryptamine (TRA) strongly accumulated (nmol/g FW vs. pmol/g FW) in our material, with a prevalence of TRA, suggesting that IAA synthesis mainly occurs via the TRA pathway. Again TRA accumulated preferentially in aerial parts (shoots and crowns) in a similar amount in both genotypes (the value obtained for the wild-type cannot be considered as significantly

Table 6. Endogenous auxin content in the roots, shoots and crowns of seedlings of Golden Promise and cytAtCKX1 transgenic line. Seedlings were harvested 8 days after sowing (DAS). Concentrations of compounds are expressed in pmol or nmol(*) / g FW. Results represent the average ± SD of 3 independent biological repeats.

Description		TRP*	TRA*	IAM	IPyA*	IAA	oxIAA	IAAsp	IAGlu	IAA-glc*	oxIAA-glc
Golden Promise	8DAS-roots	727.8 ± 68.0	1.3 ± 0.4	25.4 ± 5.8	2.1 ± 0.1	68.2 ± 7.4	29.2 ± 8.8	104.7 ± 15.2	n.d.	2.7 ± 0.4	68.2 ± 7.4
	8DAS-shoots	4015.2 ± 1291.5	12.1 ± 3.1	76.5 ± 11.1	2.5 ± 0.9	82.6 ± 16.8	154.3 ± 20.6	n.d.	n.d.	41.6 ± 1.2	82.6 ± 16.8
	crown	658.2 ± 73.5	28.4 ± 18.2	n.d.	3.8 ± 0.3	81.5 ± 27.4	1633.6 ± 403.0	369.1 ± 4.0	18.1 ± 3.1	97.0 ± 8.8	2381.5 ± 109.8
cytAtCKX1	8DAS-roots	900.7 ± 85.0	4.3 ± 0.9	53.3 ± 4.4	3.5 ± 0.4	102.0 ± 2.0	67.2 ± 0.5	64.9 ± 17.4	n.d.	8.5 ± 1.3	102.0 ± 2.0
	8DAS-shoots	2110.0 ± 532.7	12.7 ± 2.3	62.5 ± 11.9	1.7 ± 0.3	59.9 ± 11.5	171.4 ± 10.6	n.d.	n.d.	22.0 ± 9.4	59.9 ± 11.5
	crown	447.4 ± 109.9	10.4 ± 2.2	n.d.	2.8 ± 0.8	31.3 ± 7.6	1653.7 ± 295.0	345.1 ± 21.5	16.8 ± 4.9	100.6 ± 15.5	1498.7 ± 377.2

different from that obtained for the transgenic line). Again the difference is seen in roots with the transgenic line accumulating more TRA than Golden Promise. If one considers either the IAA-conjugated compounds (IAAsp, IAGlu and IAA-glc) or the products derived from the IAA degradation (oxIAA and oxIAA-Glc, major degradation products of IAA (Novak *et al*., 2012), no real significant difference could be observed between genotypes.

5.5. First step toward understanding the role of SL in crown-root

As mentioned in introduction, strigolactones (SLs), another group of phytohormones very recently identified, were suggested to be part of the mechanisms controlling crown-root initiation and development. In our study, we monitored the expression of three genes encoding proteins of the SL synthetic pathway: D27, CCD7 and CCD8.

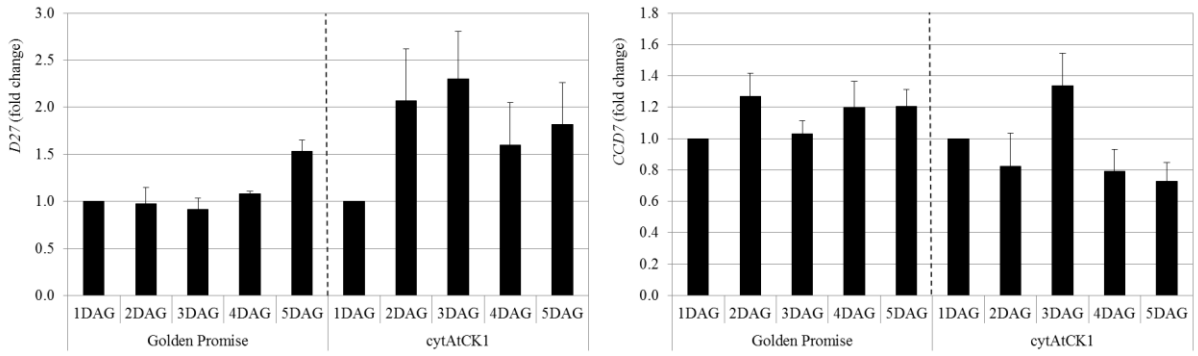

Figure 16. Expression profiles of *D27* and *CCD7* genes, involved in the strigolactone biosynthesis. Gene expression was monitored in the crown of Golden Promise and transgenic line (cytAtCKX1) seedlings harvested at different time after germination (DAG: day after germination). The results represent the geometrical average ± SEM of 3 independent biological replicates. Normalization was performed against 3 newly identified housekeeping genes („5439“, *EIF52A* and „20934“).

The *D27* gene encodes a trans/cis-β-carotene isomerase which catalyzed the first committed step of the SL synthesis. In the wild-type, its expression start to increase at 5DAG; in the crown of the transgenic line, its expression is significantly up-regulation in the crown of 2DAG-seedlings; no significant difference can be observed for the rest of the time course. In the case of *CCD7* gene, no clear conclusion can be made concerning its expression in Golden Promise. In opposite, its expression is transiently up-regulated in 3DAG-seedlings of the transgenic line.

In order to determine a possible interaction between auxin and SLs, the expression of D27 and CCD7 was studied in relation to auxin treatment.

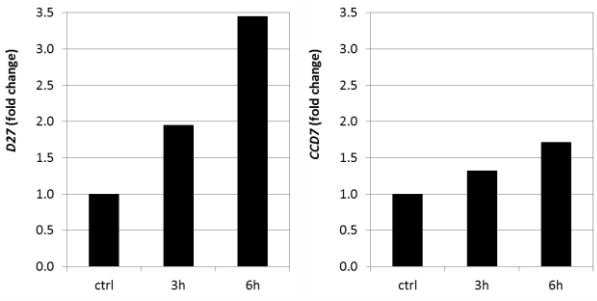

Figure 17. Effect of exogenous auxin (NAA) on the expression profiles of *D27* and *CCD7* genes. Gene expression was monitored in the crown of 5-days old Golden promise seedlings grown for 3 and 6h in nutritive solution containing 100 nM NAA. Normalization was performed against 3 newly identified housekeeping genes („5439“, *EIF52A* and „20934“). No biological replicate.

Unfortunately, we were not able to determine the endogenous SL content in our material during the time of this master study. It will be interesting not only to repeat the analysis of gene expression but also to develop methods of extraction and quantification of SLs in such small organs.

6. Discussion

In the present master thesis, we investigated the initiation and development of crown-roots in barley from a genetic and hormonal point of view. The results obtained are of great importance for several reasons. First, whereas a huge amount of studies and data is available concerning the initiation and development of lateral roots in dicots, the information concerning the initiation and development of monocot crown-roots is very limited. Lateral roots and crown-roots emerge from different tissues and there development obeys different processes (Jung and McCouch, 2013). Second, our study was conducted on barley which is poorly used in research, despite the fact that it is the 4th most important cereal crops worldwide.

6.1. Identification of suitable reference genes for barley gene expression

In this work, we studied by real-time qRT-PCR the expression of 8 barley genes orthologues to rice, maize or wheat genes encoding proteins involved in (crown-) roots initiation and development. Real-time qRT-PCR is very powerful technique to quantify the expression of targeted genes and requires stably expressed reference gene for data normalization. No universal reference gene exists. Indeed, the stability of gene is relative and depends on the tissue or conditions of the experimental design (Hua *et al*., 2015). In barley, many housekeeping genes were found and evaluated for different stress conditions or different barley developmental stages (Faccioli *et al*., 2007; Ovesna *et al*., 2012; Rapacz *et al*., 2012; Janska *et al*., 2012; Hua *et al*., 2015). Nevertheless, any research dealt with suitable reference genes for non-stressed crown-zone tissue. Based on genevestigator prediction of gene expression in the crown or root of barley seedlings, we identified 6 genes which might have a potential as reference gene in our study. These 6 genes, together with two broadly used HKGs (*actin* and *EF2*α) were evaluated for their stability in our research context. For this purpose, we used three software packages (geNorm, NormFinder, and BestKeeper) based on different algorithm and having each of them advantages (Hua *et al*., 2015). Using more than one software provide a high reliability in the prediction of genes as reference (Manoli *et al*., 2012). All three algorithms determined that 3 genes (“5439*”*, “20934*”* and *EIF52A*) were the most stable in our experimental design. Even, they were predicted to be more stable than the two well-known and used reference genes, *actin* and *EF2*α.

6.2 Genetic control of crown-root initiation and development in barley

As already mentioned several times, no information is available concerning the initiation and development of crown-roots in barley. Orman and coworkers (2011) provided a list of barley genes orthologues to genes of rice, maize, wheat and Arabidopsis with a function during root development. Among these genes, we decided to focus our attention on 8 with a very high probability to play an important role in crown-root initiation and development in barley. These 8 genes are briefly described in the table 7.

Table 7. List of candidate genes including their potential role during root initiation and development.

Genes	Function
TaRAN1	Cell division and auxin signaling pathway
RHD3	Cell expansion during root epidermis development and root tip growth
RTH1	Root elongation but not initiation
RTH3	Root hair elongation
CRL1	Crown- and lateral roots initiation, regulation by auxin
OsCKI1	Auxin metabolism
OsCSLD1	Elongation of root hairs
SLR1-1	Gibberellin regulation, initiation and elongation of roots

In the wild-type Golden Promise, genes could be divided into 2 groups based on their profil of expression during the first five days after germination. The 1st group is characterized by genes whose expression varies during the time of experiment; it includes *HvRAN1*, *HvRHD3* and *HvCKI1*. Interestingly, *HvRAN1* and *HvCKI1* are both part of the auxin response. In wheat RAN1 is involved in regulation of cell division; in rice and Arabidopsis, it alters primordial meristem, mitotic progress and sensitivity (Wang *et al.*, 2006). Its overexpression in rice demonstrated its involvement in meristem cell proliferation in the root (Xu and Cai, 2014). *HvCKI1* encodes a casein kinase I with a nuclear localization in rice, suggesting a role in the control of gene expression. Transgenic OsCKI1-deficient rice plants showed abnormal root development, including fewer lateral and crown-roots, and shortened primary roots as a result of reduced cell elongation. OsCKI1 was described to act in part through the regulation of auxin metabolism or by mediating the interplay between auxin, brassinosteroids and abscisic acid (Liu *et al.*, 2003). Finally, *RHD3* encodes a protein with GTP-binding motifs. RHD3 plays an essential role in cell wall biosynthesis and actin organization which are both known to be important for cell expansion. Its mutation resulted in

wheat in an abnormal phenotype of the root (Shan *et al*., 2005). In this regard, we can explain their role during initiation of crown-root primordia. As already mentioned, auxin is the signal that initiates the initiation of crown-root primordia (Yamamoto *et al.*, 2007). The first signal leading to the local change in auxin is unknown, but we can assume that after auxin perception, both *HvRAN1* and *HvCKI1* are stimulated in order to regulate the expression of genes involved in the process, notably cell division. The concomitant accumulation of *HvRHD3* transcripts might reflect the need of cell wall organization during cell division.

The 2nd group is characterized by genes whose expression increases over the time of experiment. It includes *HvRTH3* and *HvCSLD1*. *HvRTH3* gene encodes a COBRA-like protein that is unique to monocots. Most COBRA-like proteins contain a predicted plant-specific glycosylphosphatidylinositol (GPI) anchoring site which is connected through an amino acid designated ω to GPI anchors. COBRA-like proteins follow a GPI secretion pathway and are found in Golgi vesicles and at the outer face of the cell wall. COBRA-like proteins are involved in various types of the cell expansion and cell wall biosynthesis. The mutation of *RTH3* in maize affects root hair morphology: the mutant initiated root hair primordia but failed to elongate them properly (Hochholdinger *et al*., 2008). *OsCSLD1*, a *cellulose synthase-like D1* gene is required for root hair elongation but not initiation in rice (Kim *et al*., 2007). To summarize, both genes are involved in the process of root elongation. It is thus logical to observe that their expression increase during the time of experiment, i.e. meanwhile the crown-root primordium elongates until it will emerge from the stem.

The barley orthologue of OsCRL1/ZmRTCS is known in rice and maize, respectively, to play a pivotal role in crown-root initiation, integrating both auxin and cytokinin signal (Inukai *et al*., 2005, Majer *et al*., 2012). OsCRL1 belongs to the AS2/LOB domain protein and was characterized as transcription factor (Inukai *et al*., 2005; Husbands *et al*., 2007). The barley genome contains 16 genes encoding putative LBD proteins. They share a high degree of identity, rendering the identification of the CRL1/RTCS orthologue difficult. A first screen for the database gave a sequence homology for the CRL1-like. Nevertheless, later, the same search gave a better homology with CRL1-MLOC10784, whose expression in analyzed tissue was very low, making this EST barely detectable (data not shown). *CRL1-like* is orthologue to the maize *ramosa2*, which functions in the patterning of stem cells in axillary meristems (Bortiri *et al*., 2006). In our conditions, we could observe a strong accumulation of the *CRL1-like*/*ramosa2* transcripts in 2DAG-seedlings. This is not surprising but does not link it to the initiation of crown-root primordia. Indeed, the crown is not a well define organ, rather the junction between the roots and the shoot. In this region develop also the shoot meristems.

Histological observations of the crown at different time after germination showed that shoot meristems form also very early after seed germination (not shown). Consequently, the high accumulation of *CRL1-like/ramosa2* transcript might be related to the initiation of shoot meristems.

6.3 Hormonal control of crown-root initiation and development in barley

The barley transgenic line overexpressing the cytosolic form of the *AtCKX1* gene offers the possibility to indirectly determine the role of CK in the process of crown-root initiation and development. The root-specific expression of the transgene resulted in plants with altered endogenous CK content, higher root numbers and longer root system. The analysis of expression of all genes investigated in this study did not give satisfactory results in the fact that a strong variability was observed between the different biological replicates.

Nevertheless, trends might be determined. Concerning the CRL1-like/ramosa2, we observed that its pattern of expression did not change compare to the wild-type. Indeed the peak of expression at 2DAG was also detected in the transgenic lines. As discussed above, we can assume that *CRL1-like/ramosa2* is involved in the initiation and development of shoot meristem which might be independent from the endogenous CK content. The genes whose expression followed "a wavy pattern" (*HvRAN1*, *HvRHD3* and *HvCKI1*) in the wild-type obeyed also a "wavy pattern" in the transgenic line but earlier in the time. As already discussed, these genes play an important role in the auxin signaling pathway which leads to the initiation of crown-root primordia. An earlier stimulation of their transcription indicated that the initiation of crown-root primordia arose earlier in the transgenic line compared to the wild-type.

The endogenous phytohormon auxin is essential for crown-root development (Yamamoto *et al.*, 2007) because promotes interaction between repressor protein and ubiquitin ligase complex which frees the transcriptional regulators and allows them to bind *CRL1* gene (Coudert *et al.*, 2010). In a simple experiment based on growth of seedlings in hydroponics in the presence of different concentration of NAA, we demonstrated that auxin stimulates the production of crown-root also in barley. The response was found to be dose-dependent with a maximum with 100 nM/1 µM NAA. Interestingly, this experiment showed the dual role of IAA: increasing the crown-root number but inhibiting the overall growth of the roots. It has also been reported that genes involved in the auxin signaling pathway function in the root development (Inukai *et al.*, 2005). We investigated the potential

regulation of candidate genes by auxin using real-time qRT-PCR. Our results suggested that all investigated genes are induced by auxin and thus could be involved in auxin signaling pathway.

To complete the information about auxin metabolism in barley plant we quantified auxin levels in shoots, crowns and roots of wild type and transgenic barley. We found that TRP is highly accumulated both in roots and shoots, but to a greater extend in shoots (4-fold compared to roots) which is in agreement with the fact that auxin is known to be synthesized in aerial part of the plant, especially the young developing leaves (Ljung *et al*., 2001). Content of IAA intermediate TRA was higher than content of IPyA which is supposed to be the main route of IAA synthesis in diverse plants. This is not surprising as TRA is not only precursor for IAA, but also for indole alkaloid/serotonin in different plant species (Mano and Nemoto, 2012). Endogenous IAA content was more abundant in the roots of the transgenic line compare to roots of wild type. In this transgenic line, the *CKX* gene is overexpressed in a root-specific manner, leading to the decrease of free bases (*t*Z, *c*Z and iP) in this organ (Pospisilova *et al*., 2016). The antagonism between cytokinin and auxin is known for several physiological processes (Aloni *et al*., 2006). Plants overexpressing *CKX* genes have larger root system, due to the suppression of CK which act as repressor of root system. Furthermore, CK modulates auxin transport through decreasing the expression of *PIN1* and *PIN3* in the *Arabidopsis* root meristem (Ruzicka *et al*., 2009). We might hypothesize that in the cytAtCKX1 transgenic lines, the reduction of CK in the root releases the CK-mediated inhibition of *PIN* transcription, leading to a highly active auxin transport and its consequent accumulation in the roots. In opposite, endogenous IAA content was lower in the shoot of transgenic seedlings compared to wild-type. In this mutant, we can assume that the amount of CKs moving upward through phloem to the aerial part is lower than in the wild-type. It was reported that in reducing CK levels, either by induction of CKX or inhibition/mutation of the *ISOPENTENYL TRANSFERASE* gene, resulted in the reduction in auxin biosynthesis. Also, CK modifies the abundance of transcripts for auxin biosynthetic genes (Jones *et al*., 2010). The regulation of auxin levels by *de novo* synthesis is one important homeostatic mechanism operating in plants, but the levels of IAA can also be attenuated by conjugation and by degradation. We found no differences between levels of degradation products between wild type and mutant plant indicating that degradation (enhanced, reduced) did not caused differences between IAA contents.

Strigolactones were revealed as new hormonal players in the control of shoot branching or of tillering, an important agronomic trait in the grasses (Vallabhaneni *et al*.,

2010). A recent study showed that SLs and their derivatives play a key role in the modulation of root development (Ruyter-Spira *et al*., 2011). The biosynthetic pathway of the SLs is still not completed; nevertheless 3 genes have been identified: i) D27 which encodes a trans/cis-carotene isomerase, responsible for the first committed step of SL synthesis and producing 9-*cis*-β-carotene (Lin *et al*., 2009; Alder *et al*., 2012), ii) CCD7 is a carotenoid cleavage dioxygenase that cleaves 9-*cis*-β-carotene, producing one β-ionone and 9-*cis*-β-apo-10′-carotenal. The latter product can be further cleaved by CCD8, another carotenoid cleavage dioxygenase, to produce a strigolactone-like compound carlactone (Ruyter-Spira *et al*., 2012). From studies in *Arabidopsis*, pea and rice CCD7 and CCD8 were shown to be involved in the production of the SLs formed in roots. Also roots are the tissue where the highest levels of CCD7 and CCD8 were detected in maize (Vallabhaneni *et al*., 2010). Strigolactones inhibit adventitious root formation in *Arabidopsis* and pea (Sun *et al*., 2015); however, Arite *et al*. (2012) found in study that SLs positively regulate the length of crown-roots in rice. In Golden Promise, the expression of D27 slowly increased during the time course of the experiment with a maximum at 5DAG. In the transgenic line, its expression was found to be "rhythmic" with a first maximum observed at 3DAG. No real significant difference in the expression of CCD7 could be observed neither during the time course of experiment, nor between genotypes. In our condition, the expression of CCD8 was very low, under the limit of acceptable detection. If one considers that SLs are positive regulator of crown-root elongation, the expression pattern of *D27* might correspond to the elongation of crown-root primordia in barley. When crown-root initiation was stimulated by IAA, we could observe an increase in expression of both genes. Our data were in agreement with studies on Arabidopsis demonstrating that IAA significantly stimulates the accumulation of *D27* and *MAX3*/*CCD7* transcripts (Hayward *et al*., 2009; Waters *et al*., 2012).

7. Conclusion

The aim of the current thesis was to study the molecular and hormonal control of initiation and development of crown-root in barley. The qPCR analysis identified 6 genes with a potential role in the process. A study of in situ localization would be valuable to confirm their role.

Based on our results we suggest that alteration of endogenous CK levels in roots leads to enhanced polar auxin transport (PAT) via upregulated PIN carriers and thus increase auxin levels in roots (Figure 18). Increased auxin levels in roots upregulate genes involved in root development and thus enlarge root system of transgenic plants.

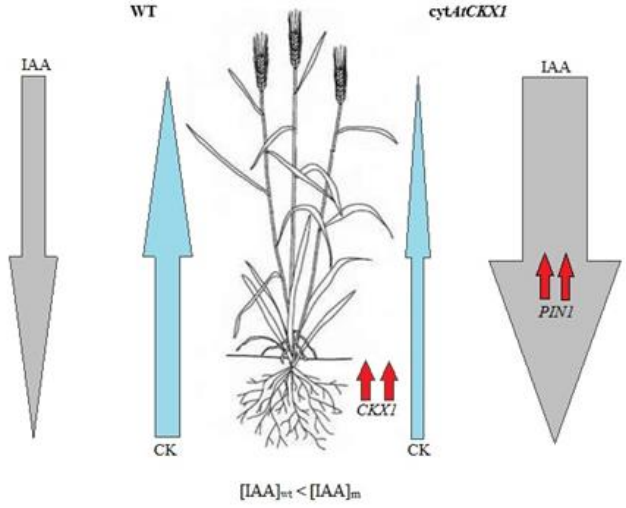

Figure 18. Auxin and cytokinin transport in wild type and transgenic barley. Grey arrow represents auxin transport, blue arrow represents cytokinin transport and red arrows represent upregulated genes.

8. Abbreviations

ABCB	ATP-Binding Casette group B
AFB	Auxin signaling F-Box
ARF	Auxin Response Factor
ARR	*Arabidopsis* Response Regulator
AS2	Asymetric Leaves 2
AUX1	AUXin resistence 1
Aux/IAA	Auxin/Indole-3-Acetic Acid
CCD	Carotenoid Cleavage Dioxygenase
cDNA	Complementary Deoxyribonucleic Acid
CK	Cytokinin
CKX	Cytokinin oxidase/dehydrogenase
CR	Crown-Root
CRL	Crown-Rootless
CV	Coefficient of Variance
DAG	Days After Germination
DAS	Days After Sowing
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide Triphosphate
dT	Deoxythimidine
FAO	Food and Agriculture Organization
GPI	Glycosylphosphatidylinositol
IAA	Indole-3-Acetic Acid
IAN	Indole-3-Acetonitrile
IAOx	Indole-3-Acetaldoxime
IPyA	Indole-3-Pyruvic Acid

LAX	Like-AUXin
LOB	Lateral Organ Boundaries
NAA	Naphthalene Acetic Acid
PAT	Polar Auxin Transport
PIN	PIN-formed
QC	Quiescent Centre
qRT-PCR	quantitative Reverse-Transcription Polymerase Chain Reaction
rpm	Round Per Minute
RAM	Root Apical Meristem
RNA	Ribonucleic Acid
ROX	6-carboxy-X-rhodamine
SCN	Stem-Cell Niche
SD	Standard Deviation
SEM	Standard Error of Mean
SG	Sybr Green
SL	Strigolactone
TIR1	Transport Inhibitor Response 1
TRA	Tryptamine
TRP	Tryptophan

9. References

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