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Department of Cell Biology and Genetics

VIGS as a tool for validation of plant gene function

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Martin Krejčí

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3. Příprava konstruktů pro umlčování genů.
4. Validace infekce a funkce konstruktů.
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Abstract — Virus-induced gene silencing has become an effective tool for plant functional genomics studies over the recent decades. However, in the past, virus induced gene silencing could be used only for dicot plants. This changed with the use of *Barley stripe mosaic virus* as a vector for gene silencing in monocots. The fungal pathogen *Blumeria graminis* is a causal agent of powdery mildew, which negatively affects the cultivation of agronomically important crops such as wheat and barley, every year. Virus-induced gene silencing finds a significant application in pathogen-host interaction studies, as in the case of *Blumeria graminis*. This thesis provides a protocol for virus-induced gene silencing that has been optimised for reliable use in future research. For this purpose, a modified *Barley stripe mosaic virus* vector was used to silence the gene encoding the phytoene desaturase, which is necessary for the synthesis of compounds that protect chlorophyll from photo-bleaching. Symptoms of photo-bleaching have appeared on leaves of infected wheat cultivars as the result of the inactivated gene.

Keywords	wheat, race-specific resistance, transformation, *in vitro* transcription, VIGS, BSMV
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Abstrakt

Virem indukované umlčování genů se za poslední desítky let stalo účinným nástrojem pro studování funkční genomiky rostlin. V minulosti mohlo být virem indukované umlčování genů uplatňováno pouze pro dvouděložné rostliny. To se změnilo s využitím *Barley stripe mosaic virus* jako vektoru, který umožnil použití této metody také u jednoděložných rostlin. Houbový patogen *Blumeria graminis* způsobuje padlí travní, které má každoročně negativní dopad na pěstování hospodářsky významných plodin, jako je pšenice a ječmen. Virem indukované umlčování genů nachází významné uplatnění při studiu interakcí mezi houbovými patogeny, jako je *Blumeria graminis*, a jejich hostiteli. Tato práce přináší protokol pro virem indukované umlčování genů, optimalizovaný tak, aby jej bylo možné spolehlivě využívat pro budoucí výzkum. Za tímto účelem byla pomocí upraveného *Barley stripe mosaic virus* vektoru přerušena funkce genu kódujícího fytoen desaturázu, která je nezbytná pro syntézu látek chránících chlorofyl před světelnou degradací. To mělo za následek vybělení listů u infikovaných kultivarů pšenice jako důsledek nefunkčnosti genu.

Klíčová slova	pšenice, rasově specifická rezistence, transformace, *in vitro* transkripce, VIGS, BSMV

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Prohlašuji, že jsem bakalářskou práci vypracoval samostatně pod vedením Mgr. Macieje Majky, Ph.D. a za použití citované literatury.

V Olomouci 29. 4. 2023   Martin Krejčí

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LIST OF SYMBOLS AND SHORTCUTS

AVR	avirulence
BAC	bacterial artificial chromosome
BMV	*Brome mosaic virus*
bp	base pairs
BSMV	*Barley stripe mosaic virus*
CP	coat protein
CSEP	candidate secreted effector protein
ETI	effector-triggered immunity
FoMV	*Foxtail mosaic virus*
HIGS	host-induced gene silencing
HR	hypersensitive response
chv	chromosomal virulence gene
LRR	leucine-rich repeats
NB	N-terminus binding site
NLR	gene with the N-terminus binding site and leucine rich repeats
NTP/CAP	mix of nucleotides producing 7-methyl guanosine cap 5'ends RNA
ORF	open reading frame
PAMP	pathogen-associated molecular patterns
pBS-BSMV	plasmid bearing the subunit of BSMV
PDS	phytoen desaturase
PRR	pattern recognition receptor
PTGS	post-transcriptional gene silencing
PTI	PAMP-triggered immunity
R	resistance
RISC	RNA-induced silencing complex
TGB	triple gene block
Ti	tumor-inducing
VIGS	virus-induced gene silencing
Vir	virulence gene
VOX	virus-mediated gene overexpression
WSMV	*Wheat streak mosaic virus*
Y/FxC	motif in *Blumeria graminis* candidate effector proteins

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1   INTRODUCTION

Species from the *Poaceae* family, such as wheat, barley, or rye, are one of the most agronomically important plants worldwide. However, the yield of these crops is being significantly reduced each year by pathogens. Powdery mildew, caused by the fungus *Blumeria graminis*, is one of the most devastating diseases globally. During the infection, the pathogen interacts with its host on many different levels. Functional genomics studies, such as virus-induced gene silencing (VIGS), are adding to the body of knowledge to better understanding of this complex interaction and help to find better solutions to the challenges surrounding this topic. The most widely used viral vector for VIGS in monocots is *Barley stripe mosaic virus* (BSMV), which has a broad experimental host range including wheat (*Triticum aestivum*). Reliable protocols are essential for the successful utilization of BSMV-VIGS. The main objective of this thesis was to optimise the protocol of VIGS, by *in vitro* transcription of BSMV and inoculation of wheat plants with functional RNAs.

2 OBJECTIVES OF WORK

The aim of the theoretical part of this thesis was to write a literature review on the topic of powdery mildew, host-pathogen interactions and methods of transformation with emphasis on virus-induced gene silencing and *Barley stripe mosaic virus*. The objective of the practical part of this thesis was to optimise the protocol of VIGS, by *in vitro* transcription of BSMV, for silencing of the phytoene desaturase gene in selected cultivars of wheat.

3 LITERATURE REVIEW

3.1 Powdery mildew (*Blumeria graminis*)

Powdery mildew is one of the most devastating diseases worldwide (Savary *et al.*, 2019). The causal agent of powdery mildew is the ascomycete fungus *Blumeria graminis* (Order Erysiphales, Family: Erysiphaceae), which can be divided into eight distinct host forms ('*formae speciales*', f. sp.) that are highly specialised to infect particular host species (Vendelbo *et al.*, 2021). In terms of cultivated crops, the most important host forms of *B. graminis* are f. sp. *tritici*, *hordei*, *avenae*, and *secalis* which cause powdery mildew of wheat, barley, oat, and rye, respectively. The other four f. sp. of *B. graminis* infect the wild grasses (Wyand *et* Brown, 2003). Plants infected by *B. graminis* have white spots or white cover of mycelium and conidia on the leaves (Wegulo, 1997). *B. graminis* also forms a haustorium, which absorbs nutrients from the host for its further growth (Zhang *et al.*, 2005). Populations of this pathogen are highly dynamic and constantly change their virulence structure (Glawe, 2008). In Western Europe, yield losses due to powdery mildew are typically around 10% (Jørgensen *et al.*, 2014), but can be significantly higher reaching over 30%. For example, yield losses of 30–35% in Russia, up to 62% in Brazil, and 30–40% in China have been reported during severe epidemics. In general, yield losses exceeding 40% are rare (Singh *et al.*, 2016).

The following section describes the mechanism of infection and reproduction of *B. graminis* f. sp. *hordei* as this is the best-studied member of this group and is used as a model organism to study the biology of the powdery mildew (Both *et* Spanu, 2004).

3.1.1 Infection of the host

B. graminis initiates its infection on the living epidermal cells of the leaf (Zhang *et al.*, 2005). The airborne asexual spores (conidia) or the sexual airborne spores (ascospores) are blown onto the leaf. The infection continues with germination of the spore and the formation of the primary germ tube (PGT) and an appressorial germ tube approximately 1-2 and 4-8 hours after infection (Thordal-Christensen *et al.*, 2000). This differentiates into apical appressorium from which the fungus attempts to penetrate the host cell wall (Haugaard *et al.*, 2002). If successful, a haustorium is formed. Nutrients absorbed by the haustorium fuel the ramification of ectopic secondary hyphae, the production of numerous secondary haustoria in other cells, and sporulation (Zhang *et al.*, 2005). Host responses and nutrient transfer determine whether a successful parasitic relationship develops and is maintained (Glawe, 2008; Panstruga *et* Dodds, 2009). During the infection, *B. graminis* causes a gradual decline in the rate of photosynthesis and premature loss of chlorophyll in infected parts of the host, while making carbohydrates and amino acids available to the fungus in the process (Both *et* Spanu, 2004).

3.1.2 Asexual reproduction of *B. graminis*

After infection of the host, hyphae elongate and branch repeatedly, forming circular colonies. As the hyphae on the outer surface of the leaves grow, they produce more haustoria in the epidermal cells underneath the colony (Both *et* Spanu, 2004).

The conidiophores, which are asexual reproductive structures, are produced from the somatic (assimilative) hyphae (Glawe, 2008). Conidial production typically begins within several days after infection of the host. Mature conidia are abstracted from the apex of the conidiophores and are readily dispersed by wind to infect other plants (Moriura *et al.*, 2006). The dispersion itself is significantly influenced by climatic conditions, as the pathogen has no active internal release mechanism (Hammet *et* Manners, 1971). These airborne conidia can be dispersed over long distances and do not need water to germinate. Hermansen *et al.* (1978) claim, that airborne conidia of *B. graminis* have travelled from the British Isles to infect plants in Denmark, which means that the spores have travelled about 700 km (Glawe, 2008). Because of the high number of propagules produced and easy dispersion, asexual reproduction is very important for the propagation of the species. Furthermore, the asexual cycle is usually repeated several times during the season (Moriura *et al.*, 2006).

3.1.3   Sexual reproduction of *B. graminis*

The sexual cycle starts when the ascospores, the sexual spores of *B. graminis*, infect the host (Koltin *et* Kenneth, 1970; Götz *et* Boyle, 1998). Germination of the ascospores is initiated and influenced by moisture (Koltin *et* Kenneth, 1970). Germinated *B. graminis* ascospores produce only a single germ tube type, therefore the ascosporic and the conidial germination patterns are different in *B. graminis* (Jankovics *et al.*, 2015). Reproduction is initiated by the production of male (antheridia) and female (askogonia) gametangia, followed by their cytoplasmic connection in a process referred to as dikaryotization, resulting in a cell consisting of two nuclei called dikaryon (Glawe, 2008). The sexual cycle ultimately results in the formation of chasmothecia (cleistothecia), which can survive an inclement environment (such as drought and low and high temperatures) without a host and produce ascospores when conditions are favourable (Both *et* Spanu, 2004). At later developmental stages, the generative mycelium progressively becomes more independent of the nutrition supply from the host (Götz *et* Boyle, 1998). Chasmothecia themselves do not contain mature ascospores, only asci filled with protoplasm. Ascospore development is induced by moist conditions as described above and is a rapid process (Turner, 1956; Both *et* Spanu, 2004). In addition to their role in survival, the sexual structures allow the fungus to undergo recombination, evolve rapidly and adapt swiftly to strong selection forces (Both *et* Spanu, 2004). On the contrary to this, Jankovics *et al.* (2015) pointed out that chasmothecia and ascospores are probably more important as survival structures under adverse conditions than genetic recombination factors in the life cycle of this pathogen. The study of ascosporic infection and sexual life cycle of *B. graminis* can be problematic in some cases, because colonies originating from ascosporic infections can quickly start sporulating in an asexual way and distinguishing these colonies from those initiated by conidia is practically impossible (Jankovics *et al.*, 2015).

3.2   Pathogen-host interaction

The interaction between a pathogen and its host does not always lead to a successful infection, because the plant is not completely defenceless (Thordal-Christensen *et al.*, 2000; Sela *et al.*, 2014; Vendelbo *et al.*, 2021). A highly specialised form of parasitism, as in the case of *B. graminis* and many other pathogens, requires specific genes to overcome the host's

immune responses and achieve successful infection. These genes are referred to as virulence genes and employ a set of secreted effector proteins. Effector proteins interfere with the host's immune system and facilitate colonisation and retrieval of nutrients from the host (Kloppe *et al.*, 2022). The effector proteins delivered by the pathogen can then be recognised by the matching resistance (R) gene. This recognition leads to the HR response in the plant, often resulting in localised cell death and disease resistance (Nowara *et al.*, 2010). Virulence genes that encode proteins which interact with R-genes in the host are called avirulence (AVR) genes. In this gene-for-gene interaction, both AVR genes in the pathogen and the corresponding R-genes must be present to initiate the host response (Dangl *et* Jones, 2001; Jones *et* Dangl, 2006; Nowara *et al.*, 2010).

3.2.1 PAMP and R-genes

Resistance can generally be divided into two types – horizontal resistance and vertical resistance (Van der Plank, 1968). Horizontal resistance is controlled by many genes and is independent on the pathogen. It is usually more stable and durable compared to vertical resistance (Robinson, 1973) and provides defence against all potential pathogens (Rouxel *et* Balesdent, 2010). This line of defence uses transmembrane pattern recognition receptors (PRRs) that respond to features termed as pathogen-associated molecular patterns - PAMPs. Recognition of PAMPs by PRRs results in PAMP-triggered immunity (PTI), which can halt further colonisation by the pathogen (Jones *et* Dangl, 2006). It is generally accepted that the resistance manifested at the early penetration stage is largely race-non-specific (Thordal-Christensen *et al.*, 2000).

On the other hand, vertical resistance is the resistance of plants against pathogens that is controlled by a single gene, and there are also single genes for parasitic ability in the parasite. This is a very important phenomenon known as gene-for-gene relationship (Flor, 1971). It is based on the specific interactions between pathogen AVR (avirulence) gene loci and alleles of the corresponding plant disease resistance (R) locus. In this process, a given effector, termed as avirulence (AVR) protein, is directly or indirectly recognised by a particular plant receptor in a highly specific manner and Effector-Triggered Immunity (ETI) is induced (Fabro *et* Alvarez, 2012). The largest class of R genes encodes a 'nucleotide-binding site at the N-terminus plus a leucine-rich repeat at the C-terminus' (NB-LRR) (Dangl *et* Jones, 2001). Pathogen effectors are recognised by NB-LRR proteins, which activate defence responses. This resistance is effective against pathogens that can grow only on living host tissue, such as *B. graminis* (Jones *et* Dangl, 2006). Vertical resistance is effective only against some races of the pathogen and is therefore highly specific (Van der Plank, 1968).

3.2.2 Race-specific resistance

As described above, race-specific resistance occurs when the R-gene is present in the host and the pathogen has the corresponding AVR gene. Race-specific resistance often triggers a hypersensitive response (HR) mediated by this gene-for-gene interaction (Prats *et al.*, 2005). HR response leads to the accumulation of hydrogen peroxide, nitric oxide, and phenolic compounds in the HR cell. This is followed by cellular collapse, which ultimately results in controlled cell death in purpose of arresting the powdery mildew fungus spread (Thordal-Cristensen *et al.*, 2000; Prats *et al.*, 2005). It is a defence mechanism that can terminate

germlings which have successfully penetrated the papillae. In some cases, a single-cell HR response is triggered rapidly after penetration. In other cases, HR may occur in response to secondary or subsequent penetrations (Thordal-Christensen *et al.*, 2000).

In barley, alleles at the *Mla* locus confer race-specific resistance against *B. graminis* f. sp. *hordei*. The first isolated *Mla* gene is *Mla*-1, which belongs to the NB-LRR class. When avirulent isolates attack *Mla*1 barley, HR occurs rapidly, leading to death and collapse of the attacked single epidermal cell (Prats *et al.*, 2005). In wheat, similar race-specific resistance against *B. graminis* f. sp. *tritici* is conferred by alleles at the *Pm* loci. At present, *Pm1 ~ Pm68* has been named as powdery mildew resistance genes, but only a few *Pm* genes have been cloned, e.g., *Pm2*, *Pm3*, or *Pm60* (Wang *et al.*, 2022). The co-evolution between the host and the pathogen means that single major R genes are often rapidly overcome in lines with widespread cultivation, and the race-specific resistance is short-lived. Therefore, breeders are constantly challenged to broaden the resistance resources in their breeding programmes (Brunner *et al.*, 2011). Another possible solution is to transfer several resistance genes to common wheat *Triticum aestivum* from its wild relatives (Tyrka *et* Chelkowski, 2004; Brunner *et al.*, 2011; Jia *et al.*, 2020).

One of the examples is the *QPm.tut-4A* locus introgressed into chromosome arm 4AL of *T. aestivum* cv. Tähti from *T. militinae*, which confers resistance to powdery mildew at both seedling and adult plant stages. When this segment is present in the host, the resistance reduces the number of secondary haustoria formed by the pathogen and promotes host cell apoptosis (Janáková *et al.*, 2019). However, the gene or genes present within *QPm.tut-4A* act in a race non-specific manner (Jakobson *et al.*, 2012).

3.2.3 *Mlo*-mediated resistance

The identification of *Mlo*-mediated resistance in the 1930s and 1940s was a major discovery in the fight against a variety of powdery mildews that infect many species, including crops. The resistance is conferred by loss-of-function of the *Mildew resistance locus o* (*Mlo*) gene (Jørgensen *et* Wolfe, 1994). In contrast to (R) gene-mediated resistance, *mlo*-based resistance is non-race specific and is recessively inherited. *Mlo*-mediated resistance is effective against the vast majority of powdery mildew isolates and is considered to be very durable (Brown, 2015; Kush *et* Panstruga, 2017). Barley *mlo*-mediated resistance is characterised by several distinctive features. In *mlo* mutants, the number and diameter of papillae formed at attempted penetration sites are increased. In addition, the accumulation of defence-associated compounds such as p-coumaroyl-hydroxyagmatine and hydrogen peroxide is stronger and faster in *mlo* null mutants (Kush *et* Panstruga, 2017).

The phenotype of *mlo*-resistant barley lines is characterised by the occurrence of occasional mildew colonies on the leaves and the tendency to necrotic and chlorotic leaf spotting, which is a pleiotropic effect of the *mlo* genes (Jørgensen *et* Wolfe, 1994). These pleiotropic effects may also affect yield through reduced grain size. The *mlo* genes do not interact with other mildew resistance genes in the plant. Therefore, *Mlo* genes can be combined with other resistance genes to produce multi-resistant plants. Due to the introgression of *mlo* resistance into a broad panel of barley varieties, more than half of the commercially grown spring barley varieties in Central Europe are largely immune to powdery mildew (Jørgensen, 1992).

Although *mlo* has recently been introgressed into a few winter barley lines, the use of *mlo* in winter barley is not recommended because it may result in year-round pathogen survival (Kush *et* Panstruga, 2017).

3.2.4 Avirulence genes in *B. graminis*

The potential proteins of avirulence in the *B. graminis* genome are referred to as candidate secreted effector proteins (CSEPs). Isolate-specific variants of these powdery mildew CSEPs are recognised by matching intracellular immune receptors (Frantzeskakis *et al.*, 2018). Some of the identified avirulence genes include, for example, *AvrPm3$^{a2/f2}$*, *AvrPm3$^{b2/c2}$*, and *AvrPm3^{d3}* which are recognised by *Pm3a/Pm3f*, *Pm3b/Pm3c*, and *Pm3d*, respectively (Müller *et al.*, 2022). The cloning and functional characterisation of AVR genes by several authors (Praz *et al.*, 2017; Bourras *et al.*, 2019; Müller *et al.*, 2022) has led to the conviction that AVR effectors have highly divergent amino acid sequences. However, there are several patterns that are similar to most of the already characterised AVR proteins. *Blumeria* AVR effectors are small proteins (102 to 130 amino acids) that contain an N-terminal signal peptide, a largely conserved Y/FxC motif, and a conserved cysteine residue towards the C-terminus (Müller *et al.*, 2022). Most of the avirulence proteins in barley and wheat, that were characterised in the last decade, also exhibit a ribonuclease-like fold (Praz *et al.*, 2016).

3.2.5 Identification of avirulence and resistance genes

The ongoing co-evolution of *B. graminis* and its hosts is one of the reasons why it is important to focus not only on R-genes in plants, but also on AVR-genes in the pathogen. The identification and functional characterisation of powdery mildew avirulence genes have significantly broadened our understanding of race-specific resistance and resistance gene breakdown in the wheat–mildew pathosystem (Müller *et al.*, 2022). The ability of *B. graminis* to keep a delicate balance between maintaining virulence and avoiding host recognition is probably based on a rapid evolutionary turnover of effector genes (Müller *et al.*, 2019).

There are several possible methods to test the pathogen AVR candidates. One of them is to test cell death mediated by matching NLR/AVR pairs. It is possible to deliver pathogen effectors into resistant hosts via the bacterial type-III secretion system. A commonly used alternative to this is the *in planta* co-expression of AVR and matching NLR genes. To determine AVR-dependent NLR activation, transgenic plants expressing pathogen effectors are crossed with plants encoding matching NLR resistance. In successful crosses, cell death is then observed, usually in the form of seedling lethality.

The main limitations of this method are the time required (several months) and the difficulty of generating stable transgenic cereal plants (Saur *et al.*, 2019).

Another possible way to identify avirulence genes is to use sequencing methods. For this purpose, BAC (bacterial artificial chromosome) sequencing can be used. BAC sequencing is based on cloning DNA sequences into bacterial cells to amplify the inserted DNA. BAC DNA can then be isolated and sequenced, which is followed by data analysis and gene annotation (Zhang *et* Wu, 2001; Praz *et al.*, 2016; Bourras *et al.*, 2019). Another possible way is to sequence the proteins directly isolated from the extracellular fluids of the infected plant.

Isolated proteins can then be sequenced using Edman degradation or tandem mass spectrometry (Rep, 2005).

3.3 Methods of transformation

Plant genetic transformation offers an important tool in breeding programmes by producing novel and genetically diverse plant material. Genetic transformation is a term used to describe the directed transfer of a desired gene from one organism to another and the subsequent stable integration and expression of this foreign gene (Keshavareddy *et al.*, 2018). The desired gene that is transferred to another plant is known as transgene, and organisms successfully altered by genetic transformation are known as transgenics (Cheng *et al.*, 1997). There are several DNA delivery methods available for the production of transgenic plants. Delivery methods can generally be divided into two classes of delivery systems: non-biological (direct) and biological (indirect) (Keshavareddy *et al.*, 2018). Well-known and widely used biological methods are *Agrobacterium*-mediated plant transformation and transformation mediated by viral vectors. The most important non-biological method is particle bombardment/biolistics (Hensel *et al.*, 2010; Keshavareddy *et al.*, 2018).

3.3.1 *Agrobacterium*-mediated plant transformation

The era of plant transformation began in the early 1980s with the report of *Agrobacterium tumefaciens*-mediated gene delivery for the production of transgenic plants (Cheng *et al.*, 1997). The method exploits the natural ability of the bacterium *A. tumefaciens* to transform host plants. DNA transferred from *Agrobacterium* into the nucleus of plant cells is located in the region of a large tumor-inducing (Ti) plasmid. The transferred DNA (T-DNA) is called the T-region when it is located on the Ti plasmid and is approximately 10 to 30 kbp in size (Gelvin, 2003). Plant genetic transformation begins with the induction of the *Agrobacterium* virulence (*vir*) region by specific host signals. This induction ultimately leads to the release of the single-stranded T-DNA molecule. Chromosomal genes (*chv*) are essential for this process as they are responsible for T-DNA transport. Once inside the host cell cytoplasm, the T-strand presumably exists as a nucleoprotein complex and is integrated into the nucleus of the host cell (Gelvin, 2003; Tzfira *et al.*, 2004).

Agrobacterium-mediated transformation remains popular and is one of the most effective, especially in most dicotyledonous plants where *Agrobacterium* is naturally infectious (Keshavareddy *et al.*, 2018).

Monocotyledonous species are less easily infected by *Agrobacterium* spp. and it is less straightforward to regenerate shoots from somatic tissue *in vitro*. The generation of stably transgenic monocotyledonous plants has long been considered difficult (Hensel *et al.*, 2010).

However, reproducible and efficient methods have been established for example for rice, corn, wheat or barley (Hensel *et al.*, 2010; Keshavareddy *et al.*, 2018). Either tissue cultures or living plants (*in planta* method) can be used as transformation targets. For tissue cultures, immature embryos or embryogenic callus can be used. Transgenic plants are then regenerated from the

explants (Cheng *et al.*, 1997). *In planta* method eliminates tissue culture steps and relies on simple protocols (Tzfira *et al.*, 2004; Keshavareddy *et al.*, 2018).

3.3.2 Biolistic method

The biolistic method was first demonstrated in the late 1980s by Klein *et al*, 1987, and was described as a delivery of nucleic acids into plant cells using high-velocity microprojectiles. The term biolistics is derived from the words *biology* and *ballistics* and is a physical method of delivering DNA into plant tissues (Matsumoto *et* Gonsalves, 2012). In this method, the desired genetic material is precipitated onto micron-sized metal particles. These metal particles are then accelerated into target cells at speeds sufficient to penetrate the cell wall but not to cause lethal damage to the cell. The desired DNA is therefore transported into the cell where it detaches from the microprojectile and integrates into the nuclear or plastid genome (Taylor *et* Fauquet, 2002). Metal particles are usually made of tungsten or gold and embryogenic cell cultures are often used as targets for transformation due to their ability to recover and regenerate (Kikkert *et al.,* 2004).

The biolistic method is not dependent on host specificity or species limitation, such as *Agrobacterium*-mediated transformation. It has therefore been used successfully to produce transgenic plants in a wide range of different species. Nevertheless, the use of biolistics is not limited only to plants. Adaptations of this technology can be used to transfer DNA into bacteria, fungi, insects, and even mammals (Taylor *et* Fauquet, 2002; Matsumoto *et* Gonsalves, 2012). Disadvantages of biolistics are that the transformation efficiency may be lower than with *Agrobacterium*-mediated transformation and the device and consumables are expensive. Random integrations are also a notable concern. Furthermore, multiple copy insertions, that are often made during the process, can lead to gene silencing (Kikkert *et al.*, 2004).

3.4 Methods using viral vectors

These methods use specifically designed viral vectors to deliver foreign genes *in planta*. The use of viral vectors provides an efficient alternative to *Agrobacterium*-mediated transformation or biolistic method (Zaidi *et* Mansoor, 2017). The advantages include high copy number resulting in rapid production of the desired product and typically efficient expression in susceptible hosts. Other advantages include systemic spread due to autonomous replication of the viral vector and the ability to express genes at specific plant growth stages. However, expression is mainly transient and not stable. This means that there is usually no transmission to subsequent generations. The risk of transmission to other susceptible crops or wild hosts is another limitation when considering the use of viral vectors.

Applications of virus-based vectors include the production of human/animal therapeutic proteins in plant cells and the study of plant biochemical processes. Additionally, virus-induced gene silencing (VIGS) has been exploited as a powerful tool to study the functions of the host genes (Zaidi *et* Mansoor, 2017; Abrahamian *et al.*, 2020).

3.4.1 Virus-induced gene silencing (VIGS)

The term VIGS was first used by Van Kammen (1997) to describe the resistance event against viral infection in plants. This method is based on post-transcriptional gene silencing (PTGS), which is a sophisticated defence system in plants for the detection of exogenous and altered endogenous nucleic acids (Dhariwal *et al.*, 2022). VIGS is a powerful tool for plant functional genomic studies by rapidly generating gene knockdown phenotypes (Lu *et al* 2003; Scofield *et* Nelson, 2009). Originally, gene silencing in response to genetically manipulated RNA viral vectors was observed in *Nicotiana benthamiana* in the mid-1990s. Since then, VIGS has been reported in other dicot plant species such as tomato and *Arabidopsis*. More recently, VIGS has also been applied to monocot crop species such as barley, rice, wheat and maize. However, vector preparation is more complex for the study of monocots using VIGS (Becker *et* Lange, 2010).

The mechanism of VIGS is based on the exploit of RNA-mediated defence in plants. The plant is inoculated with a viral vector carrying an inserted fragment of a sequence from a gene of the host plant. The viral ssRNA replicates by processing dsRNAs intermediates, which are then found by dicer-like proteins. These dicer-like proteins cleave viral dsRNAs into short interfering RNAs (siRNAs), which are about 21-25 nucleotides in length. Newly made siRNAs are incorporated into the RNA-induced silencing complex (RISC). This complex targets and degrades specific mRNA transcripts that have sequence complementarity with the specific siRNA. This ultimately leads to the degradation of both the viral RNA and the transcript of the target gene, since a fragment of this gene was part of the viral vector (Lu *et al.*, 2003; Unver *et* Budak, 2009; Dhariwal *et al.*, 2022). The gene that was chosen to be silenced is referred to as the gene of interest or target gene (Unver *et* Budak, 2009).

VIGS has several advantages that make it useful for functional genomic studies. The experimental procedure is faster compared to stable transformation. It is not necessary to know full-length cDNA sequences for VIGS to work, which is particularly useful when full sequence information is lacking. Gene silencing is mainly transient and not stable. This can also be seen as an advantage because the loss-of-function phenotype affects only part of the plant and is usually not lethal, which is important for the evaluation of the gene function (Scofield *et* Nelson, 2009). Other advantages of VIGS are its reliability and the relatively low cost. In addition, since this method does not require plant transformation, it is particularly useful for plants that are difficult or impossible to transform (Unver *et* Budak, 2009).

The main limitation of VIGS is the lack of suitable vectors for different plant species (Scofield *et* Nelson, 2009). Another limitation is the fact that complete loss-of-function by VIGS might not be achieved (downregulation of expression is generally around 75-90%) and the low level of gene expression can be sufficient to produce a functional protein.

Another limitation of VIGS can also be the fact that the method relays on at least partial information about the gene sequence. Furthermore, symptoms of the viral infection may also mask the desired loss-of-function phenotype of the host (Unver *et* Budak, 2009). The most commonly used vectors for VIGS in cereals are *Barley stripe mosaic virus* (BSMV) and *Brome mosaic virus* (BMV) (Jackson *et al.*, 2009).

3.4.2 Host-induced gene silencing (HIGS)

HIGS has emerged as a powerful alternative to chemical treatments for crop protection. The potential of this technology has been demonstrated over the last two decades and it has been shown to be effective against a wide range of viruses, insects and fungi. Similarly to VIGS, HIGS uses RNA-mediated plant defence, but instead of silencing host genes, it silences pathogen genes. This method is based on the insertion of a dsRNA-producing transgene construct into plant cells. The construct carries the sequence of the pathogen gene and induces RNA-mediated defence. dsRNAs are processed by dicer-like proteins into siRNAs, which are then incorporated into RISC. This complex silences the pathogen genes that are homologous to the inserted sequence, resulting in plant resistance to the pathogen. It is possible to use HIGS to produce transgenic plants expressing antifungal RNA interference (RNAi) constructs. The main problems with chemical treatment against pathogens and pests are the loss of biodiversity and the increasing resistance of pathogens to the treatment. HIGS offers a pesticide-free alternative to chemical treatment and can therefore contribute to solving the challenges associated with this issue (Nowara *et al.*, 2010; Koch *et* Wassenegger, 2021).

3.4.3 Virus-mediated gene overexpression (VOX)

Virus-mediated overexpression is based on the transient expression of recombinant proteins *in planta* using plant virus vectors. Viral vectors used for VOX have been modified to rapidly produce foreign peptides and proteins at a high level. Viruses with positive-sense ssRNA genomes are mainly used as vectors. For cereals, *Barley stripe mosaic virus* (BSMV) and *Wheat streak mosaic virus* (WSMV) are the most commonly used (Lee *et al.*, 2015). There are several advantages to using plant virus expression vectors for this technology. Full-length vectors can spread systemically within a plant and are easily transmitted to new plants. Furthermore, massive-scale expression of the desired product is achieved within a few days and purification steps are simple. VOX has great potential for studying metabolic pathways, cellular protein localisation, or functional characterisation of resistance genes and effector proteins (Lee *et al.*, 2015).

3.4.4 Viral vectors

The discovery of post-transcriptional RNA silencing (PTGS) and the development of modern sequencing tools have led to the widespread use of viral vectors in functional genomic studies. Plant viruses with positive sense ssRNA are have been widely used for both VOX and VIGS, with each vector system having its advantages and limitations (Bouton *et al.*, 2018).

As mentioned above, the most widely used viral vector is *Barley stripe mosaic virus* (BSMV). More recently, *Brome mosaic virus* (BMV) VIGS system has been developed for rice, maize and barley. BMV has a tripartite genome and one of the broadest monocotyledonous host ranges among viruses. For years, BMV has been a useful model for studying the interactions of positive-strand RNA virus with host factors. The possibility of using BMV in VIGS systems was demonstrated by knocking down the *phytoene desaturase* (PDS) genes in maize and barley, and the actin and Rubisco activase genes in rice (Ding *et al.*, 2006). Another viral vector used in VIGS and VOX is *Foxtail mosaic virus* (FoMV).

The fact that FoMV has a monopartite genome is an advantage when compared to other VIGS systems. Another advantage of the FoMV-based vector is its mild symptoms (Bouton *et al.*, 2018).

3.4.4.1 Barley Stripe Mosaic Virus (BSMV)

The possibility of using BSMV as a vector for VIGS in monocots was first demonstrated by Holzberg *et al.* (2002) by silencing the PDS gene in barley. Since then, BSMV has become the most widely used VIGS vector in grasses (Scofield *et* Nelson, 2009). BSMV is a single-strand positive RNA virus of the genus *Hordeivirus*. It has a tripartite genome consisting of the *α* (3.8kb), *β* (3.3 kb) and *γ* (2.8kb) RNAs (Solovyev *et al.*, 1996) (Fig. 1), that are individually encapsidated as short, rigid rods composed of proteins and RNA. BSMV is easily transmitted by mechanical means from plant to plant or via seeds and has no known biological vectors. The main natural hosts of BSMV are two important monocot crops, wheat and barley. Upon infection, this virus causes mild to moderate mosaic symptoms on the leaves of most cultivars (Jackson *et al.*, 2009).
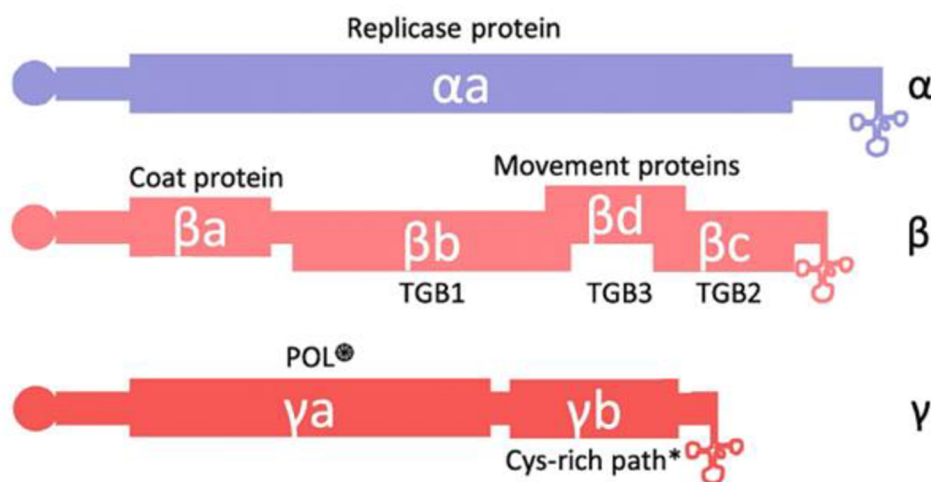
Figure 1: Genome organisation and genetic modifications of barley stripe mosaic virus (BSMV) (adapted from Dhariwal *et al.*, 2022).

α, β and γ represents the RNA subunits of BSMV. Full circles are representing the cap on the 5´end. Rectangular boxes are representing open reading frames (ORFs) for different proteins and enzymes. RNAα is coding a replicase protein, which is necessary for the replication of the virus. RNAβa is coding a coat protein, which can be removed from the genome. Triple gene blocks (TGB) are a multifunctional movement proteins. RNAγa is coding the polymerase component of replicase. RNAγb is a cysteine-rich viral pathogenicity region and can be modified for cloning target gene fragment. An t-RNA-like structure is present at the 3´end.

The popularity of BSMV-based VIGS has increased mainly due to the availability of full-length infectious BSMV clones and increasing knowledge of the molecular and biological functions of its genome components (Lee *et al.*, 2012). RNAα encodes a replicase protein containing methyltransferase and helicase domains. RNAβ encodes the coat protein (CP) and TGB movement proteins. The expression of the TGB movement proteins is mediated by two subgenomic (sg) RNAs, designated sgRNAβ1 and sgRNAβ2, while each of the TGB proteins is essential for local and systemic movement. RNAγ encodes the polymerase component of the replicase and a Cys-rich protein involved in viral pathogenicity. All three genomic RNAs are required for plant infection, but the RNAβ CP gene is not required for systemic infection (Holzberg *et al.*, 2002; Jackson *et al.*, 2009; Lee *et al.*, 2012).

In the laboratory, BSMV can be mechanically transmitted to many dicot and monocot species by rub inoculation. Several variants of BSMV-VIGS vectors are available. Most systems rely on the introduction of the foreign gene fragment into RNAγ using cloning sites downstream of the γb ORF. Another possibility is to introduce the foreign fragment into the RNAβ ORF (Lee *et al.*, 2015). It is also possible to use a BSMV vector carrying foreign fragments inserted into both in RNAβ and RNAγ cloning sites. This can be used to silence two unrelated genes in the host simultaneously (Kawalek *et al.*, 2012). BSMV vectors can be further modified by deletion of the gene encoding viral CP, which enhances silencing efficiency at the cost of increased severity of viral symptoms (Holzberg *et al.*, 2002).

BSMV vectors have several advantages. Unlike other VIGS vectors, BSMV can be transmitted by seed and pollen, allowing silencing at these stages of plant development. On top of that, BSMV has a wide experimental host range, and there is considerable potential for the application of VIGS (Lee *et al.*, 2015). The knowledge of BSMV genome components creates an opportunity to make new variations of the BSMV vectors, which can further improve the BSMV-based VIGS systems. Furthermore, it is also possible to use BSMV vectors for both HIGS and VOX systems (Lee *et al.*, 2012). The main disadvantage of BSMV vectors is that their genome consists of α, β, and γ RNAs, all of which need to be present in the same plant cell to initiate infection. Another limitation is the size of the inserted fragment. In general, inserts larger than 450-500 bp are relatively unstable and show reduced cell-to-cell and systemic spread in the plant. It is also worth mentioning that symptoms caused by BSMV infection can sometimes hinder the screening for the desired phenotype or interfere with the silencing results (Bouton *et al.*, 2018).

3.5 Transmission of viral constructs

Successful transmission of the viral construct into the desired host is a crucial step in all vector-based approaches. Each of the plant hosts presents challenges for the design and use of delivery methods to achieve local and systemic infection. One of the common methods of delivery is mechanical inoculation of purified plasmid DNAs or RNA transcripts. This method is relatively simple but has its limitations (Abrahamian *et al.*, 2020). Another widely used method is agroinoculation. Agroinoculation is based on the use of *A. tumefaciens* to efficiently introduce a DNA copy of the viral vector into the plant cell, where all the processes necessary to generate the functional vector take place. This approach is commonly used for *in planta* production of infectious vectors in *N. benthamiana* and secondary inoculation of the desired plants

with *N. benthamiana* leaf homogenate. In case of RNA viruses, agroinoculation also represents an inexpensive alternative to *in vitro* transcription (Gleba *et al.*, 2004; Dhariwal *et al.*, 2022).

3.5.1 *In vitro* transcription

In vitro transcription has been used in many studies and is still commonly used today. This method is based on the production of infectious RNA from linearised plasmid DNA using appropriate RNA polymerase (Abrahamian *et al.*, 2020). In the case of BSMV, at least three separate *in vitro* transcription reactions are required (for α, β and γ subunits), as BSMV has a tripartite genome. After the reaction, all three transcripts are mixed in a 1:1:1 ratio, diluted in a suitable buffer, and transferred directly to plant leaves by rub inoculation (Unver *et* Budak, 2009; Dhariwal *et al.*, 2022). Most plant species can be infected by mechanical inoculation of *in vitro* transcripts, which is an advantage, as not all plant species are susceptible to agroinoculation. The biggest limitation of this method is lower efficiency compared to alternative methods and a relatively high cost of the reagents needed (Gleba *et al.*, 2004; Lim *et al.*, 2010).

3.5.2 Abrasion of leaves

Leaf abrasion is necessary for successful inoculation of the plant, as the virus itself is not able to penetrate the cell wall. It is recommended to continue with inoculation right after successful *in vitro* transcription for better results. An inoculation mix consisting of a BSMV vector diluted in a suitable buffer (containing cell wall disruptors) is pipetted onto the upper (adaxial) surface of the leaf. Mechanical rub inoculation is performed by squeezing the leaf between two fingers and drawing the mixture over the surface in small circles, resulting in abrasion (Dhariwal *et al.*, 2022). Most protocols rely on the use of the leaf abrasion method for virus inoculation. The main advantages of this method are that it is fast and efficient. Limitations of this method include uneven distribution of gene silencing and very limited success in infection of the seed tissues (Cheuk *et* Houde, 2017). To overcome the limitations of the traditional abrasion method, Cheuk *et* Houde (2017) demonstrated a new procedure of BSMV inoculation – imbibition at the seed stage, which allows for more uniform gene expression in different plant tissues.

4 MATERIALS AND METHODS

4.1 Biological material

To produce the RNAs, the following plasmids (carried by *Escherichia coli* strain DH5α) were provided by Javier Sánchez-Martin (University of Zurich) and were stored as bacterial glycerol stock (-80°C):

- #1147: pBS-BSMV- α
- #1148: pBS-BSMV-β
- #1150: pBS-BSMV- γ (wild type)
- #1151: pBS-BSMV-γ-PDS

A mix of viral RNAs was inoculated into two wheat (*T. aestivum*) cultivars: Bobwhite and Fielder (both susceptible to BSMV).

4.1.1 Plant growth and conditions

Seeds were sown seven days before the inoculation procedure into black pots (12,5x12,5x13) filled with a good quality substrate (Terrasan, cat. no. 18808530) and were grown in a phytotron with a16h day/8h night cycle with 60% humidity. The temperature was stable at 23°C during the day and 16°C during the night.

4.2 Used chemicals, kits and solutions

Chemicals used

- Agarose wide range low melting (200 bp - 25 kb) (VWR, cat. no. 444152G, Radnor, USA)
- Ampicillin (Serva, cat. no. 1339701, Heidelberg, Germany)
- Ethanol 70% and 96%
- Ethidium bromide (Sigma-Aldrich, cat. no. 46067, St. Louis, USA)
- Isopropanol (Sigma-Aldrich, cat. no. 67-63-0, St. Louis, USA)
- Molecular weight marker GeneRuler 1kb plus (Invitrogen, cat. no. 10787018, Waltham, USA)
- Gel loading dye, purple 6x (New England Biolabs, cat. no. B7024, Ipswich, USA)
- Nuclease-Free Water, for Molecular Biology (Sigma-Aldrich, cat. no. W4502-1L, St. Louis, USA)
- Restriction enzyme BssHII 5 U/µl (New England Biolabs, cat. no. R0199S, Ipswich, USA)
- Restriction enzyme BtgI (10 U/µl) (New England Biolabs, cat. no. R0608S, Ipswich, USA)
- Restriction enzyme ClaI (10 U/µl) (New England Biolabs, cat. no. R0197S, Ipswich, USA)
- Restriction enzyme HincII (10 U/µl) (New England Biolabs, cat. no. R0103S, Ipswich, USA)
- Restriction enzyme MluI-HF (20 U/µl) (New England Biolabs, cat. no. R3198S, Ipswich, USA)

- o Restriction enzyme NdeI (20 U/μl) (New England Biolabs, cat. no. R0111S, Ipswich, USA)
- o Restriction enzyme NsiI (20 U/μl) (New England Biolabs, cat. no. R3127S, Ipswich, USA)
- o Restriction enzyme PacI (10 U/μl) (New England Biolabs, cat. no. R0547S, Ipswich, USA)
- o Restriction enzyme PstI-HF (20 U/μl) (New England Biolabs, cat. no. R3140S Ipswich, USA)
- o Restriction enzyme PvuI-HF (20 U/μl) (New England Biolabs, cat. no. R3150S Ipswich, USA)
- o Restriction enzyme PvuII-HF (20 U/μl) (New England Biolabs, cat. no. R3151S, Ipswich, USA)
- o Restriction enzyme SpeI-HF (10 U/μl) (New England Biolabs, cat. no. R3133S, Ipswich, USA)
- o Restriction enzyme StyI-HF (20 U/μl) (New England Biolabs, cat. no. R3500S Ipswich, USA)
- o Restriction enzyme XbaI (20 U/μl) (New England Biolabs, cat. no. R0145S, Ipswich, USA)

Kits used

- o Nucleobond Xtra Midi kit (Macherey-Nagel GmbH & Co. KG, cat. no. 740410.5, Düren, Germany)
- o mMessage mMachine T7 Transcription kit (Thermo Fisher Scientific, cat. no. AM1344, Waltham, USA)

Solutions used

- o 2YT medium
  - Kasein enzyme 1.6 g/100 ml
  - Yeast autolysate 1 g/100 ml
  - Sodium chloride 0.5 g/100 ml (Sigma-Aldrich; cat. no. S9888-500G, St. Louis, USA)
  - Agar 1.6 g/100 ml

- o GET buffer
  - 50 mmol/l glucose
  - 10 mmol/l EDTA (Sigma-Aldrich, St. Louis, USA)
  - 25 mmol/l Tris-HCl, pH 8 (Sigma-Aldrich, St. Louis, USA)

- o Neutralising solution (pH 4.8–5.3)
  - Potassium acetate 5 mol/l 60ml/100 ml
  - chilled Acetic acid 11.5 ml/100ml
  - ddH_2O 28.5 ml

- o 3M sodiumacetate (pH 5.2)
  - Sodium Acetate 61.52 g/100ml (Sigma-Aldrich, cat. no. S2889-1KG, St. Louis, USA)
  - Acetic acid (99%) to adjust pH
  - ddH$_2$O 250 ml
  - add 250 μl DEPC (Sigma, cat. no. D5758-25ML, St. Louis, USA)
  - Incubate at 37°C over night, autoclave to inactivate the DEPC

- o TElight (RNAse-free)
  - 0.01 ml 0,5M EDTA (final concentration 0.1 mM)
  - 0.5 ml 1M Tris/HCl (pH 8) (final concentration 10 mM)
  - Adjust to 50 ml with RNase-free ddH$_2$O (Sigma-Aldrich, cat. no. W4502-1L, St. Louis, USA)

- o 20× SB buffer (pH 8.0)
  - 800 ml ddH$_2$O
  - 8 g Sodium hydroxide (Sigma-Aldrich, cat. no. S5881-500G, St. Louis, USA)
  - 45 g Boric acid (H$_3$BO$_3$) to Ph ~ 8.0
  - Fill to 1L with ddH$_2$O (Sigma-Aldrich, W4502-1L, St. Louis, USA)

- o 10× GP buffer
  - 18.77 g Glycine (Roth, cat. no. HN07.2, Karlsruhe, Germany)
  - 26.13 g Dipotassium hydrogen phosphate (Merck, cat. no. 1051041000, Darmstadt, Germany)

- o FES buffer
  - 50 ml 10× GP buffer
  - 2.5 g Sodium Pyrophosphate Decahydrate (Sigma-Aldrich, cat. no. 221368-100G, St. Louis, USA)
  - 2.5 g Bentonite (Sigma-Aldrich, cat. no. 285234-500G, St. Louis, USA)
  - 2.5 g Celite 545 (Merck, cat. no. 1026930250, Darmstadt, Germany)
  - Fill up to 250 ml with water (Sigma-Aldrich, cat. no. W4502-1L, St. Louis, USA)
  - Autoclave

- o RNA cleaning solution
  - 0.1 M Sodium hydroxide and 1 mM EDTA
  - 4 g Sodium hydroxide (Sigma-Aldrich, cat. no. S5881-500G, St. Louis, USA)
  - 2 ml 0.5M EDTA

- o Viral cleaning solution
  - 2.5% Sodium hypochlorite (Mychem, cat. no. 1122-250, Zürich, Switzerland)

- o Alkaline SDS
  - • 0.2mol/l Sodium hydroxide (Sigma-Aldrich; St. Louis, USA)
  - • 1% SDS v/v (Sigma-Aldrich; St. Louis, USA)
  - • ddH$_2$O (Sigma-Aldrich, cat. no. W4502-1L, St. Louis, USA)

- o 5× TBE buffer
  - • 450 mmol/l Tris (Sigma-Aldrich; St. Louis, USA)
  - • 450 mmol/l Boric acid (Lach-Ner; Neratovice, Czech republic)
  - • 10 mmol/1 EDTA, pH 8.0 (Sigma-Aldrich; St. Louis, USA)

4.3 List of instruments and equipment
- o horizontal laminar box (Jouan; Thermo Fisher Scientific, Waltham, USA)
- o fridge ARDO (JP Industries, Italy)
- o shaker (Thermo Fisher Scientific, Waltham, USA)
- o centrifuge Heraeus Multifuge X1R (Thermo Fisher Scientific, Waltham, USA)
- o table vortex Reax Control (Heidolph Instruments, Germany)
- o centrifuge Mega Star 600R (VWR Collection; Leicestershire; Great Britain)
- o microwave (DAEWOO, KOR-6C2B, Korea)
- o microvolume UV-Vis Spectrophotometer NanoDrop™ One/OneC (Thermo Scientific, USA)
- o C1000 Touch Thermal Cycler (Bio-Rad, Hercules, USA)
- o Mini-Sub Cell GT Horizontal Electrophoresis System and PowerPac Basic Power Supply (Bio-Rad, Hercules, USA)
- o freezer (-80°C; ultrafreezer -80°C, Thermo Fisher Scientific, USA)
- o phytotron (Fytoscope, FS-SI-4600, Photon Systems Instruments, Czech Republic)
- o freezer (-20°C; Nord Line, Czech Republic)
- o UV transilluminator, InGenius system (Syngene; Bengaluru, India)
- o laboratory scale Vibra AJ-820CE (Shinko Denshi; Tokyo, Japan)
- o magnetic stirrer IKA RCT Basic (IKAMAG™, Germany)
- o biological thermostat BT 120 (Labo MS s.r.o., Czech Republic)

4.4 Methods
The aim of this bachelor thesis was to develop an optimised protocol for *in vitro* transcription of BSMV and infiltration of wheat plants. The first step was to multiply the BSMV virus subunits inserted into appropriate plasmids and to generate a restriction map for the selection of suitable colonies for further steps, i.e., linearization, *in vitro* transcription and inoculation of plants.

4.4.1 Spread on Petri dishes and growth of colonies

1. Each culture of *E. coli* carrying different plasmid (α, β, γ or γ-PDS subunits) was spread on Petri dishes with 2YT medium and ampicillin using a sterile bacteriological loop.
2. Petri dishes were incubated overnight at 37°C in a thermostat.

4.4.2 Extraction of plasmid DNA for restriction and selection

1. 3 ml of 2YT medium and 3 μl of antibiotic (ampicillin) were pipetted into sterile tubes.
2. A grown colony was picked up with a sterile tip and the tip was dropped into the prepared tubes with a medium.
3. The tubes were placed on a shaker and incubated overnight at 37°C, 220 rpm.
4. On the second day, the GET buffer was thawed and placed on ice.
5. The GET buffer was mixed with RNAse: 100 μl of GET with 1 μl of RNAse multiplied by the number of samples.
6. The contents from the shaker were pipetted from the tubes into 1.5 ml microtubes.
7. The samples in microtubes were centrifuged at 10 000 rpm for 5 min at 4°C. This was repeated for the rest of the samples.
8. The supernatant was poured off and the remaining supernatant that could not be poured off was removed by pipetting.
9. 101 μl of the mixture of GET-RNAse was added to each microtube and the pellet was resuspended by pipetting.
10. 200 μl of alkaline SDS was added and the sample was pipetted thoroughly to form a mucous solution.
11. Samples were incubated for 5 minutes at room temperature.
12. 75 μl of neutralisation solution was added. Samples were gently pipetted until a white protein precipitate was formed.
13. The samples were incubated for 30 min on ice.
14. After incubation, the samples were centrifuged at 13 000 rpm for 10 min at 4°C.
15. The supernatant was transferred into new 1.5 ml microtubes.
16. 225 μl of isopropanol was added to the samples.
17. The samples were incubated for 30 minutes at room temperature.
18. Samples were centrifuged at 9 000 rpm for 10 min at 4°C.
19. The supernatant was poured off and the pellet was washed carefully with 300 μl of chilled 70% ethanol.
20. The samples were centrifuged for 2 min at 9 000 rpm at 4°C, the ethanol was then carefully poured off.
21. The pellet was washed carefully with 96% ethanol with the same procedure as for 70% ethanol.
22. The ethanol was poured/pipetted off and the samples were allowed to dry for 10 min. The pellet could not be left to overdry.
23. The dried pellets were dissolved in 20 μl of water each and transferred to new 0.5 ml microtubes.

4.4.3 Restriction map

A restriction map of the plasmids was prepared to determine which colonies are suitable for further experiments. For each plasmid (α, β, γ and γ-PDS) a set of restriction enzymes was selected using the NEBcutter tool v.3.0.16 (https://nc3.neb.com/NEBcutter/). Enzymes used for the restriction of each plasmid are listed below:

α: ClaI (10 U), NsiI (20 U), BtgI (10 U), HincII (10 U), StyI-HF (20 U), PvuI-HF (20 U)

β: XbaI (20 U), NdeI (20 U), PacI (10 U), PvuII-HF (20 U)

γ and γ-PDS: PstI-HF (20 U), HincII (10 U), ClaI (10 U), StyI-HF (20 U), PvuI-HF (20 U)

4.4.3.1 Restriction reaction
1. The restriction reaction was set for the isolated plasmids. The scheme of the reaction for each plasmid is presented in Table 1:
2. The conditions of the reaction was set to 37 °C for 2 hours.

Table 1: Reaction mixture for the restriction of the α, β, and γ/ γ-PDS plasmids.

	α x1	β x1	γ/γ-PDS
DNA	2 µl	2 µl	2 µl
CutSmart buffer	5 µl	5 µl	5 µl
Enzymes: 1 µl each according to the list	6 µl	4 µl	5 µl
H₂O	37 µl	39 µl	38 µl

4.4.3.2 Agarose gel electrophoresis
1. A solution consisting of 1 g of agarose and 100 ml of 0.5× TBE buffer was boiled in the microwave.
2. Subsequently, an electrophoretic bath was prepared, boiled gel cooled to 40-50 °C was poured into the bath and a comb has been added. The gel was allowed to solidify at room temperature for 30 minutes.
3. Comb was removed and the gel was placed in the electrophoresis bath filled with 0.5× TBE buffer. Samples were separated for 90 min at 3-5 V/cm. The gel was stained in 0.01% ethidium bromide for 20 min. Separated fragments were visualised with a documentation device.

4.4.4 Extraction of plasmid DNA for *in vitro* transcription

For the extraction of a large amount of each plasmid (BSMVα, BSMVβ, BSMVγ and BSMVγ-PDS), the Nucleobond Xtra Midi kit (Macherey-Nagel GmbH & Co. KG) was used.

1. 3 ml of 2YT medium and 3 µl of antibiotic (ampicillin) were pipetted into sterile tubes.
2. A grown colony was picked up with a sterile tip and the tip was dropped into the prepared tubes with medium (starter culture).
3. The tubes were placed on a shaker and incubated for 8 hours at 37°C, 220 rpm.
4. 3 µl of starter culture were pipetted to 100 ml of 2YT medium with 100 µl of ampicillin.
5. The samples were placed on a shaker and incubated overnight at 37°C, 220 rpm.
6. After incubation, the tubes were centrifuged at 5 000 rpm for 15 min at 4°C.
7. The supernatant was completely discarded and the pellet was resuspended in 8 ml of RES buffer by inverting the tube five times.
8. 8 ml of LYS buffer was added to the suspension to lyse the cells, and the tube was gently mixed by inverting up and down.
9. The mixture was incubated at room temperature for 5 min.
10. The column filter was inserted into NucleoBond Xtra Column and the column was equilibrated by adding 12 ml of EQU buffer onto the rim of the column filter.
11. The column was allowed to empty by gravity flow.
12. 8 ml of NEU buffer was added to the suspension and the lysate was immediately mixed gently by inverting the tube until the samples turned colourless.
13. The lysate was mixed by inverting the tube three times to make a homogeneous suspension before continuing with the procedure.
14. The suspension was loaded on an equilibrated NucleoBond Xtra Column Filter and the column was allowed to empty by gravity.
15. The column was washed with 5 ml of EQU buffer two times.
16. The filter from the column was discarded and the column was washed with 8 ml of WASH buffer two times.
17. The plasmid DNA was eluted with 5 ml of the ELU buffer into the 50 ml centrifugation tube.
18. The following steps were performed in the flow box.
19. The eluted plasmids were precipitated with 3.5 ml of isopropanol and the tubes were mixed thoroughly by vortexing.
20. The samples were centrifuged at 5 000 rpm for 30 minutes at 4 °C and the supernatant was carefully discarded.
21. The pellet was washed with 2 ml of 70% ethanol and the sample was centrifuged at 5 000 rpm for 5 min at room temperature.
22. Ethanol was removed by pipetting and the pellet was allowed to dry at room temperature for 10 min.
23. The DNA pellet was dissolved in 200 µl of RNAse-free TElight buffer.

4.4.5 Linearization of BSMV plasmids

To linearize the plasmids, a restriction reaction with the corresponding restriction enzymes (Table 2) was performed.

4.4.5.1 Restriction reaction
1. The plasmids were diluted to 1 µg/µl.
2. The restriction reaction was set up to 80 µl following the scheme in Table 2.
3. SpeI-HF and BssHII enzymes were inactivated by heat at 80°C for 20 min. MluI-HF cannot be deactivated by heat.

Table 2: Scheme of the restriction reaction for the plasmid linearization.

	pBS-BSMVα	**pBS-BSMVβ**	**pBS-BSMVγ/ pBS-BSMVγ-PDS**
Plasmids	30 µl	30 µl	30 µl
CutSmart Buffer	8 µl	8 µl	8 µl
Enzyme	2.5 µl of MluI-HF (20 U/µl)	2.5 µl of SpeI-HF (20 U/µl)	6 µl of BssHII (5 U/µl)
Water	39.5	39.5	36
Incubation	2 h at 37°C	2 h at 37°C	2 h at 50°C

4.4.5.2 Gel electrophoresis and purification of the linearized plasmids

The linearized plasmids were purified by RNAse-free Sodiumacetate precipitation. Complete linearization was confirmed by gel electrophoresis on a 0.7% agarose gel.

1. 0.7% agarose gel was prepared by dissolving 0.7 g of agarose in 100 ml of 0.5× TBE.
2. 0.5 µl of linearized plasmids were loaded to the gel, and the undigested plasmids were run aside as a control.
3. 8 µl of 3M Sodiumacetate and 200 µl of 96% ethanol were added to each of the plasmids and the solution was mixed by inverting.
4. Plasmids were incubated at -20°C for 30 min and then centrifuged at 10 000 rpm for 10 min at 4°C.
5. The supernatant was discarded and the pellet was washed with 70% ethanol.
6. Samples were centrifuged for 1 min at 10 000 rpm, the supernatant was discarded and the pellet was allowed to dry at room temperature for 10 min.
7. The pellet was resuspended in 30 µl of RNAse-free TElight.

4.4.5.3 Determination of DNA concentration

The concentration of the plasmids was measured using Microvolume Spectrophotometer NanoDrop™ (Thermo Scientific). Plasmids were then diluted to 500 ng/µl and stored at -20°C until further use.

4.4.6 *In vitro* transcription

To produce BSMV RNAs from linearised plasmids, *in vitro* transcription was performed using the mMessage mMachine T7 Transcription kit (Thermo Fisher Scientific). Successful *in vitro* transcription was verified on a 0.7% agarose gel (with SB buffer). As *in vitro* transcription is a crucial step in VIGS, the acquired transcripts were immediately used for the inoculation of the plants.

1. The nucleotides from the kit were thawed, vortexed until completely solubilized and kept on ice.
2. The buffer from the kit was thawed, vortexed and kept at room temperature.
3. The enzyme mixture from the kit was immediately put on ice and left there throughout the whole procedure.
4. The *in vitro* transcription reaction was set up for each plasmid at room temperature.
5. Assembling the reaction mixture was done in the exact order as can be seen in Table 3.
6. The transcription mixture was mixed by flicking and incubated for 2 h at 37°C.
7. 0.7% agarose gel was prepared by dissolving 0.7 g of agarose in 100 ml of SB buffer.
8. 0.2 µl of the *in vitro* transcription was mixed with 5 µl of water and 1 µl of gel loading dye purple and were loaded on a prepared gel and separated for 15 min at 300 V.
9. The rest of the transcripts were kept on ice until inoculation.

Table 3: The reaction mixture for *in vitro* transcription

Reagent	Volume
Nuclease free H_2O	0.1 µl * x
Nucleotides (2x NTP/CAP)	0.25 µl * x
Buffer	0.05 µl * x
Linearized plasmid	0.05 µl * x
Enzyme mix	0.05 µl * x

* x – multiplied by the number of plants

4.4.7 Inoculation of viral RNA

1. The transcripts were mixed in a 1:1:1 ratio and FES buffer was added to the mixture.
2. The mastermix was prepared for inoculation of each plant: 0.5 µl of each transcript (α, β, and γ or γ-PDS) were mixed with 23.5 µl of FES buffer.
3. The first leaf of a 7 day old seedling was inoculated with 24 µl of the RNA mixture.
4. Inoculation was performed by taking the leaf between the thumb and the index finger and pipetting the mixture on top.
5. Leaf was squeezed between the two fingers and the mixture was drawn in small circles over the surface of the leaf four times.
6. Two to four plants were chosen as negative control and were inoculated only with 24 µl of FES buffer.
7. Plants were covered with plastic bags sprayed with water and kept in the room temperature overnight before placing in the phytotron.

4.4.8 Observation of viral symptoms

Viral symptoms were observed after about a week post-inoculation on the 2nd and 3rd leaves. Successful VIGS was confirmed 10–14 days post inoculation on the 3rd and 4th leaves of the inoculated plants. No viral symptoms, and no photo-bleaching should be observed on the negative controls.
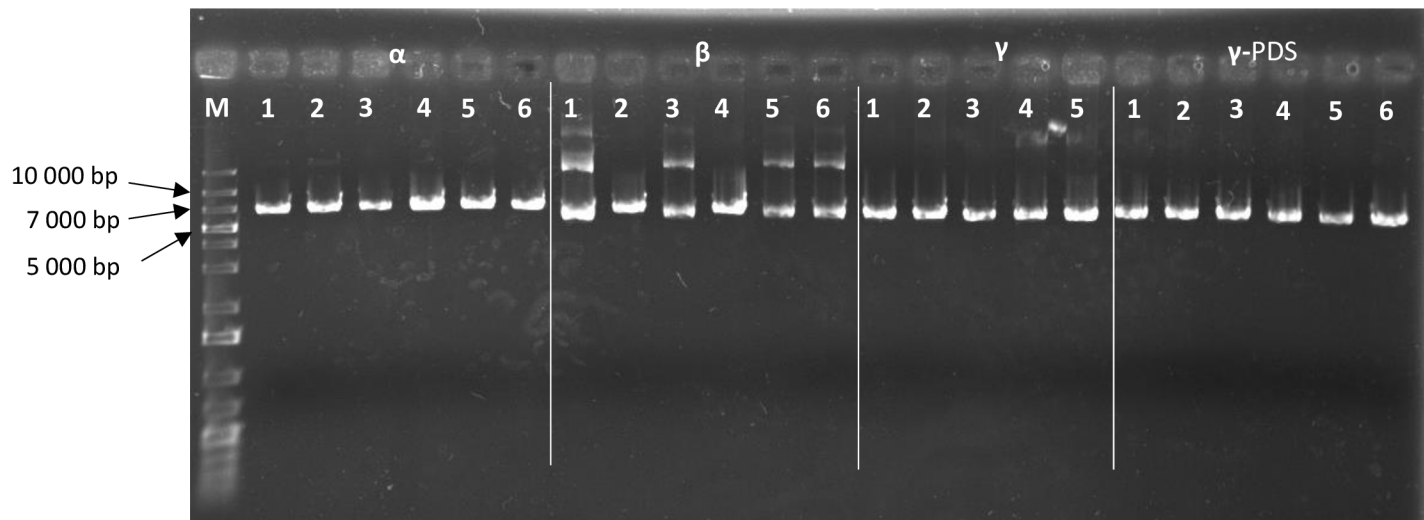
5 RESULTS

5.1 Extraction of plasmid DNA

The extracted plasmid DNA from 5-6 colonies of *E. coli* with the appropriate BSMV subunit were separated on a 1% agarose gel. The size of each plasmid is presented below:

pBS-BSMV-α: 6602 bp
pBS-BSMV-β: 6054 bp
pBS-BSMV-γ: 5629 bp
pBS-BSMV-γ-PDS: 5826 bp

Figure 2: Electrophoresis of plasmid DNA extracted from single colonies of *E. coli* bearing subunits of BSMV.

M is a molecular weight marker (GeneRuler 1kb plus); **α** are pBS-BSMV-α; **β** are pBS-BSMV-β; **γ** are pBS-BSMV-γ; **γ-PDS** are pBS-BSMV-γ-PDS; each number represents a different colony of *E. coli* carrying the relevant plasmid.

5.2 Restriction map

A restriction map was prepared to determine which colonies were suitable for further experiments. The restriction was first simulated *in silico* by the NEBcutter tool v.3.0.16 to see the expected results. For restriction of each plasmid bearing different subunits of BSMV (α, β, γ, and γ-PDS), three colonies (marked by numbers) were selected as shown in Figure 3. Based on the comparison between expected and experimental restriction map, colonies α-**1**, β-**2**, γ-**1,** and γ-PDS-**1** were chosen as suitable for further experiments.
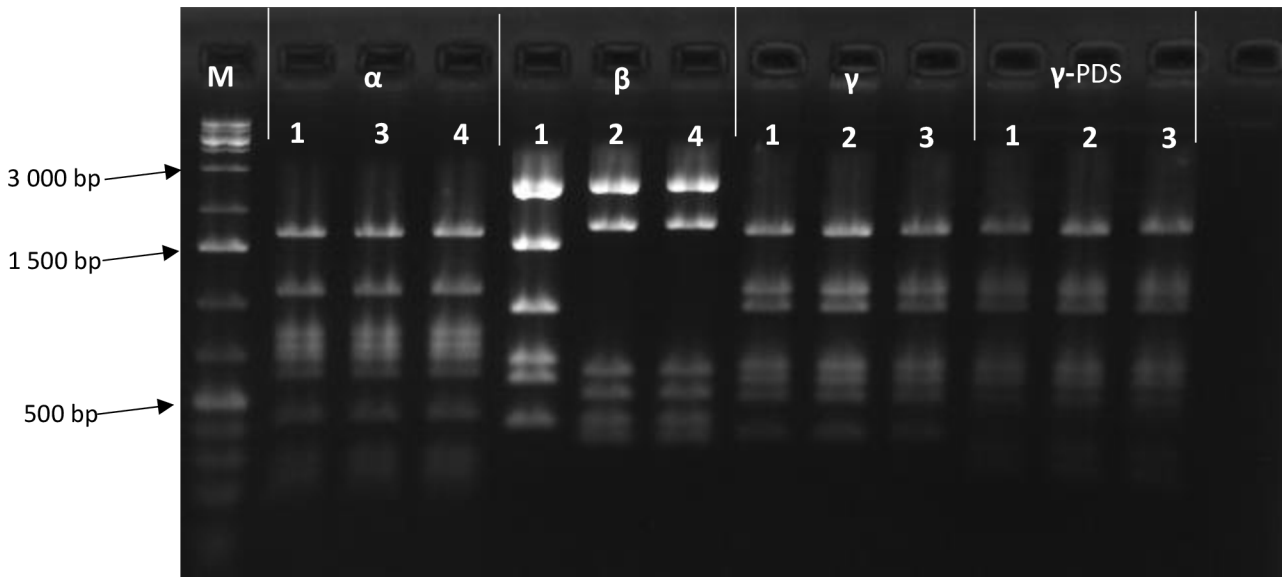
Figure 3: Restriction map of the plasmids bearing the subunits of BSMV.

M is molecular weight marker (GeneRuler 1kb plus); **α** are pBS-BSMV-α; **β** are pBS-BSMV-β; **γ** are pBS-BSMV-γ; **γ-PDS** are pBS-BSMV-γ-PDS; each number represents a colony of *E. coli* carrying the relevant plasmid.

5.3   Linearization

Complete linearisation of the plasmids isolated from the suitable colonies was verified by gel electrophoresis on a 0.7% agarose gel. Undigested (circular) plasmids were run alongside the linearised plasmids as a control (Figure 4).
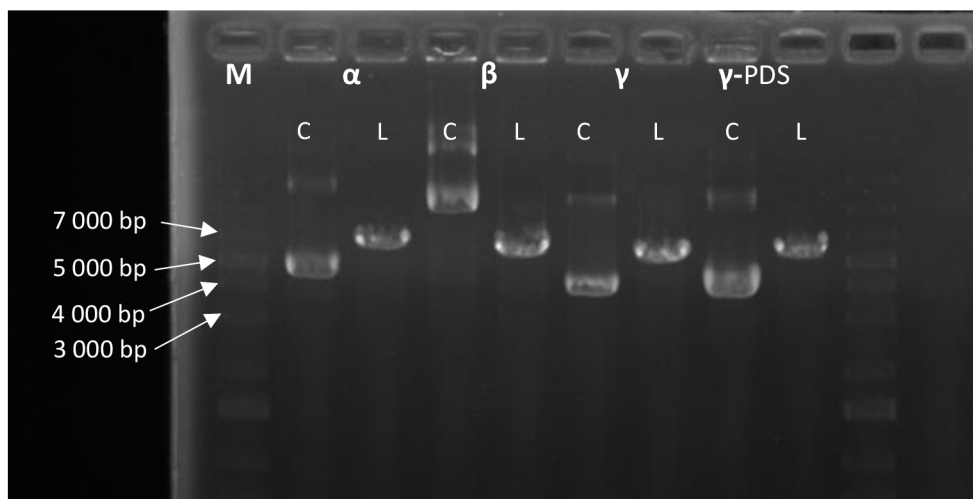
Figure 4: Electrophoretic separation of the circular and linearized plasmids carrying the subunits of BSMV.

M is a molecular weight marker (GeneRuler 1kb plus); **α** is pBS-BSMV-α; **β** is pBS-BSMV-β; **γ** is pBS-BSMV-γWT; **γ-PDS** is pBS-BSMV-γ-PDS; **C** – circular plasmids; **L** – linearized plasmids.
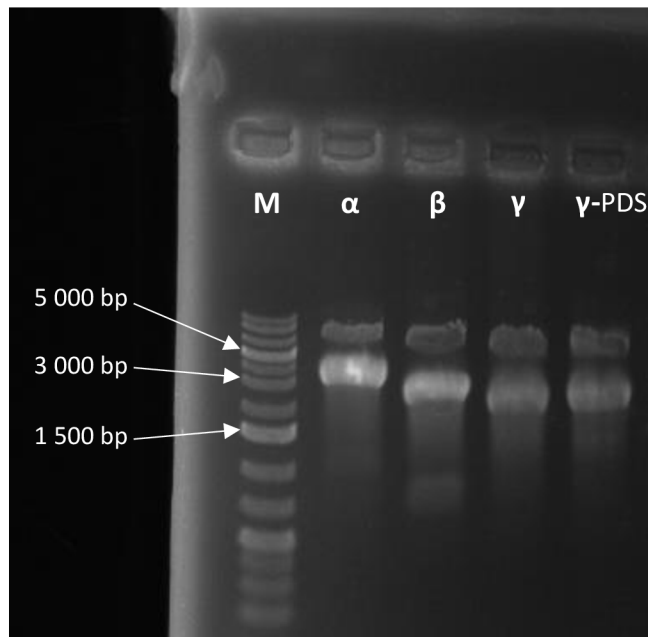
5.4 *In vitro* transcription

The outcome of the *in vitro* transcription of the linearised plasmids was verified by gel electrophoresis on a 0.7% agarose gel with SB buffer (Figure 5). The size of the transcripts is presented below:

BSMV-α: 3 549 bp
BSMV-β: 3001 bp
BSMV-γ: 2 572bp
BSMV-γ-PDS: 2 769 bp

Figure 5: Verification of the *in vitro* transcription of the linearized plasmids.

**M** is a marker of the molecular weight; **α** is the α subunit of BSMV; **β** is the β subunit of BSMV; **γ** is the γ wild type subunit of BSMV without insert; **γ-PDS** is the γ subunit of BSMV with the PDS insert.

5.5 Viral symptoms

For this experiment, 20 plants of *T. aestivum* were inoculated. Ten plants of cultivar Bobwhite and ten plants of cultivar Fielder. One plant of each cultivar was not inoculated and served as a negative control. One plant of each cultivar was inoculated only with FES buffer and served as a second negative control. One plant of each cultivar was inoculated with the BSMV without the PDS insert into the γ subunit – BSMV:00 (α: β: γ; 1:1:1) and served as the positive control. The rest of the plants were inoculated with the BSMV with PDS inserted into the γ subunit – BSMV:PDS (α: β: γ-PDS; 1:1:1).

The efficiency of VIGS was confirmed 10-14 days post inoculation on the 3rd and 4th leaf of the plants inoculated with BSMV:PDS by significant photo-bleaching (Figure 6). The photo-bleaching appeared as a result of the silenced PDS gene in the plant and shows systemic spread.

Negative control plants have shown no signs of viral symptoms or gene (PDS) silencing (Figure 7). Viral symptoms of BSMV were not clearly visible on the positive controls inoculated with the BSMV without PDS insert (BSMV:00).
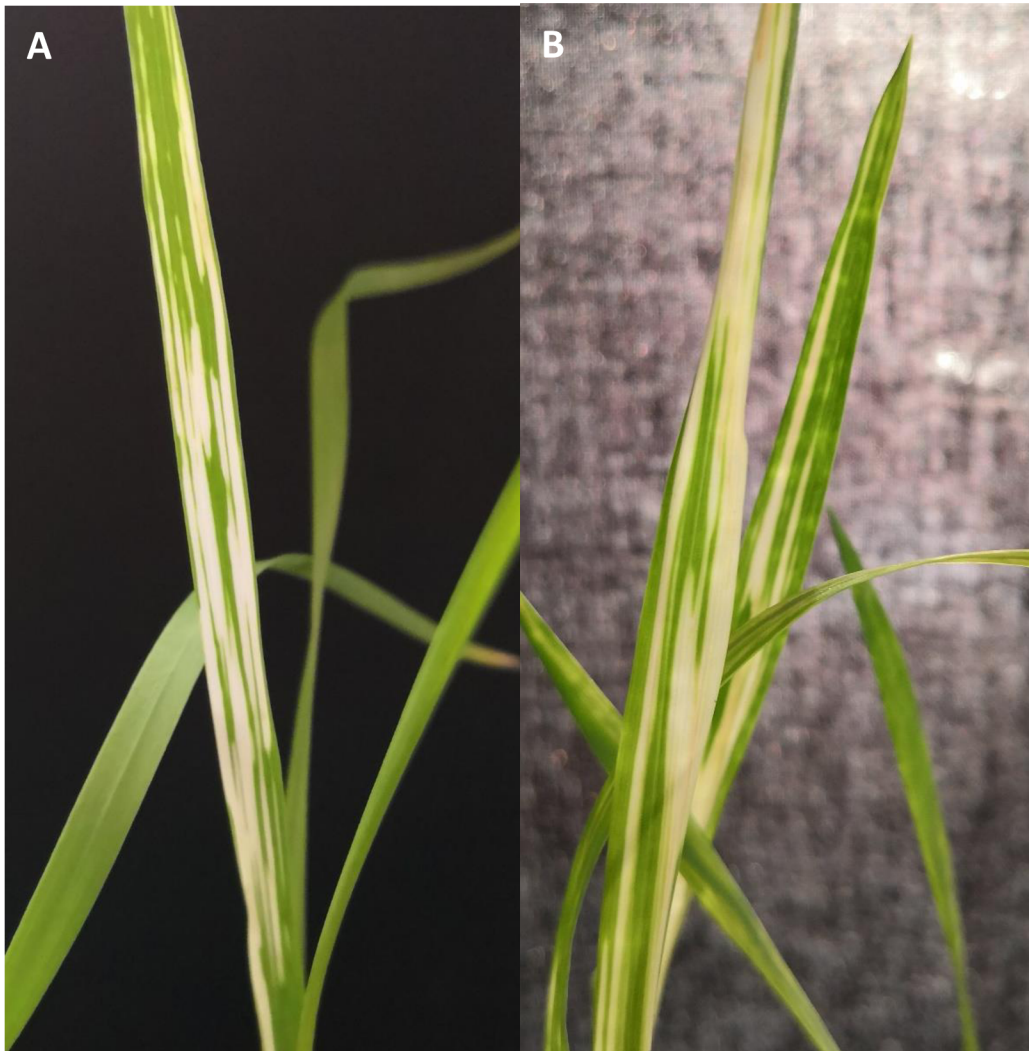
Figure 6: Photo-bleaching as a symptom of the PDS gene silencing with VIGS.

A) wheat cultivar Fielder inoculated with BSMV:PDS; **B)** wheat cultivar Bobwhite inoculated with BSMV:PDS.

Figure 7: Negative control plant (wheat cultivar Fielder) inoculated with FES buffer without the viral symptoms or VIGS symptoms (PDS gene silencing).

6 DISCUSSION

The main aim of this bachelor thesis was to optimise the protocol for VIGS using BSMV as a vector to be a reliable and repeatable procedure, so that it could be used extensively in future experiments. Particular attention was paid to *in vitro* transcription and plant inoculation, as these two steps are critical for successful gene silencing.

The first step was to extract the plasmids bearing different subunits of the BSMV genome from the colonies of the *E. coli* strain DH5α. The sizes of the extracted plasmids were comparable to the data described by Scofield *et* Brandt (2012) (Fig. 1). The control of the extracted plasmids showed variable and unexpected results in the case of plasmids bearing the β-subunit (Fig. 2). To select colonies of *E. coli* carrying the pBS-BSMV-β that were suitable for further experiments, a restriction map was prepared. The restriction was first simulated using the NEBcutter tool to determine the optimal results. Restriction reaction was performed several times, because some of the plasmids bearing the β-subunit of BSMV, showed restriction patterns that repeatedly differed from the expected results. Using the restriction map, suitable plasmids bearing the β-subunit with a restriction pattern comparable to the predicted one were finally selected (Fig. 3, samples 2 and 4). The restriction patterns of the rest of the plasmids bearing the α or γ/ γ-PDS subunits were comparable to the *in silico* prediction of the restriction.

The next step was to linearise the extracted plasmids. Complete linearisation is necessary for the whole procedure because T7 RNA polymerase preferentially transcribes supercoiled templates, so partial linearisation may result in inferior production of the viral RNA (Scofield *et* Brandt, 2012). Moreover, circular, non-linearised plasmid DNA is a much better template for transcription than linear DNA, which would result in the production of even more unspecific RNA products (Lu *et al.*, 2003). The obtained results showed complete linearisation of BSMV plasmids (Fig. 4).

For this experiment, the *in vitro* transcription was chosen over the agroinfiltration-based method (successfully used, for example, by Cheuk *et* Houde, 2017) for several reasons. Agroinfiltration has been used for VIGS experiments at IEB CAS in the past but often failed to deliver the desired results. Symptoms of the BSMV were observed only in the immediate vicinity of the infection site and did not spread to the rest of the plant. *In vitro* transcription is a more straightforward approach of VIGS and has been extensively used over the years (Holzberg *et al.*, 2002; Scofield *et al.*, 2005; Sánchez-Martín *et al.*, 2021). The mMessage mMachine T7 Transcription kit (Thermo Fisher Scientific) was used because of the positive results acquired by Sánchez-Martín *et al.* (2021). The result of the verification of the *in vitro* transcription (Fig. 5) is in accordance with the results presented by Scofield *et* Brandt (2012) and the *in vitro* transcription was considered successful.

Plants inoculated with BSMV:PDS showed significant photo-bleaching on the leaves of both cultivars (Fig. 6), which appeared as a result of successful silencing of the PDS gene. These observations are in agreement with the results acquired by Holzberg *et al.* (2002) and Scofield *et al.* (2005). Furthermore, gene silencing appeared also on the 3rd and 4th leaves of the plants inoculated with BSMV:PDS, which is a proof of the systemic spread in the plants, which was also described by Holzberg *et al.* (2002). Negative control plants (inoculated only with FES buffer) have shown no signs of viral symptoms or gene (PDS) silencing (Fig. 7), which confirms no contamination during the inoculation of the plants. Positive control plants inoculated with BSMV:00 has shown no changes in phenotype that could be confidently

considered as symptoms of BSMV infection. This fact is particularly interesting, given the overall positive outcome of the experiment. The possible cause of this may be unfavourable conditions in phytotron necessary for the development of sensitive reactions. Scofield *et al.* (2005) claim that the symptoms associated with infection with the BSMV:00 were significantly less severe in wheat than in barley. This could be another possible explanation considering the different physiological changes associated with BSMV infection in wheat and barley as hosts. Unfortunately, no barley plants were used as another control in this thesis. Different controls help to confirm the correctness of the obtained results, which is the reason why three for each cultivar were used in this study. The importance of using different types of controls was described, for example, by Scofield *et* Brandt (2012).

Both selected cultivars of *T. aestivum*, namely Bobwhite and Fielder, were confirmed to be susceptible to BSMV and can be considered suitable for VIGS studies. This is in agreement with the results obtained by Bennypaul *et al.* (2012) and Buhrow *et al.* (2016).

Experiments with the usage of BSMV to generate short interfering RNAs were planned to be a part of this thesis. It was intended to silence the candidate genes within the *QPm.tut-4A* region (introgressed into wheat from *T. militinae*) conferring full resistance to powdery mildew infection. With this approach it was expected to find a gene of interest. However, this study had to be postponed because of the limited efficiency of the previously used four-subunit BSMV vector (Cheuk *et* Houde, 2017). Therefore, the approach was changed to the *in vitro* synthesis of viral RNA according to Sánchez-Martín *et al.* (2021) and optimisation with silencing of the PDS gene. Based on the data obtained during the experiments, this thesis proves that the use of BSMV-VIGS is a valuable option for future research. When optimised, it is a straightforward and reliable method that can be used to screen wheat candidate genes conferring *B. graminis* resistance, which is the main goal of future experiments.

7 CONCLUSIONS

This bachelor thesis describes an optimised protocol for VIGS by *in vitro* transcription using BSMV as a vector. For this purpose, the phytoene desaturase gene was silenced in selected cultivars of wheat.

The suitable colonies of *E. coli* carrying the pBS-BSMV-α, pBS-BSMV-β, pBS-BSMV-γ, and pBS-BSMV-γ-PDS, were selected using restriction maps.

Plasmids from suitable colonies were extracted and linearised. Complete linearisation was verified by agarose gel electrophoresis.

Linearised plasmids were transcribed *in vitro* and these transcripts of viral RNA were inoculated into wheat cultivars Bobwhite and Fielder.

Symptoms of photo-bleaching as a result of the silenced PDS gene were observed 10-14 days post-inoculation on the 3rd and 4th leaves. Systemic spread of the virus and silencing was confirmed. Negative control plants have shown no signs of viral symptoms or gene (PDS) silencing. Symptoms of viral infection were not visible on the BSMV:00 positive control. Because of this, the growing conditions of the inoculated plants and the time of post-inoculation screening of the symptoms may be altered in future experiments.

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