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„Transgene expression and plant regeneration in the Brassicaceae *Aethionema arabicum*"

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Contents

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Abstract

The herbaceous plant *Aethionema arabicum* develops two distinct fruit and seed morphs on the same plant, termed heterocarpy. It is assumed to represent an adaptation to unpredictable environment. While genetic and genomic information necessary to investigate the molecular regulation behind this phenomenon is available, an essential tool in reverse genetics is still missing: transformation of *Aethionema* species was not achieved yet.

In this thesis I describe the application of several methods to introduce transgenes into *Aethionema* and show successful transient transformation, using either *Agrobacterium*-mediated transformation approaches or a biolistic method.

By applying the leaf infiltration method or particle bombardment, single cells within the treated tissue expressed the GFP marker gene for a couple of days. The efficiency was dependent on the construct and the *Agrobacterium* strain.

Virus-induced gene silencing (VIGS) with the *Tobacco Rattle Virus* (TRV) and a *PHYTOENE DESATURASE (PDS)* insert led to photobleaching in *N.benthamiana*, as a proof of concept. To establish the method in *Ae. arabicum*, I generated inserts with its own genes for *PDS* and *INDEHISCENT (IND),* a gene involved in fruit formation, were tested by leaf infiltration. Bleaching was not observed; some phenotypic modifications were observed for the IND construct, which have to be confirmed by determining the down-regulation of the target transcript.

Performing a co-cultivation of seedling explants with GFP-transmitting *Agrobacterium* on callus-inducing medium led to fluorescent callus, transiently expressing the marker gene. I could maintain the callus culture over several months by sub-cultivation, using hygromycin selection medium and/or selection for the expression of the visible marker.

In parallel, I could establish shoot differentiation from mature leaf explants using a medium with a high amount of cytokinin. Future combination of this shoot differentiation protocol with the successful transformation by co-cultivation provides a promising future prospect to generate stable transgenic *Aethionema* plants.

Introduction

Heteromorphism in seeds and fruits

While most angiosperm species form one type of seed and fruit (homomorphism) that is optimally adapted to a certain habitat, a few angiosperm families with seed and fruit heteromorphism have evolved, producing two or more different seed and/or fruit morphs by the same individual. Morphological difference in seeds is defined as heterospermy, while heterocarpy refers to heteromorphism in fruits. Seed heteromorphism is most common in Asteraceae, Chenopodiaceae and Brassicaceae, and can affect different properties (Imbert, 2002).

Diverse types of propagation units (diaspores) can lead to different fates of the progeny, e.g. due to distinct opportunities for distribution or germination. Under some circumstances, this flexibility may result in a fitness advantage. Subsequently, diaspore heteromorphism is a typical bet-hedging strategy in adaptation to unpredictable environments, as it was first suggested by Slatkin (1974) and later revisited by Philippi & Seger (1989). Diversifying bet-hedging reduces short-term reproductive success in favor of decreasing the long-term risk of extinction (Venable, 2007), or, in other words, bet-hedging is a trade-off between the mean and variance of fitness, as Slatkin (1974) described in his commentary `Hedging one's evolutionary bets`.

Heteromorphic species are mainly annual plants, frequently growing in highly disturbed or stressful habitats such as arid and semi-arid environments. There, bet-hedging might be a successful strategy to preserve a species (Imbert, 2002; Venable, 2007). Cohen (1966) described a classical model for desert annuals, where seeds, dependent on environmental conditions, can either germinate immediately after release and thereby rapidly produce more seeds, or stay dormant for a longer period. Delayed germination may lead to a higher long-term success if growth-supporting conditions reappear only later, but it bears the risk of decreased viability of dormant seeds in the soil.

Aethionema arabicum as model system

In the tribe Aethionemeae (family Brassicaceae), diaspore heteromorphism has been described for six species in the same evolutionary clade. However, only five annual species form a monophylum, while heteromorphism in the sixth species – the perennial *Ae. saxatile* – is of independent origin. One of the annual heteromorphic species is *Aethionema arabicum*, a small, diploid, herbaceous species adapted to arid and semi-arid environments of the Irano-Turanian region (Lenser et al., submitted).

Heteromorphism of *Ae. arabicum* was reported for the first time by Solms-Laubach (1901). More than 100 years later, the ´SeedAdapt´ consortium (www.seedadapt.eu) consisting of six European

research groups suggested the plant as a model system to investigate the basis for heteromorphism, phenotypic plasticity and adaptation to environmental changes. *Ae. arabicum* shows clear heterocarpy as well as heterospermy, without intermediates between the two types, confirmed for the shape of fruits/seeds, germination and dispersal behavior by morphometric analyses (Lenser et al., submitted).

The larger fruit morph opens easily along a septum (dehiscence with low applied force, DEH type) and is strongly attached to the infructescence (no natural abscission). It releases 2-6 seeds per fruit that can germinate rapidly and become mucilaginous upon imbibition (M^+ seed morph; myxospermy). The smaller fruit morph tightly encloses only one seed per fruit (in dehiscence, IND type), does not contain a septum, and abscission is the primary way to disperse this diaspore. Seeds from these fruits do not develop mucilage during imbibition (M^- seed morph). Germination occurs within the fruit, which may lower germination speed and vitality and has been interpreted as the non-risk strategy of the bet-hedging model for heteromorphism. Accordingly, DEH fruits and M^+ seeds may represent the high-risk strategy, because non-dormant germination in unpredictable environment tends to be risky for annuals (Fig. 1).

Therefore, *Ae. arabicum* evolved two distinct dispersal strategies: fruit dehiscence with sticky seeds immediately germinating upon water supply, versus abscission of single-seed diaspores with delayed germination of the enclosed seed (Lenser et al., submitted).

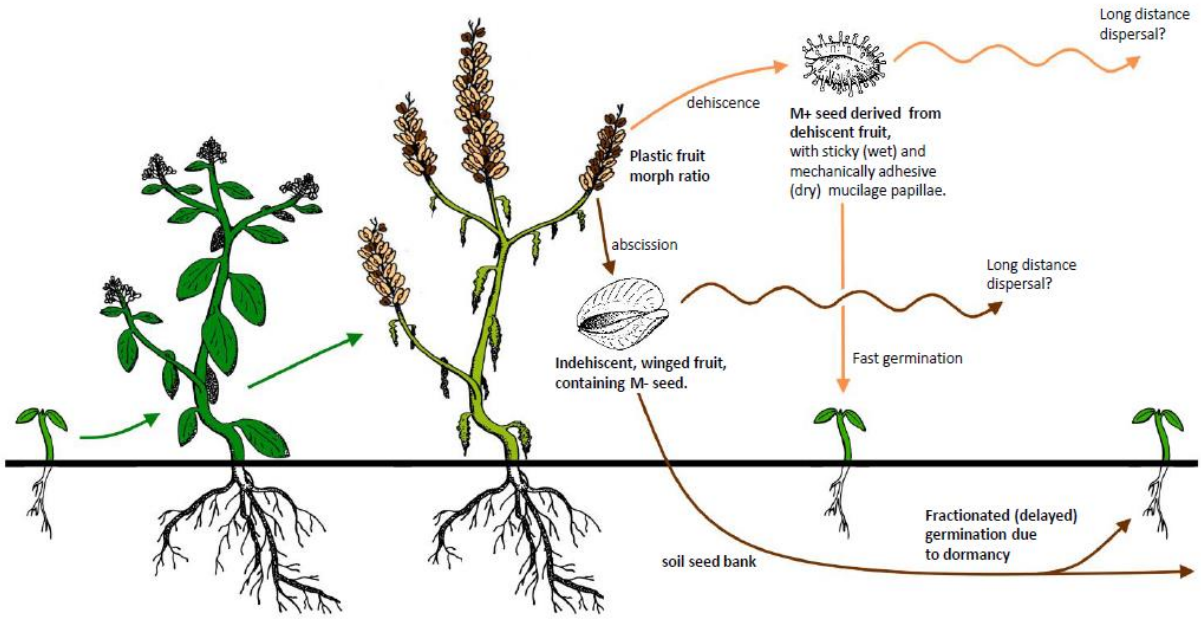

Fig. 1. Scenario of different life-history strategies of *Ae. arabicum* seed morphs (Lenser et al., submitted). After fruit maturation, indehiscent fruits with enclosed M^- seeds abscise from the mother plant while dehiscent fruits open to release M^+ seeds. M^+ seeds germinate quickly after water uptake, while M^- seeds remain in the soil seed bank and show delayed germination within the fruit itself. Both diaspores may further be subject to long-distance dispersal due to the presence of adhesive mucilage (M^+) or enclosure in winged indehiscent fruits (containing M^- seeds).

Interestingly, both seed morphs give rise to plants with the same habitus upon maturity. Both fruit morphs can appear at the same infructescence with a rather stochastic distribution. However, there is a developmental component, as higher order side branches produce more indehiscent fruits than the main branch. Additionally, environmental conditions like temperature can modify the ratio between the fruit morphs: plants grown at 20 °C form more indehiscent fruits compared to those grown at the 25 °C (Lenser et al., submitted).

The genetic and molecular control behind this heteromorphism respectively the environmental regulation is still not clear, but several tools and resources already established suggest *Ae. arabicum* as appropriate model system to investigate the interplay between molecular, evolutionary and ecological aspects of heteromorphism and adaptation in general. Beside of the clear dimorphism, the plants are small and easy to grow, have a relatively short life cycle of 2-3 months, are closely related to the well-established model plant *Arabidopsis thaliana*, and the genome has been sequenced recently (Haudry et al., 2013; Lenser et al., submitted). The plants are diploid and propagate mainly by selfing. There are different accessions that display natural variation of phenotype and genotype (Klaus Mummenhoff, Eric Schranz, personal communication). However, an important tool for genetic and molecular research is still missing – the possibility to modify gene expression or edit the genome by producing transgenic plants! Work described in this thesis attempted to fill this gap in the tool box.

Generating transgenic plants

There are several ways of plant transformation and numerous protocols for a wide range of plant species, especially model and crop plants. Besides the biolistic particle bombardment, many *Agrobacterium* mediated approaches are established, where disarmed bacteria function as a "gene shuttle" to introduce foreign genes. Vectors based on the Ti plasmid (tumor inducing) are used. This plasmid is harboring the disarmed transfer DNA (T-DNA) without any tumor inducing genes, and the virulence genes (vir). Upon interaction with host proteins, the vir proteins suppress the host innate immune system and support the integration of T-DNA into the host cell genome. The vector can be used in conjunction with any binary transformation vector (Klee et al., 1987; Pitzschke & Hirt, 2010). For *Ae. arabicum*, there was no hint which strategy would work. Therefore, I have applied several techniques in parallel, and I will describe the rationale of the different approaches in the following.

Floral dip method

As *Ae. arabicum* is a member of the Brassicaceae family, it was obvious to apply the ´floral dip method´ that is routinely used to transform its relative *Arabidopsis thaliana*, the "workhorse" model of plant science. Since Bechtold et al. (1993) established the protocol for vacuum infiltration of

disarmed *Agrobacterium tumefaciens* into inflorescences, this method has been simplified and represents a reliable and straightforward technique to create stable transgenic Arabidopsis lines (Clough & Bent, 1998). In short, floral buds are dipped for a few seconds in an *Agrobacterium*-sucrose suspension supplemented with a surfactant, such as Silwet L-77, and plants are then covered with a lid to maintain humidity for 24 hours. Plants are then allowed to set seeds, and these can be screened for the expression of the transgenic reporter gene. While the first generation is usually hemizygous for the inserted genes, self-fertilization of these plants generates the homozygous transgene genotype in the next generation.

The advantage of the technique is its straightforwardness and reliability. It does not require expensive laboratory facilities, can be achieved by non-experts and shows fast transformation success (Clough & Bent, 1998). The transformation event is assumed to take place in the gynoecium in *Arabidopsis* flowers, a vase-like structure with a gap that fuses roughly 3 days prior to anthesis. *A. tumefaciens* is supposed to access through this gap to the interior of the developing gynoecium prior to closure of the carpel. In a mutant line that maintains an open gynoecium, a 6-fold higher transformation rate can be obtained (Desfeux et al., 2000). The transformation efficiency rate for T1 seeds of *A. thaliana* ranges from 0.5 up to 4% and is determined by the *Agrobacterium* strain, the plant ecotype and the physiology of the dipped plant (Clough & Bent, 1998; Ghedira et al., 2013).

Although the benefits of the method are evident, the big drawback of the technique over many years was the restriction to *A. thaliana*. However, since a couple of years, the floral dip method has been successfully applied to many other plants species such as *Brassica campestris* subsp. *Chinensis* (pakchoi), *Medicago truncatula*, tomato, wheat, maize and flax (Desfeux et al., 2000; Bastaki & Cullis, 2014).

Leaf infiltration

By using an *Agrobacterium* suspension to infiltrate plant leaves, transient expression of marker genes in plant tissue was already reported 20 years ago in *Phaseolus vulgaris*. Although not integrated into the genome, T-DNA copies remain transiently present in the nucleus, get transcribed and lead to transient expression of the transgenes. Successful gene expression can be detected very shortly after infiltration without the need to create stable transformed plants (Kapila et al., 1997).

While initially *Agrobacterium* was introduced by vacuum infiltration (Kapila et al., 1997), direct inoculation by applying the suspension with a syringe onto the leaf blade is commonly used these days. Especially the model plant *N. benthamiana* is well established for testing the transient expression of new constructs in epidermal cells via simple inoculation of bacteria carrying the construct of interest.

A potential drawback of any transient expression system is the overexpression of marker genes and thus saturation, which can alter the subcellular distribution or trafficking. Additionally, RNA silencing

can block the expression of transgenes and may lead to a rapid decrease of expression after 2-3 days. However, efficient induction of *Agrobacterium* vir genes prior to infection with the phenolic compound acetosyringone induces higher virulence, and including silencing suppressors can also be used to reduce gene silencing (Kapila et al., 1997; Sparkes et al., 2006; Wydro et al., 2005). Beyond transient transformation, protocols for *N. benthamiana* exist to generate stably transformed plants from infiltrated leaves via tissue culture by immediate induction of shoots and roots, circumventing callus induction (Sparkes et al., 2006).

Unexpectedly, *A. thaliana* turned out quite recalcitrant to routine transient transformation assays working in N. benthamiana, although some success was shown recently (Sanchez-Serrano & Salinas, 2014). For other species, the leaf infiltration method for transiently expression of transgenes works, including some important crops like *Solanum lycopersicum, Lactuca sativa* and *Theobroma cacao* (Wroblewski et al., 2005; Fister et al., 2016). Nevertheless, *N. benthamiana* is still the best model to test new constructs in leaf tissue and is routinely used for this purpose.

Particle bombardment

Since *Agrobacterium* shows a host-range restriction, a rapid and simple alternative procedure to introduce foreign DNA into plant cells is particle bombardment. The concept to use high-velocity microprojectiles to deliver DNA into living plant cells was first achieved in 1987 with small tungsten particles, carrying DNA into epidermal tissue of *Allium cepa* (Klein et al., 1987). The particles pierce cell walls and membranes without killing the cells, and the delivered DNA gets transiently expressed, regardless of species or tissue type. Microprojectiles can be defined as any small particle capable of being accelerated and binding DNA, such as high-density metals like tungsten or gold between 0.4 and 2.0 µm in diameter (Sanford, 1990).

Several accelerating mechanisms have been used to shoot microprojectiles onto target cells, such as the original gunpowder device, electric discharge-based instruments, a micro-targeting apparatus or pneumatic devices (Christou, 1993). In the latter case, helium pressure is applied to accelerate DNA-coated microprojectiles within a partial vacuum chamber to penetrate and thus transform the cells. Fig. 2 shows the assembling of the pneumatic device of BioRad® (Takeuchi et al., 1992; catalog BioRad, 1996) used in this study.

Beyond transient expression, bombardment of totipotent tissue such as pollen, embryos and meristems may even lead to stable transformed mature plants (McCabe et al., 1988b; Cao et al., 1992), and organelles have also been biolistically transformed (Daniell et al., 1990). However, this procedure often gives rise to chimeric plants, which need to be sorted out and stabilized in next generations. On the other hand, the phenomenon of somaclonal variation can be minimized by targeting totipotent tissue, as extended tissue culture phases are avoided (Sanford, 1990).

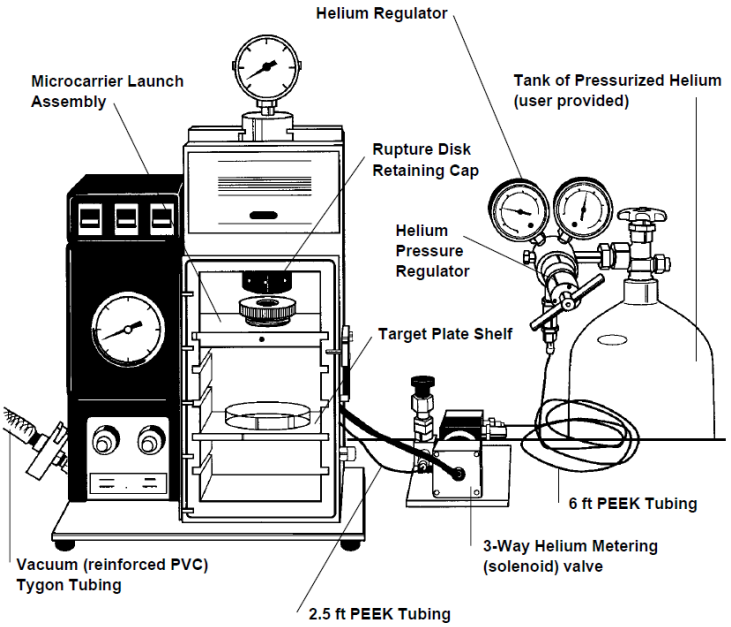

Fig. 2. Unit components BioRad Biolistic® PDS-1000/He Particle Delivery System (out of Catalog Numbers 165-2257 and 165-2250LEASE to 165-2255LEASE, BioRad, Molecular Bioscience Group)

A major advantage of the technique is obviously its broad utility. Particle bombardment represented a breakthrough in transformation of monocots, followed by successful transformation of many important crops and model plants (Christou, 1993; Sanford, 1990).

Virus-induced gene silencing (VIGS)

In addition to the intended expression of foreign genes, it is equally important to modify the expression of endogenous genes, especially if they should be turned off. Virus-induced gene silencing (VIGS) is a method, based on the phenomenon of RNA-interference (RNAi), to rapidly silence plant genes of interest. A VIGS vector cassette containing viral genes (as cDNA) and the target gene insert is introduced into the plant cells by agroinfiltration or biolistic treatment as described. The subsequently produced transcript is recognized and degraded due to natural immune response against viruses in plants. The signals of a virus attack initiate a spreading mechanism throughout the whole plant; short interfering RNAs (siRNA) are produced to trigger – together with a multiprotein effector complex, called RNA-induced silencing complex (RISC) – the homology-dependent degradation of target transcripts within the entire plant (Fig. 3). The pathway is related with post-transcriptional gene silencing (PTGS) that can occur also without viral infection. No matter what, it results in the sequence-specific degradation of endogenous mRNA and is a useful tool in genetic research to silence genes of interest (Purkayastha & Dasgupta, 2009).

The first VIGS vector was established using the RNA virus *Tobacco Mosaic Virus* (TMV) engineered to contain the plant phytoene desaturase gene (PDS). PDS is an enzyme involved in the biosynthesis of

carotenoids, which are supposed to protect chlorophyll from photo-bleaching. Silencing of the gene via the VIGS approach leads to white leaves in *N. benthamiana* (Kumagai et al., 1995).

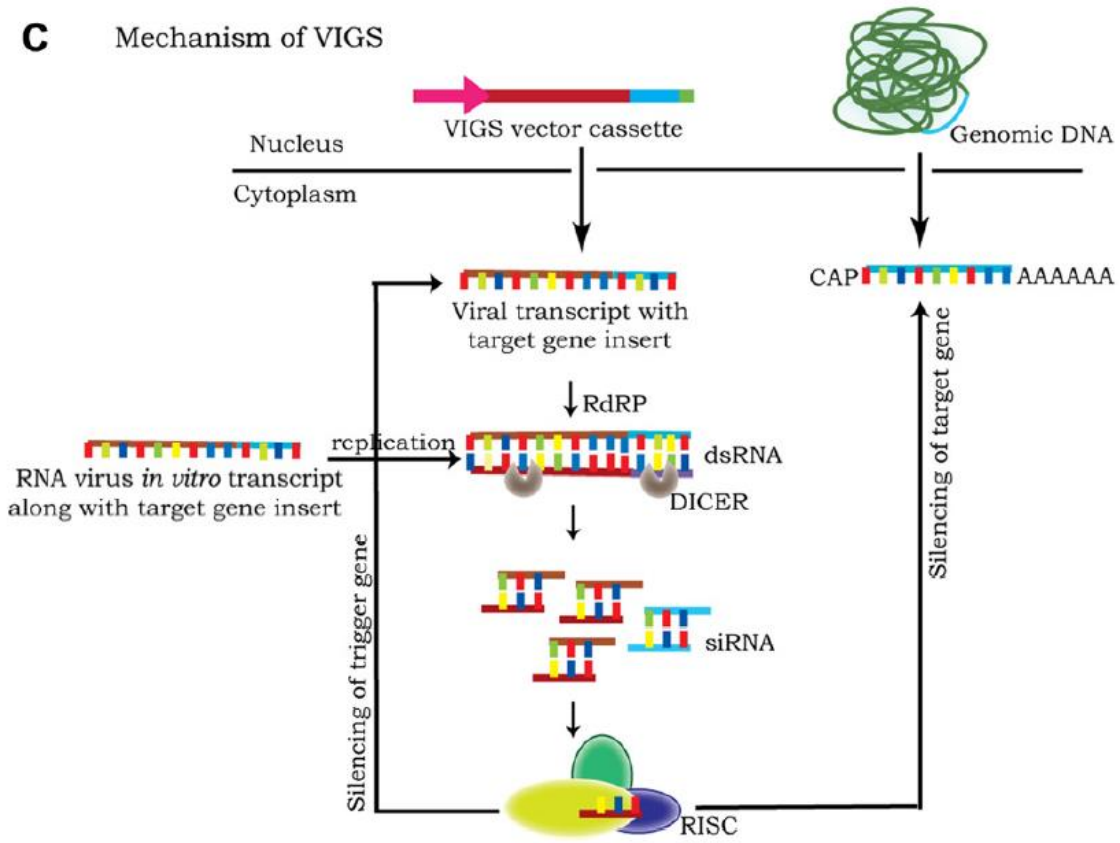

Fig. 3. Mechanism of VIGS (virus-induced gene silencing). RNA transcripts derived from a cDNA-based construct with homology to the target gene gets expressed and amplified to dsRNA by host RdRP. DICER mediates production of 21 – 24 nt siRNAs, which act as guide molecules within the whole plant. siRNAs trigger - together with the RISC complex - homology-dependent degradation of target transcripts. RdRP, RNA dependent RNA polymerase; dsRNA, double-stranded RNA; siRNA, short interfering RNA; RISC, RNA-induced silencing complex, (Purkayastha &

Many virus vectors, originating from distinct plant viruses, were developed to be effective for this "knock-down" methodology (Purkayastha & Dasgupta, 2009; and references within). A commonly used VIGS vector derived from *Tobacco Rattle Virus* (TRV) is able to spread vigorously throughout the entire plant, including meristem tissue, but is less virulent compared to other viruses (Bruch-Smith et al., 2004). TRV is a 2-component, single-stranded, positive-polarity RNA plant virus (ssRNA as direct transcript for translation) and encodes only for a couple of proteins to operate within plants (Donaire et al., 2008).

Two independent VIGS vectors need to be created for successful silencing. TRV1 consists of three coding regions for a RNA-dependent RNA polymerase (to transcribe RNA from a RNA template), a movement protein and a cysteine-rich protein, while TRV2 includes a coat protein and the insert

designed to match the target gene, as indicated in Fig. 4 (Zhang et al., 2014). The size of TRV1 is around 6.8 kb and TRV2 is around 4.5 kb long, dependent on the insert. The viral RNA has to be translated to cDNA within the cells. T-DNA vectors with both templates are introduced into *Agrobacterium* and can be transferred to the plants by leaf infiltration (Senthil-Kumar & Mysore, 2014).

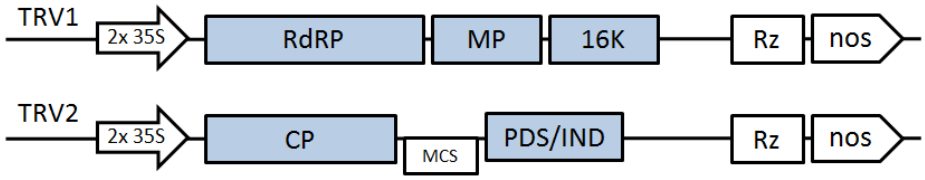

Fig. 4. Organization of TRV-VIGS vectors. TRV cDNA clones were placed between two CaMV 35S promoter (2x 35S) and a nopaline synthase terminator (nos) in a T-DNA vector. TRV1, TRV RNA, first part. TRV2, TRV RNA second part. RdRP, RNA-dependent RNA polymerase. MP, movement protein. 16K, cysteine-rich protein. Rz, self-cleaving ribozyme. CP, coat protein. PDS, phytoene desaturase insert. IND, indehiscence gene insert. MCS, multi cloning site.

Viral infection and amplification is influenced by growth conditions, and temperature seems to be one of the most important factors for effective VIG-silencing. TRV in tomato works best at or below 21 °C, while TRV in *N. benthamiana* has its optimum around 25 °C, indicating the need to setup temperature conditions for each plant species (Burch-Smith et al., 2004).

The great advantages of the system are the simple application, the fast output of successful silencing within a single generation, the potential to silence multi-copy genes or whole gene families by targeting highly conserved regions, and the broad species range of single VIGS vectors, even for non-host plants. VIGS avoids transformation to study gene function, but can also complement functional gene studies in transgenic plants. As it is carried out in mature plants or in parts of plants, loss-of-function phenotypes can be observed that would be lethal in early stages. Surprisingly, meristem-expressed genes can be targeted, although viruses are usually excluded from meristematic regions (Lu et al., 2003). However, as for the transient expression approaches, a notable limitation is the non-inheritance of the functional “knock-down”. Moreover, the decreased transcript level may still be sufficient to produce enough functional protein to conceal any altered phenotype. Furthermore, VIGS does not always result in uniform silencing throughout the entire plant (Lu et al., 2003; Purkayastha & Dasgupta, 2009).

Tissue culture – cocultivation with *Agrobacterium*

Cocultivation of protoplast with *Agrobacterium* followed by selection and regeneration was the first method used to successfully generate transgenic plants. In 1983 the first transgenic plants were

reported by three independent research groups, led by Marc Van Montagu, Mary-Dell Chilton and Robert Fraley. These groups transformed either antibiotic-resistant transgenic *Nicotiana tabacum, Nicotiana plumbaginifolia* or *Petunia x hybrida* by using disarmed *Agrobacterium* vectors (Wang, 2015). Because the procedures require an appropriate, species- and variant-specific regeneration protocol and additionally high laboratory expertise, they were first restricted to solanaceous plants such as petunia and tobacco, as these are most easily regenerated from dedifferentiated cells into fertile whole plants. Establishing equivalent protocols for other plant species or varieties remained a challenge and is still laborious. However, the benefit of generating transgenic plants has justified such investment, and many plant species were successfully transformed with tissue culture techniques. Regeneration from protoplasts has the advantage that the resulting plants are usually genetically homogeneous, due to single-cell origin.

The second basic approach that requires usually less tissue culture expertise than the protoplast regeneration is a cocultivation of plant tissue with *Agrobacterium*, stimulation of dedifferentiation and callus formation, followed by plant regeneration out of the callus (Klee et al., 1987). The initiation came from Horsch et al. (1985) using the leaf disc transformation- and regeneration method in petunia, tobacco and tomato, by cultivating inoculated leaf discs on a regeneration medium to generate genetically modified plants. Subsequently, *Agrobacterium* infection of wounded plant tissue to induce callus proliferation and create whole plants via somatic embryogenesis, were established. Prior to the floral dip method, it was the method of choice to transform Arabidopsis from root explants that regenerated whole plants easily (Martínez-Zapater & Salinas, 1998). Callus from scutellum (seed component) worked best for rice (Hiei at al., 1994). In *Brassica spp*., cotyledonary explants were used; immature embryos could be regenerated into barley plants, and half seeds worked well to obtain transgenic soybean (Wang, 2015; and references within).

However, co-cultivation can give rise to chimeric plants. Further, extended tissue culture during the differentiation of callus can result in the phenomenon of somaclonal variation: heterogeneity of phenotype or genotype that is not linked to the T-DNA insertion. Especially the exogenously applied hormones like the synthetic auxin 2,4-Dichlorophenosyacetic acid (2,4 D) seems to cause somaclonal variation (Martiínez-Zapater & Salinas, 1998). In addition, *Agrobacterium*-mediated transformation of tissue explants as well as of protoplasts may lead to multiple T-DNA insertion events in complex structures which often lead to gene silencing upon further propagation of the plants (Matzke et al., 1994).

The supplementary table 1 summarizes the different transformation methods and shows advantages as well as drawbacks for each technique.

Marker genes

All transformation systems for generating transgenic plants require processes to identify or select those cells that have integrated the introduced DNA into the genome. Development of several marker genes was a great support in transformation technologies. Two important groups of marker genes are commonly used. While selectable marker genes allow only transformed cells to grow on media containing certain chemicals, reporter or visible marker genes indicate transformation events by showing a fluorescent or other color signal.

Selectable markers can be divided into positive and negative selection systems according to maintenance and growth, or on the other hand, growth arrest or death of transformed cells. Both principles can be applied conditionally or non-conditionally, depending on the way the selecting agents such as antibiotics, herbicides and drugs are administered (Miki & McHugh, 2004). Two of the most frequently used conditional-positive selection systems are addition of the antibiotics kanamycin and hygromycin to the growth media. Plants are usually sensitive to the drugs, but expression of genes coding for an enzyme that converts the antibiotics to non-toxic derivatives confers resistance to the antibiotic and protects transformed cells from the toxicity of the chemical (Klee et al., 1987; Miki & McHugh, 2004). In case of kanamycin resistance the *E. coli* gene *NEOMYCIN PHOSPHOTRANSFERASE II* (*NPT II*) codes for a phosphotransferase that catalyzes phosphorylation of certain aminoglycosides including kanamycin (Beck et al, 1982). Hygromycin B can be detoxified by another *E. coli* enzyme, called *HYGROMYCIN B PHOSPHOTRANSFERASE* (*HPT*), catalyzing an ATP-dependent phosphorylation (Waldron et al., 1985).

Visible markers represent the second main group of genes helping to identify transformed cells and are often combined with selectable marker genes, particularly during protocol establishment for transformation methods. The *GREEN FLUORESCENT PROTEIN* (*GFP*) from the jellyfish *Aequorea victoria*, has been first reported as a non-conditional reporter gene in 1994 by a group of Chalifie et al. (1994) by showing expression in living *Caenorhabditis elegans* cells. Since then it became a powerful and commonly used reporter gene in any model in cell biology. The direct visualization of GFP in living tissue in real time without the need of additives rendered this protein a significant improvement over the conditional systems such as β-*GLUCURONIDASE (GUS)* or *LUCIFERASE (LUC)*. GFP does not exert any cytotoxic effects on plant cells; it is non-destructive and shows a bright fluorescence signal when excited at an absorbance maximum of 480 nm (Chalfie et al., 1994; Miki & McHugh, 2004). Many *Aequorea* fluorescent protein derivatives (xFP), as well as fluorophores from other origins have been developed to obtain a broad assortment of fluorescent marker proteins in different colors. However, only a few of them are routinely used for plant research, such as the original GFP (green), YFP (yellowish green), CFP (cyan) and mCherry (red), to name the major ones (Sanchez-Serrano & Salinas, 2014; Miki & McHugh, 2004).

Materials and Methods

Plant material and plant growth

For all experiments I used two different accessions of *Aethionema arabicum* L.: one ecotype from Turkey and another one from Cyprus (Kato Moni, 410 m). The abbreviations TUR and CYP are constantly used within the whole thesis. Germination of *A. arabicum* seeds was done at 14°C temperature in dark on wet filter paper or, if axenic material was needed, on sterile ½ MS medium (Murashige & Skoog, 1962). After cotyledons appeared, seedlings were planted into soil (4:1 mixture Einheitserde®:Perlite; one plant/pot), and plants were grown in a growth chamber under long-day conditions (16 h light/8 h dark) with a light intensity of approx. 150 µM/s*m^2 supplied by four Osram Cool white light color 840 and two Osram Interna light color 827. The temperature was set to 19°C during daytime and 16°C at night, humidity adjusted to 60 %.

Nicotiana benthamiana Domin. plants were germinated from seeds and grown on soil (1 plant/pot) at 21°C under short-day conditions (16h dark/8h light) with a light intensity of approx. 120 µM/s*m^2 (2x Osram Cool white light color 840; 2x Osram Interna light color 827).

Seedling tissue and callus for *in vitro* experiments were grown on sterile medium in petri dishes at two different conditions: at 23°C in permanent darkness or at 21°C and long-day light regime (8 h dark/16 h light) with a light intensity of approx. 30 µM/s*m^2 (1x Osram Cool white light color 840; 1x Osram Fluora light color 77).

Plant and bacterium media

For germination, I used ½ MS medium (Murashige & Skoog, 1962). Callus was induced on GMCI medium (GM Callus Induction) with an auxin to cytokinin ratio of 7:1. It was derived from the germination medium (GM), used in the Mittelsten Scheid lab (GMI). The protocol for the GM medium is available online: https://www.gmi.oeaw.ac.at/research-groups/ortrun-mittelsten-scheid/resources. The GMCI medium was prepared with the following final sugar and plant hormone concentrations: 30 g/l sucrose, 40 g/l glucose, 2 mg/l 1-naphtalene acetic acid (NAA), 0.15 mg/l 6-benzylaminopurine (BAP), 0.15 mg/l 6-furfurylaminopurine (Kinetin), 0.05 mg/l 2,4-dichlorophenoxyacetic acid (24 D).

For shoot induction out of callus, I used GMSI medium (GM Shoot Induction), with the following plant hormone concentrations: 0.05 mg/l auxin (2,4-dichlorophenoxyacetic acid) and 0.3 mg/l cytokinin (half 6-benzylaminopurine - BAP, half 6-furfurylaminopurine - Kinetin), resulting in a 1:6 ratio.

For shoot induction out of mature leaves I prepared a medium with an even higher content of cytokinin, called SIJan (Shoot Induction Janos Bindics). It consists of ½ MS (pH 5,6 – 5,8),

supplemented with 2 % sucrose, 0.8 % Merck microbiology agar, 2 mg/l BAP and 0.05 mg/l NAA, resulting in a cytokinin to auxin ratio of 40 : 1.

For callus selection upon induction on GMCI medium, four different selection agents were added in the following concentration ranges. Selection agent was added to cooled medium (around 56°C), before pouring the plates.

Hygromycin – 10 mg/l; 25 mg/l; 50 mg/l

Kanamycin – 50 mg/l; 100 mg/l; 150 mg/l

BASTA® – 15 mg/l; 25 mg/l; 50 mg/l

Sulfadiazine – 5 mg/l; 10 mg/l; 20 mg/l

For selection regarding germination, ½ MS medium supplemented with the following selection agent concentrations was used.

Hygromycin – 2.5 mg/l; 5 mg/l; 7.5 mg/l; 10 mg/l; 25 mg/l; 50 mg/l

Kanamycin – 50 mg/l; 100 mg/l; 150 mg/l

BASTA® – 15 mg/l; 25 mg/l; 50 mg/l

Sulfadiazine – 5 mg/l; 10 mg/l; 20 mg/l

Gentamycine – 15 mg/l; 25 mg/l; 50 mg/l

For *Agrobacterium* and *E. coli* growth either liquid LB (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter water) or solidified LB with Bacto® Agar in petri dishes was used. Additional antibiotics for selection were added: rifampicin, kanamycin, gentamycin, carbenicillin, tetracyclin in concentrations according to the *Agrobacterium* strain and the introduced plasmid DNA:

Bacteria medium I (BM I)	LB + 100 mg/l rifampicin, 50 mg/l gentamycin, 100 mg/l carbenicillin
Bacteria medium II (BM II)	LB + 50 mg/l rifampicin, 100 mg/l carbenicillin, 25 mg/l kanamycin
Bacteria medium III (BM III)	LB + 100 mg/l rifampicin, 50 mg/l gentamycin, 25 mg/l kanamycin
Bacteria medium IV (BM IV)	LB + 100 mg/l rifampicin, 10 mg/l tetracyclin, 50 mg/l kanamycin
Bacteria medium V (BM V)	LB + 25 mg/l rifampicin, 50 mg/l kanamycin, 50 mg/l gentamycin

Seed sterilization

Seeds were surface-sterilized by exposure to chlorine gas using the reaction between hydrochloric acid (HCl) and sodium hypochlorite (bleach) in an approx. ratio of 5:1. Seeds (in Eppendorf tubes) and a reaction beaker were placed in a closed container under the fume hood and seeds were treated for 10 min.

Agrobacterium strains

Three different *Agrobacterium* strains were used. The GV3101 strain (Koncz and Schell, 1986) selected with 100 mg/l rifampicin, 50 mg/l gentamycin, 5 mg/l tetracyclin, the AGL1 strain (Lazo et al., 1991), which can be selected with 50 mg/l rifampicin and 100 mg/l carbenicillin and the C58C1 strain with a selection at 100 mg/l rifampicin and 10 mg/l tetracyclin (Kerényi et al., 2008).

Plasmid constructs

The following plasmid vectors were used to introduce the gene of interest into *Agrobacterium* and later into the plant genome. Fig. 5 lists all used constructs, labelled from #1 to #7, and including the original backbone plasmid used for cloning.

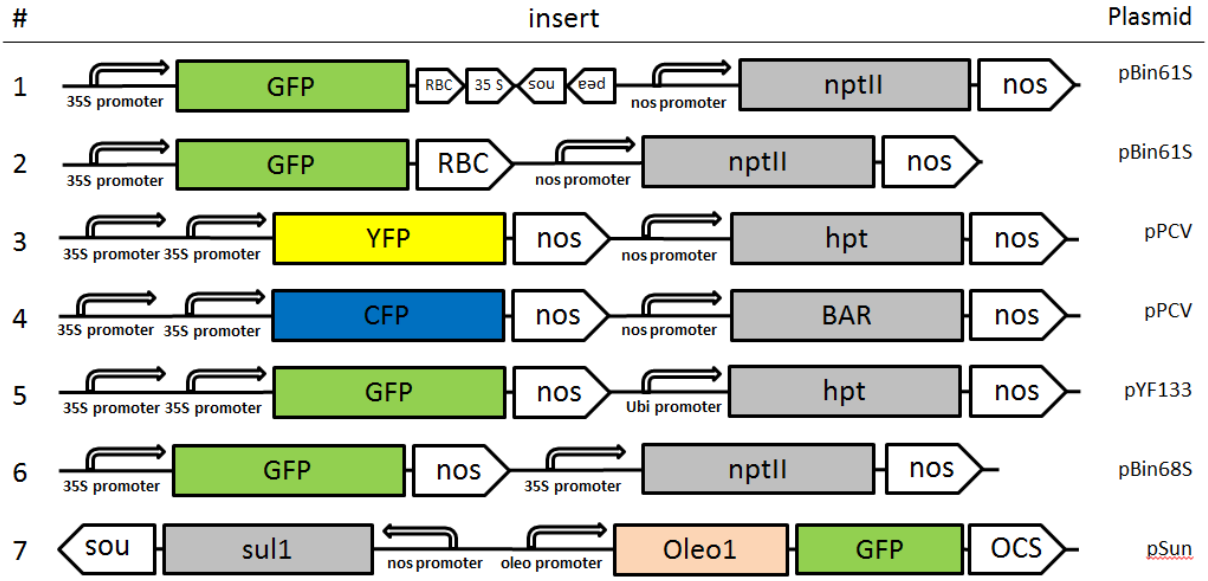

Fig. 5. List of constructs used for transformation, derived from plasmid precursors indicated in the right column. GFP, green fluorescent protein; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; Oleo1, Oleosin protein of *Arabidopsis*; Ubi promoter, promoter of ubiquitin gene; oleo promoter, promoter of oleosin gene (seed-specific expression); RBC, RubisCo terminator; nos, nopaline synthase terminator, 35S, Cauliflower Mosaic Virus 35S terminator; pea, RubisCo terminator *Pisum sativum*; OCS, octopine synthase terminator; nptII, neomycin phosphotransferase II gene;

pBin61S carrying the kanamycin resistance gene NPTII (Silhavy et al., 2002) was used for cloning of either construct #1, #2 or #6 (cloned and kindly provided by Zsuzsanna Mérai, GMI). All three constructs contain also the GFP marker gene with different promoter and/or terminator regions. Koncz & Schell (1986) first described a pPCV plasmid which was used to clone construct #3 and #4**.** Construct #3 consists of the YFP visible marker gene and the hygromycin resistance gene HPT; while construct #4 harbors the CFP visible marker and the Basta resistance gene BAR (Bauer et al., 2004).

An aliquot of each *Agrobacterium* culture with the plasmid was kindly provided by Janos Bindics, GMI.

The pYF133 harboring construct #5 is assembled of GFP and the hygromycin resistance gene HPT (Fang et al., 2002). An aliquot of an *Agrobacterium* culture carrying the construct was kindly provided by Tilo Guse, GMI.

Plasmid #7, derived from pSun (Thomson et al., 2011) was modified by Mattia Donà (GMI) by introducing an Oleosin-GFP construct aiming at seed-specific expression (pElvis, Donà M., unpublished).

Fluorescence microscopy

For callus dissection, the stereo microscope ZEISS Stereo Discovery.V8 (with appropriate emission filters) and the fluorescent lamp lumencor light engine® were used. Taking pictures of calli, I used the stereo microscope Leica MZ16 FA with a Kübler ebo100 fluorescent lamp. Pictures were acquired with a Leica DFC300FX camera and viewed with LAS software (version 4.5.0). For cytological analysis, I used a ZEISS Imager Z2.Stativ + fluorescent lamp lumencor light engine® and took photos with Camera Andor Zyla. Images were viewed and processed (pseudocoloring of B&W images) with Metamorph Version 7.8.8.0 software. Brightfield and fluorochrome images were merged with the open source software Fiji (Schindelin et al., 2012).

Transformation of electrocompetent *A. tumefaciens*

For transformation of *Agrobacterium tumefaciens* with the plasmid DNA of interest the electroporation method by Mersereau et al. (1990) was used. A 500 µl aliquot of electrocompetent *A. tumefaciens* strain GV3101 or AGL1 was mixed with 0.5 µl of plasmid DNA (1 µg/µl) and kept on ice until the mixture was transferred to a pre-chilled electroporation cuvette (0.2 cm gap). An electric pulse at a field strength of 12.5 kV/cm with the BioRad Pulser (settings: 2.5 kV; 25 µF; 400 Ω) was applied for 8–9 ms. Back on ice, 1 ml SOC (Sigma) was added for further incubation in a 2 ml Eppendorf tube for at least 2 h at 28°C and 180 rpm shaking. Hundred µl of the *Agrobacterium* suspension was then plated on selection plates containing appropriate antibiotics (5 g/l tetracycline, 50 g/l gentamycin, 25 g/l rifampicin) for selection of cells amplifying the electroporated plasmid.

Floral dipping

Agrobacterium strain GV3101 (Koncz and Schell, 1986) harboring the plasmid pElvis (Donà, unpublished), which carries an GFP-tagged oleosin gene and a sulfonamide resistance protein (Fig. 5), was used for the floral dipping method. An aliquot of fresh bacteria culture on plate (appropriate

antibiotics supplemented; 25 mg/l rifampicin, 50 mg/l kanamycin, 50 mg/l gentamycin) was grown overnight (16–24 h, 28°C, 180 rpm shaking) in 5 ml sterilized LB. Further inoculation of 250 ml LB + antibiotics with the 5 ml preculture and again overnight growth with the same conditions led to a bacteria suspension in stationary phase. Hundred fifty µM acetosyringone were added, the suspension was grown for another 2 h before measuring the OD_{600} against sterile LB, which was then adjusted to a value of 0.8 by diluting with LB. The suspension was centrifuged (4°C, 10 min, 4000 g) and gently resuspended in 5 % sucrose solution with 0.03 % Silvet L-77.

Three different protocols for dipping were used: either a single-dip of floral buds in suspension for 3 seconds, repeated dipping for half of the plant batch after 2 weeks, or by immersing floral buds in the suspension, placing them in an exsiccator and applying vacuum (vacuum pump vacuubrand 230V, 9 mbar) until bubbles in the solution appeared (5–10 s). One-month-old and 2-month-old plants were used for all three protocols. After dipping, plants were transferred back into the growth chamber (16 h light/8 h dark, 19°C) and were covered with a lid for 24 h to keep the humidity high. GFP fluorescence of mature seeds from dipped floral buds was checked under a stereo microscope (approx. 2 month after dipping).

Sulfadiazine spraying

Seeds were germinated with a soil cover at 14°C, and appearing seedlings were further propagated under long day conditions under a lid to maintain humidity. When the first pair of true leaves appeared, sprayings were performed with either 500 mg/l sulfadiazine + 0.03 % Silvet L-77 or 1000 mg/l sulfadiazine and 0.05 % Silwet L-77 (Thomson et al., 2011), four times over 10 days.

Leaf infiltration

Agrobacterium inoculation solution (AIS): LB; 10 mM MES pH 5.6; 20 µM acetosyringone; appropriate antibiotics (but no rifampicin): 5 g/l tetracyclin, 50 g/l kanamycin, 50 g/l gentamycin.

Agrobacterium resuspension solution (ARS): Sterile distilled water; 10 mM $MgCl_2$; 10 mM MES pH 5.6; 150 µM acetosyringone. Solutions can be stored at 4°C for 2 weeks.

Before inoculation, the bacteria were plated on LB plates containing appropriate antibiotics (BM I – III) dependent on the *Agrobacterium* strain and the plasmid DNA (see below) and were grown overnight (16–24 h) at 28°C. Four ml ABI were inoculated with fresh bacteria and were further propagated overnight on 28°C and 180 rpm shaking. After centrifugation (10 min, room temperature, 3000 rpm) the pellet was resuspended in 4 ml ARS and incubated for at least 3 h at room temperature. OD_{600} was set to 0.4 (if the bacteria suspension was too concentrated, it was diluted

10x for measuring). The infiltration was done by filling a syringe (without any needle) with the bacteria suspension, pressing the opening on the bottom side of the leaf and gently injecting the suspension until the liquid fills up the whole leaf. Two to three leaves per plant were infiltrated. Expression of the marker gene could be detected 3 days post infiltration (dpi) for the first time, after peeling off the lower epidermis or preparing cross sections and checking them under the fluorescence microscope.

The following six *Agrobacterium*-plasmid combinations were tested for leaf infiltration (see also Fig. 5 for visualization of constructs) and grown before on suitable Bacteria Medium (BM):

AGL + pBin61S (GFP-RBCt, Kan^R)	BM II
AGL + pBin61S (GFP-4t, Kan^R)	BM II
GV3101 + pBin61S (GFP-RBCt, Kan^R)	BM III
GV3101 + pBin61S (GFP-4t, Kan^R)	BM III
GV3101 + pPCV (YFP-nost, Hyg^R)	BM I
GV3101 + pPCV (CFP-nost, $Basta^R$)	BM I

Particle bombardment

Particle preparation

Sixty mg gold particles (BioRad® Microcarrier, 1.6 µm diameter) were suspended in 1 ml chloroform (100 %), vortexed for 3 minutes and centrifuged for 1 min at maximum speed. The supernatant was discarded. For washing, the particles were suspended in 1 ml absolute ethanol, vortexed for 1 min at maximum speed and the supernatant discarded. This step was repeated twice to remove all greasy contamination and residual chloroform. The same washing procedure was done three times with sterile water. One ml 50 % glycerol was used to resuspend the gold particles and 25 µl aliquots were stored at -20°C.

DNA attachment

QIAprep® Spin Miniprep Kit was used to prepare plasmid DNA (pBinS61 GFP-nost, Kan^R) from *E. coli* cultures grown overnight in 2 ml LB + 50 g/l kanamycin starting with a single colony. DNA concentrations were measured by NanoDrop® ND-100, samples were combined to get approx. 4 µg DNA, and liquid was reduced with an Eppendorf concentrator 5301 until 2.5–5 µl were remaining. This plasmid DNA solution was added to 25 µl gold particles (previously prepared) and vortexed for 3 min, before 25 µl $CaCl_2$ (2.5 M) were added and vortexed for another 3 minutes. Afterwards 10 µl spermidine (0.1 M) were added, the suspension was vortexed for 3 min, 1 ml 100 % ethanol was added (plus vortexing for 1 min) and gold particles were collected by 10 sec spinning and removing the supernatant. One washing step with 70 % ethanol followed (10 sec spinning, remove

supernatant) and particles were finally resuspended in 70 µl 100 % ethanol and gently vortexed before bombardment.

Bombardment

Bio Rad Biolistic® PDS-1000/He Particle Delivery System was used for bombardment of hypocotyl tissue of 1-week-old etiolated seedlings (approx. 2 cm long). Seedlings were attached on microscope slides with FixoGum glue and roots were kept in water before bombardment. Six µl of DNA-loaded gold particles in 100 % ethanol (prepared previously) were pipetted on the microcarrier, and a 900 PSI rupture disk was used for pressure control.

After bombardment, seedlings were kept in dark (roots in water) for 24 h prior to analysis of marker gene expression under a fluorescence microscope.

Cloning

The phytoene desaturase gene (PDS) and the indehiscent gene (IND) were amplified from genomic DNA (1:10 dilution) of both accessions (CYP and TUR) by PCR (Biometra® T3000 Thermocycler) using the following primers, that included an EcoRI restriction site for further cloning: PDS-For – ATGTTAACAATATTACGATCCGAATCGAT; PDS_Rev – ATGAATTCCTGAACAATAGCTTGTGAGC; IND_For – ATGTTAACCATCTCATCACTCCACACTTC; IND_Rev – ATGAATTCTGTCGTTTCAAGAACTTGGC.

PCR fragments were purified with NucleoSpin® Gel and PCR Clean Up Kit, digested with *EcoRI* in Fast Digest Buffer (10x stock) and purified again before fragments were checked on a 1 % agarose gel.

The TRV2-PDS vector (Kerényi et al., 2002) was purified out of the *Agrobacterium* strain C58C1 by Quiagen® Miniprep, then digested with *EcoRI*, treated with Shrimp alkaline phosphate (SAP) to remove phosphate groups and prevent self-ligation and finally purified with phenol-chloroform extraction.

The inserts were ligated with the TRV2-PDS vector plasmid, using ligase 4T (and appropriate ligase buffer 4T) and incubating for 2 h at 16°C, resulting in *Aethionema*-specific VIGS plasmids.

Ligated plasmid was transformed into *E.coli* (DH5α) by adding 5 µl of ligation reaction to 100 µl *E.coli,* and keeping the suspension 10 min on ice before initiation of the plasmid incorporation (1 min at 42°C in a water bath). Afterwards 400 µl LB was added and suspension was incubated for 1 h at 37°C and 180 rpm. A 100 µl aliquot was plated on LB + 50 mg/l kanamycin and bacteria were grown overnight at 37°C. Ten colonies per gene were inoculated in 2 ml LB + 50 mg/l kanamycin and Quiagen® MiniPrep was used for plasmid purification. Each plasmid was checked again by *EcoRI* digestion and gel separation, before it was introduced into electrocompetent *Agrobacterium* strain GV3101. Fig. 6 shows cloned constructs used in the VIGS experiment.

Virus-induced gene silencing (VIGS)

The leaf infiltration protocol (see above) was used to introduce *Agrobacterium* (carrying plasmids with virus sequence) into plant leaves by pressing the syringe on the lower epidermis and gently infiltrating the suspension (3-4 leaves/plant). Either 1-month-old or 2-month-old *Ae. arabicum* plants and 1-month-old *N. benthamiana* plants were used (Fig. 16, A). Leaves were penetrated with several punctures using a needle to create potential infection sides.

A three compound system based on the two-component *Tobacco Rattle Virus* (TRV) was used to trigger specific mRNA decay in plant cells, which was described previously by using a pBin61S vector within the *Agrobacterium* strain C58C1 (Fig. 4). The two virus compounds were contributed by separate plasmids: TRV1 and TRV2 with phytoene desaturase (PDS) sequence attached (sequence of the target gene to be silenced). Additionally, the silencing suppressor myc-p14 was used in a third plasmid to suppress the silencing of the virus at the infection side and allow a spread of the virus within the first days (Kerényi et al., 2008). The system was kindly provided by Zsuzsanna Mérai, GMI.

As described above, two constructs harboring *Aethionema* genes were generated based on the existing TRV2 construct (Kerényi et al., 2008) and were introduced into *Agrobacterium* strain GV3101 by electroporation. Either phytoene desaturase (AaPDS) or indehiscent gene (AaIND) sequence was attached to existing *N. benthamiana* TRV-PDS construct (Fig. 6).

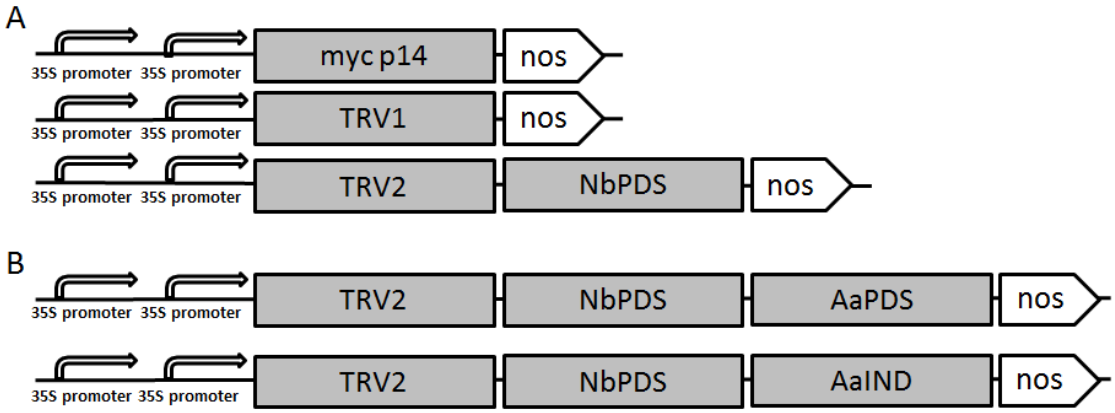

Fig. 6. Organisation of constructs used for virus-induced gene silencing (VIGS). A, original system by Kerényi et al. (2008). B, subcloned constructs with *Ae. arabicum*-specific inserts (AaPDS, AaIND). 35S promoter, CaMV 35S promoter; nos, nopaline synthase terminator; myc p14, silencing suppressor; TRV1, Tobacco Rattle Virus part 1; TRV2, Tobacco Rattle Virus part 2; NbPDS, phytoene desaturase *N. benthamiana*; AaPDS, phytoene desaturase *Ae. arabicum*; AaIND, indehiscent gene *Ae. arabicum*.

Before infiltration of the three component mixture, the final OD_{600} had to be adjusted. For the myc-p14 *Agrobacterium* suspension, the final OD_{600} was set to 0.2; for the rest of the constructs a final

OD_{600} of 0.4 was adjusted. OD_{600} for each suspension was adjusted to 3 times the final value, so that subsequent mixing of the three suspensions lead to a mutual dilution of each strain to the required final OD_{600}.

Another VIGS infiltration approach, the so-called leaf suspension infiltration with amplified virus material, was performed besides the direct *Agrobacterium* infiltration. *N. benthamiana* leaves, 5 dpi after infiltration with VIGS constructs, were ground in 2 – 3 ml (depending on amount of leaf material) 1x PDS at room temperature. The suspension was filtered with Fisherbrand 40 µm Nylon Mesh to remove suspended matter. The flow-through suspension was infiltrated into *Ae. arabicum* leaves using a syringe, as described above. A similar approach was reported previously in *N. benthamiana* (Lu et al., 2003).

RNA extraction

For RNA extraction, a maximum of 100 mg leaf material was frozen in liquid N before grinding with mortar and pestle in the appropriate amount of buffer (use hood – strong smell). The Qiagen® RNeasy® Plant Mini Kit was used. By following the protocol of the kit, the RNA could finally be eluted in 50 µl RNase-free water and stored at -20°C. Samples were thawed at 70°C (important not to thaw at 37°C – RNase is most active at this temperature) before using NanoDrop® ND-1000 to determine the concentration of RNA in each sample.

To load equal amounts on the gel, the total amount of RNA per sample has to be calculated dependent on the lowest measured concentration, which required RNA concentration measurement by nanodrop and subsequently calculating the appropriate volume for each sample (Table 1). The samples were loaded on a 1.2 % gel with 1x

leaf material	sample	concentration (ng/µl)	purity value 260/280	purity value 260/230	calculated volume (µl)
N. benthamiana	wt	301,23	2,07	2,31	2,46
	wt	424,60	2,05	2,30	1,74
	AePDS	468,46	2,01	2,31	1,58
	AePDS	482,39	2,01	2,30	1,53
	AeIND	278,58	2,06	2,25	2,66
	AeIND	803,38	2,11	2,02	0,92
Ae. arabicum	wt TUR	191,41	2,08	2,11	3,87
	wt CYP	192,45	2,13	0,74	3,85
	AePDS TUR	238,03	2,10	1,87	3,11
	AePDS CYP	218,59	2,07	0,97	3,39
	AeIND TUR	199,73	2,09	0,88	3,71
	AePDS CYP	148,00	2,07	2,00	5,00

Table 1. Concentrations of total RNA per leaf extraction in *N. benthamiana* and *Ae. arabicum* & calculated volume for sample loading on the gel. wt, wildtype. AePDS, phytoene desaturase *Aethionema*. AeIND, indehiscence gene *Aethionema*. CYP, Cyprus accession. TUR, Turkey accession.

TAE buffer. Before loading samples, 5 µl RNA loading dye was added and samples were denaturated at 65°C for 5 min.
To remove all potential RNase, all gel chamber devices were cleaned with soap, new buffer was used and contamination was generally avoided.

Tissue culture: co-cultivation with *Agrobacterium*

For tissue culture, 10-d-old seedlings (germinated at 14°C and kept for 1 day before use in a growth chamber at 21°C under long-day conditions) were used for callus induction on GMCI medium, by cutting them in four anatomical parts (cotyledons; upper greenish part of hypocotyl; transparent lower part of hypocotyl in 3-4 pieces; root without root tip; and the root tip itself) and ensuring good contact with the medium.
For *Agrobacterium* preparation, an aliquot of bacteria on plate was incubated overnight (16-24 h, 28°C, 180 rpm) in 10 ml of appropriate Bacteria Medium (BM) (described above – methods bacterium media). Acetosyringone (150 µM) was added and the culture incubated for another 2 h on the same conditions. The OD_{650} was set to 0.1 by diluting with LB.
For co-cultivation, the seedlings were simply dipped in *Agrobacterium* suspension for 1 second, cut into parts as described above and placed onto the GMCI medium.
Tissues were co-cultivated for 3 days on GMCI in dark at 23°C with *Agrobacterium* before bacteria were washed off by rinsing plant parts in sterile water and transferring them to GMCI medium containing 100 mg/l timentin to kill the bacteria.

The following *Agrobacterium*-plasmid combinations were tested for co-cultivation (see also Fig. 5 for visualization of constructs):

AGL + pBin61S (GFP-RBCt, Kan^R)	BM II
GV3101 + pBin61S (GFP-4t, Kan^R)	BM III
GV3101 + pPCV (YFP-nost, Hyg^R)	BM I
AGL + pPYF133 (GFP-nost, Hyg^R)	BM II
GV3101 + pBin61S (GFP-nost, Kan^R)	BM III

Fluorescence marker in callus tissue was detected under a stereo microscope (with fluorescent lamp and appropriate filter). Fluorescent callus parts were chopped of and transferred onto fresh GMCI medium with 100 mg/l timentin and 20 mg/l hygromycin to select for transformed calli.
GMCI medium was changed every 2 weeks, and all plates were sealed with micropore tape and kept in dark at 23°C.

Shoot differentiation out of mature leaves

Mature leaves of 1-month-old plants were surface-sterilized with 0.5 % sodium hypochlorite (bleach) and approx. 100 droplets/l Tween 20 for 10 min. Two washing steps with sterile water followed (2 min each). Leaves were cut in 3-5 slices transversal to the longitudinal axis and transferred onto SIJan medium. Leaf slices were kept tightly attached to the medium for proper nutrient and hormone supply. Plates were sealed with micropore tape and kept at 21°C and long day conditions. Medium was changed every two weeks.

Results

Selection system

To establish an appropriate selection system for *Ae. arabicum*, I tested seed germination on commonly used selection agents in ½ MS medium and callus induction in different selective GMCI medium. The media were supplemented with hygromycin, kanamycin, BASTA®, gentamycin or sulfadiazine (concentrations see in Materials and Methods).

On hygromycin medium, seedlings and also callus show a remarkable growth reduction effect, while for all other agents no sensitivity was detected. Ten mg/l hygromycin supplemented to ½ MS medium inhibited the seedling growth approx. 10 fold (Fig. 7 and Fig. 8). A slight growth reduction was already observed for low hygromycin content between 2.5 mg/l and 7.5 mg/l (Fig. 8, B).

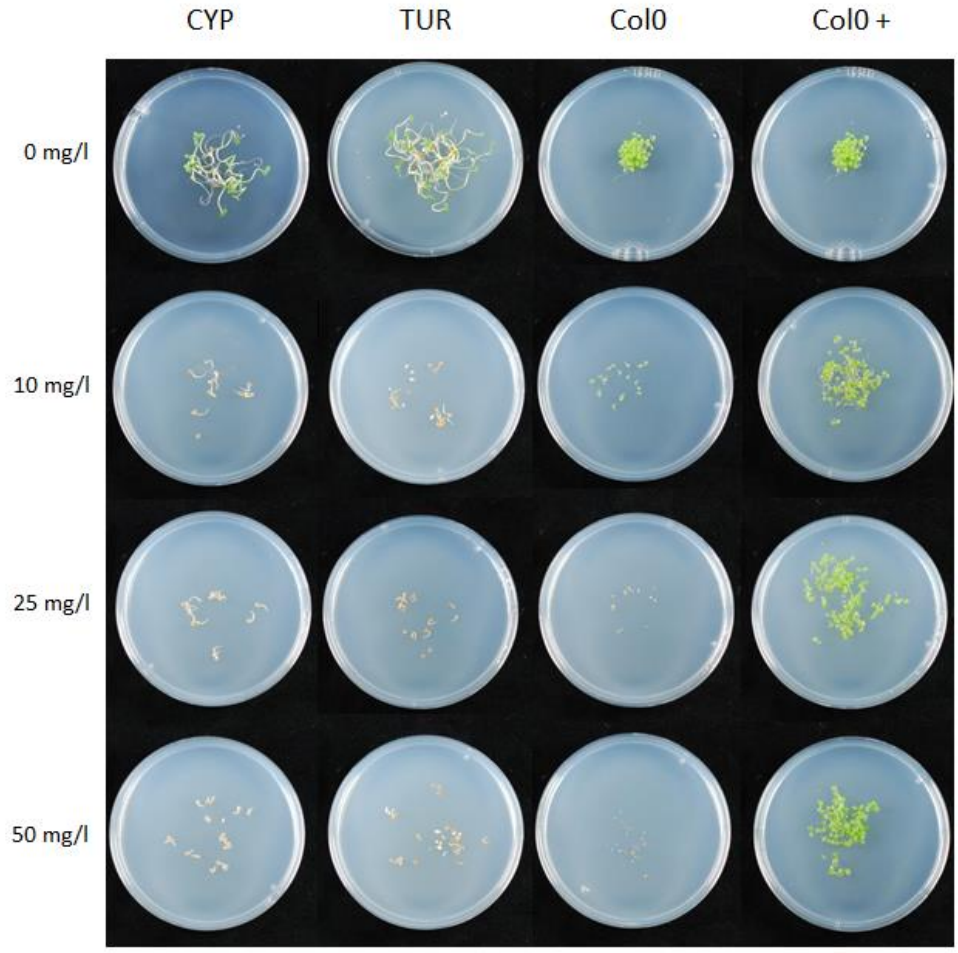

Fig. 7. Germination of *Ae. arabicum* and *A. thaliana* upon hygromycin. Concentrations are indicated on the left side from 0 to 50 mg/l hygromycin. *A. thaliana* Col0 as negative control. Col0 +, positive control – T-DNA insertion line that carries a hygromycin resistance gene. Seedlings 15 days after plating.

Callus induction on GMCI medium could be inhibited with 7.5 mg/l hygromycin (Fig. 8, A). Above of 7.5 mg/l, the explants were dying, and subsequently no callus could be induced. For further tissue culture, 20 mg/l hygromycin was used, resulting in an unambiguous selection system.

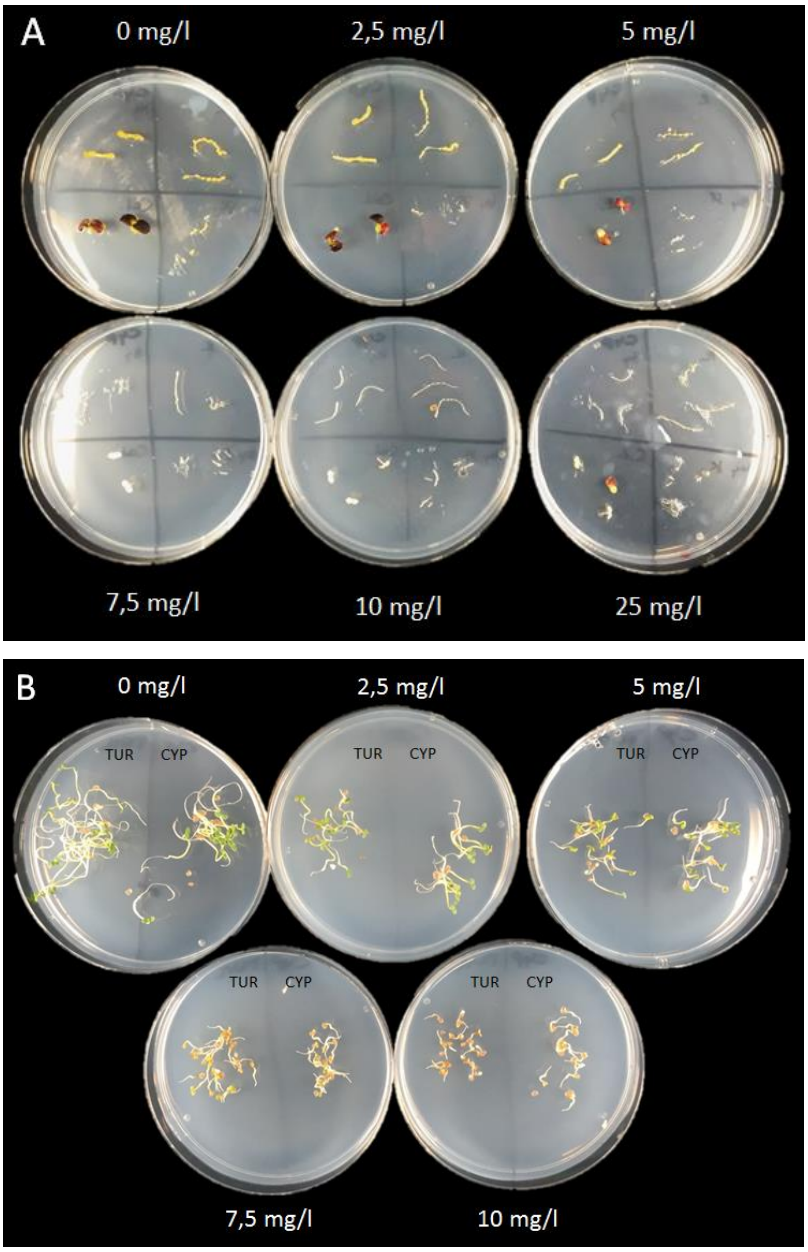

Fig. 8. Germination and callus induction on medium supplemented with hygromycin. A, Cut *Ae. arabicum* explants from 10 d-old seedlings on GMCI supplemented with hygromycin B; 35 dpi. B, Seedlings of *Ae. arabicum* (TUR/CYP) on ½ MS medium supplemented with hygromycin B, 12 days after plating.

Floral dipping

Three different dipping approaches with *Agrobacterium* suspension were attempted to target floral tissue of 1-month-old and 2-month-old *Ae. arabicum* plants, to subsequently integrate an oleosin-GFP construct into genomic DNA. One-month-old plants just started flowering, to target a very early stage of flower development, and 2-month-old plants represent already all stages of flowers including the first fruits, to widen the range of potential target tissues for *Agrobacterium* (Fig. 9, G).

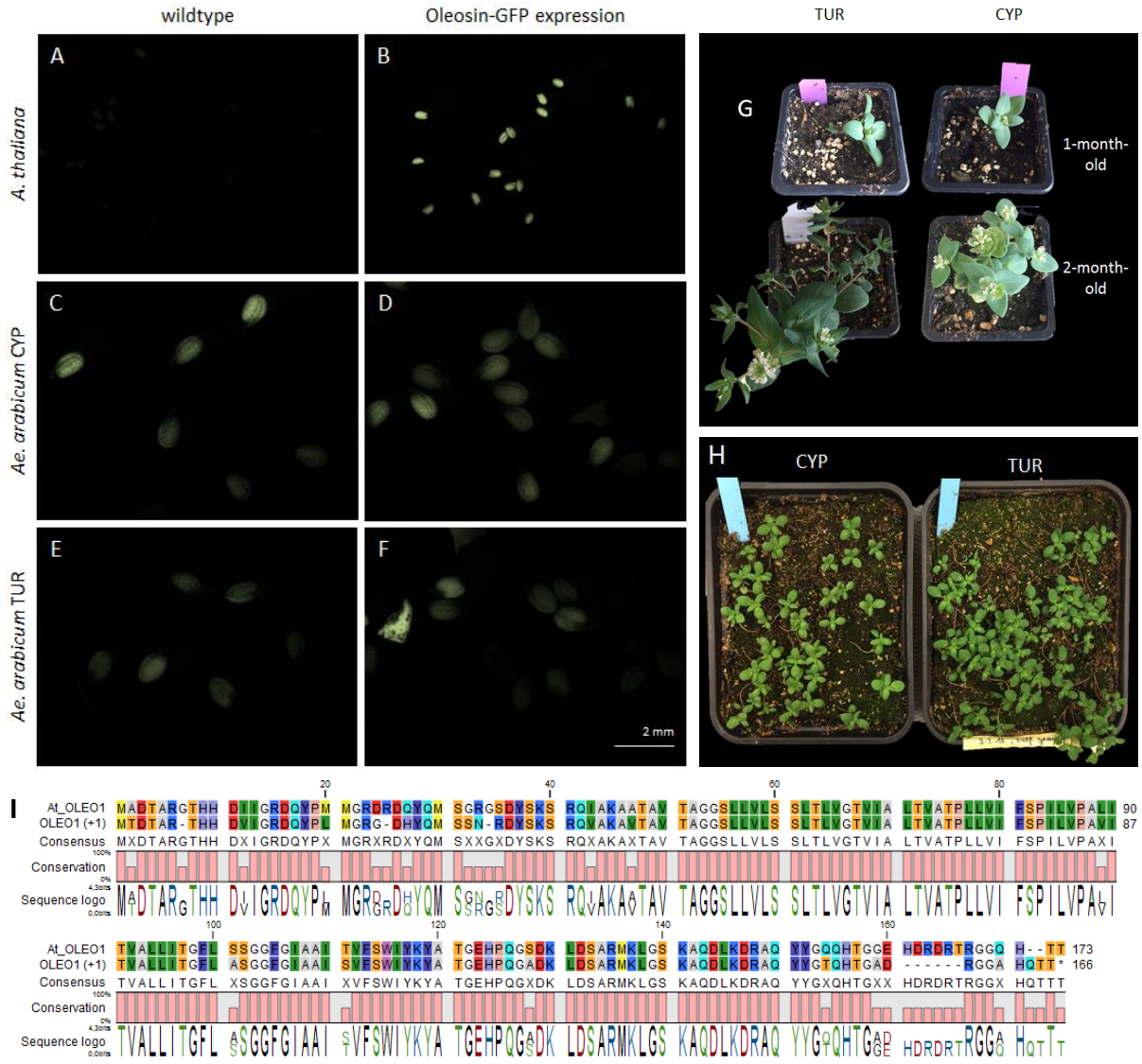

Fig. 9. Floral dip method in *Ae. arabicum*. A–F, GFP-fluorescence of *A. thaliana* and *Ae. arabicum* seeds, images taken with the same exposure time and magnification. A, C, and E, seeds from non-dipped control plants. B, positive control seeds of single dipped *A. thaliana* plants expressing GFP-tagged oleosin protein. D and F, seeds from double dipped *Ae. arabicum* plants (repeated dipping after 1 week). G, Plants from both accessions, Cyprus (CYP) and Turkey (TUR), either 1 month or 2 month after germination. H, seedlings 13 days post sulfadiazine spraying; 4 sprayings within 10 days; 1st spray when first true leaves appear. I, Protein sequence comparison between *A. thaliana* oleosin protein (upper line; At_OLEO1) and *Ae. arabicum* TUR oleosin protein (lower line; OLEO1).

For the marker gene, I have chosen an approach that provides an easy selection system for transformed seeds in *Arabidopsis*, as the fusion of a GFP-tagged oleosin protein expressed specifically in seeds allows identification of transgenic seeds already in the progeny of the dipped plants (Fig. 9, A and B). I blasted the protein sequence of *Arabidopsis* oleosin against that of *Aethionema,* and it showed high similarity, indicating that the *Arabidopsis* specific construct might also work for *Aethionema* seeds (Fig. 9, I). However, the approach in *Aethionema* was hampered as the distinction of transformed seeds from wildtype was problematic due to high autofluorescence at a similar spectrum as GFP in seeds in both accessions (Fig. 9, C–F). Whether the infection worked at all and the GFP gene was expressed in mature seeds, therefore cannot be answered. Changing the fluorophor would not help as Aethionema seeds also showed a slight autofluorescence when excited with YFP- and mCherry-specific wavelengths, although not as strong as for GFP.

As the T-DNA insert in the plasmid harbors also a sulfadiazine selection marker to select for transformants in the T1 generation, I attempted to spray seedlings with two different concentrations of sulfadiazine (500 mg/l + 0.03 % Silvet; 1000 mg/l + 0.05 % Silvet). However, wild type seedlings of *Ae. arabicum* were not affected in growth (Fig. 9, H), although the sulfadiazine concentration was twice as high as commonly used for *Arabidopsis* selection (Thomson et al., 2011). An appropriate and still economically efficient sulfadiazine selection system needs to be established to test T1 seeds for the selectable marker gene.

Leaf infiltration

Leaf infiltration is a commonly used tool to test transient expression of constructs in the model plant *N. benthamiana*, which serves as reference also for this study. Two different GFP constructs, which differ in their terminator sequence (see Materials and Methods – Fig. 5), were introduced in two distinct *Agrobacterium* strains, which lead to four different combinations tested for leaf infiltration. In lower epidermis cells of *N. benthamiana,* the GFP expression led to a strong fluorescent signal two days post infiltration (dpi), regardless of the *Agrobacterium* strain-construct combination. The expression was abundant all over the epidermis indicated by strong signals for nuclei and along the cell membrane at 10 ms exposure time (Fig. 10).

For both *Ae. arabicum* accessions, the expression was weaker than in *N. benthamiana,* requiring a prolonged exposure time between 200 and 600 ms to document the signal. Furthermore, less cells than in *N. benthamiana* expressed GFP. Cross sections showed fluorescence for a group of cells at the lower leaf side (Fig. 11), and surface peels of the lower epidermis showed single positive cells. Eight days after infiltration, the signal could still be observed in single cells of CYP epidermal tissue, when infected with the GV3101 strain (Fig. 10). For the TUR accession, positive cells could be observed in leaf surface cuts only sporadically over time, but in cross sections, signals could be

detected even nine days after infiltration. Fig. 11 shows leaf cross sections of TUR leaves, whereas surface cuts for TUR are not shown due to lack of data over the time period.

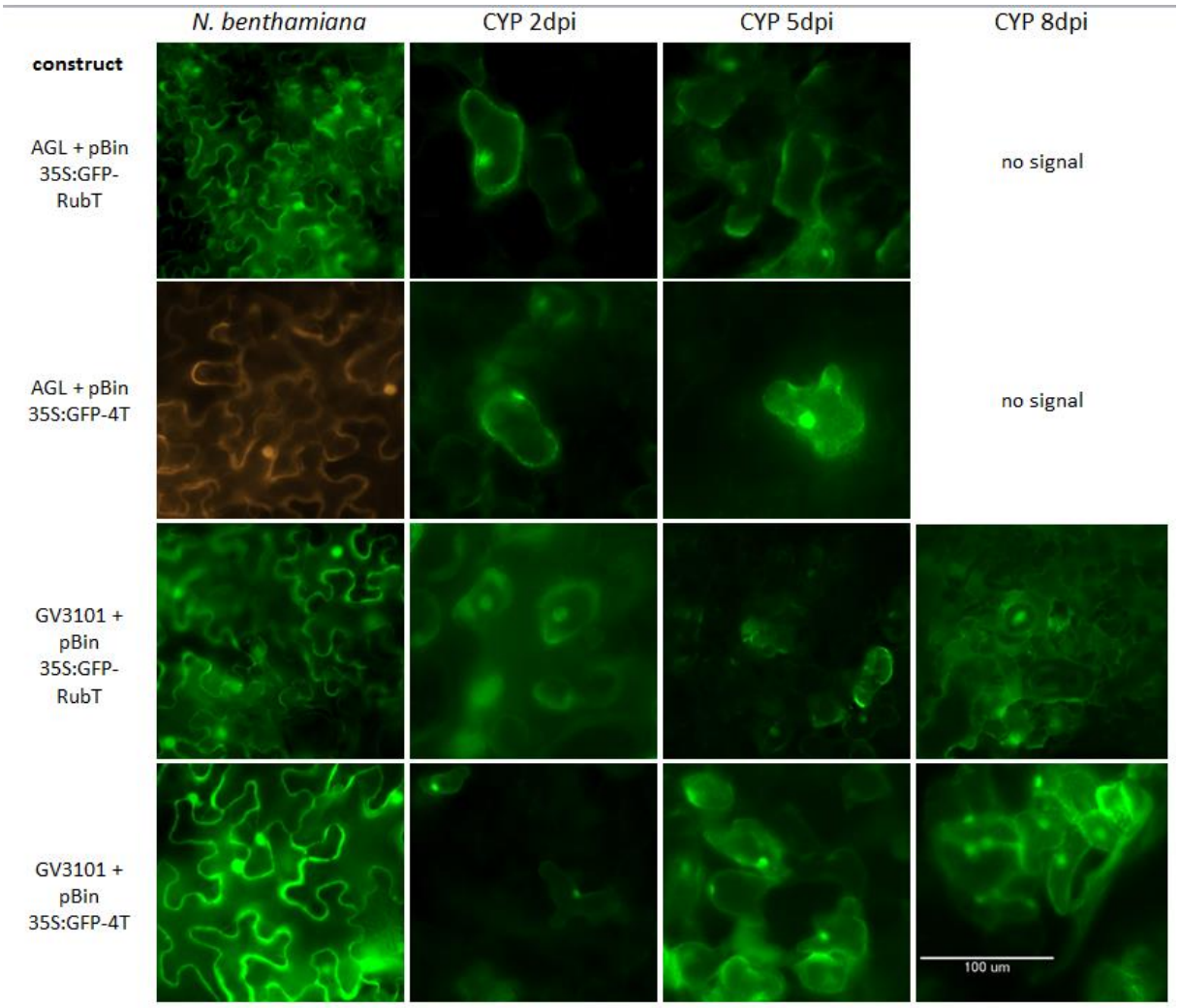

Fig. 10. GFP fluorescence signal of epidermal *N. benthamiana* and *Ae. arabicum* CYP cells after leaf infiltration. Images taken with optimized exposure time for each sample (signal strength not comparable), for *N. benthamiana* 10 ms; for *Ae. arabicum* 200 – 600 ms. Two different GFP constructs in 2 distinct *Agrobacterium* strains were used. *N. benthamiana* at 2

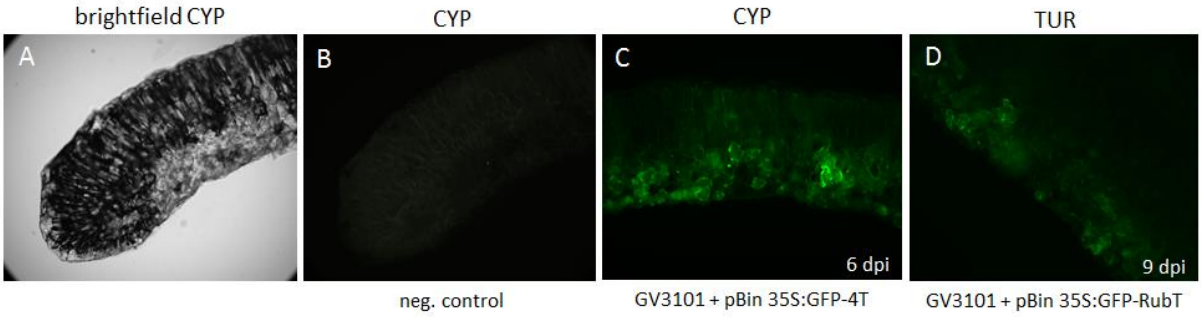

Fig. 11. GFP fluorescence in leaf cross sections in *Ae. arabicum* after leaf infiltration. A, brightfield image of non-infiltrated CYP leaf. B, non-infiltrated leaf as negative control. C, GFP signal in CYP 6 dpi. D, GFP signal in TUR 9 dpi.

In general it seems that CYP shows a stronger transgene expression compared to TUR, with more positive cells per cut and longer persistence over time. To confirm this, more replicates should be statistically tested than the two independent experiments with 3 biological replicas for each ecotype in this study.

Particle bombardment

Two independent bombardment experiments for 10 biological replicates per accession and experiment were performed with the same settings. The biolistic approach resulted in transient expression of single cells in hypocotyl tissue of etiolated seedlings for both accessions (Fig. 12).

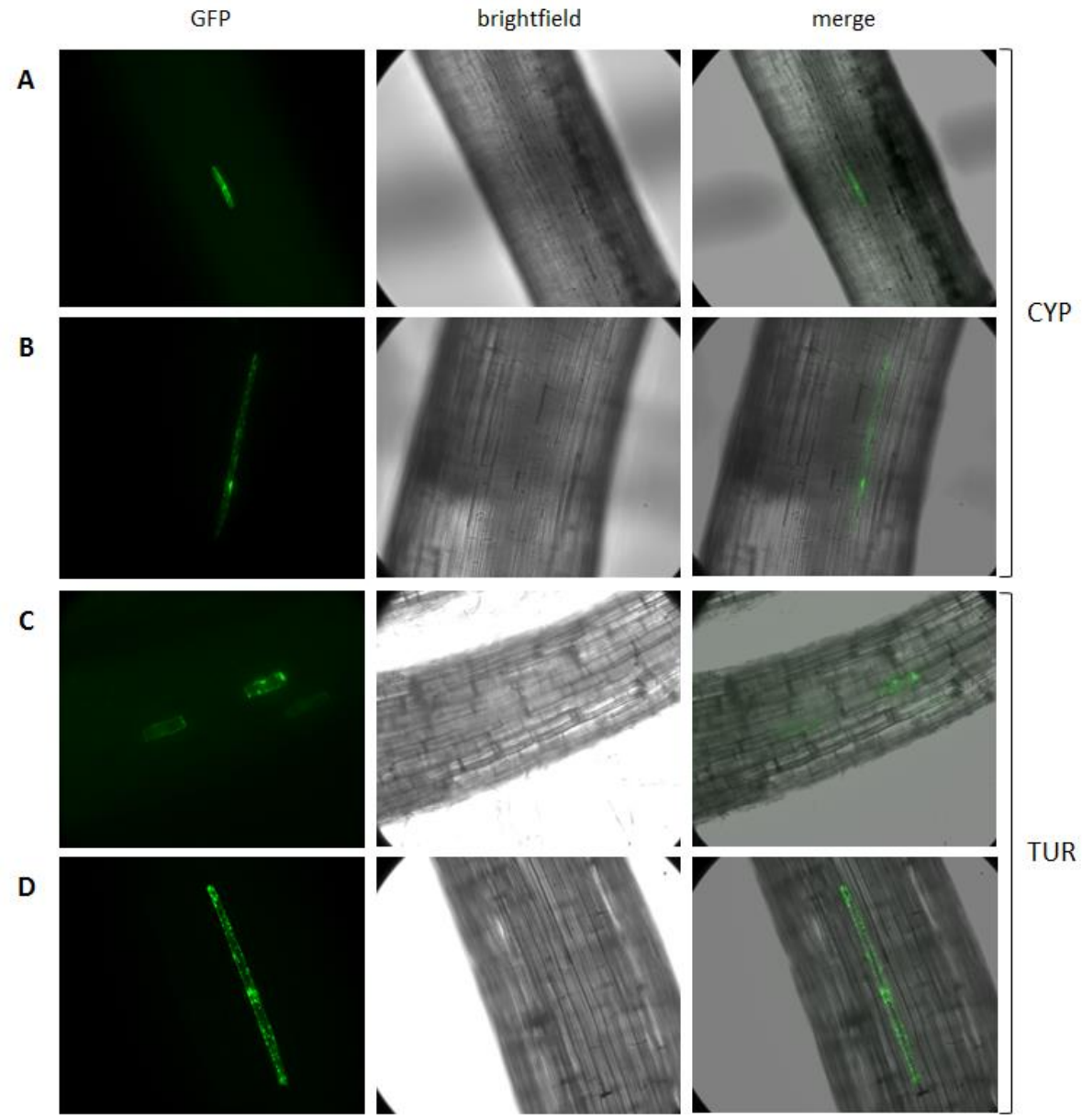

Fig. 12. GFP expression in single cells in hypocotyl tissue of *Ae. arabicum* CYP (A and B) and TUR (C and D) after particle bombardment. A and C, expression in cylindrical cells from pericycle, endodermis, cortex or epidermis. B and D, expression in tube-like sieve vessels with GFP conglomerates within the cell.

Approximately 1-3 cells per hypocotyl showed GFP expression. The low number is explained by the fact that the DNA from the coated gold particle must enter the nucleus to initiate expression of the introduced plasmid. Interestingly, the majority of the observed fluorescent cells are sieve vessel cells of the vascular system. Sieve vessels are elongated tube-like cells. Within these cells, I observed GFP conglomerates (Fig. 12, B and D). The rest of the fluorescent cells (approx. 25 %) showed a cylindrical shape with the GFP signals throughout the whole cell and stronger expression within the nucleus. Those cells rather belonged to the pericycle, endodermis, cortex or epidermis tissue layer (Fig. 12, A and C).

Targeting meristematic tissue, combined with further setting adjustments of pressure, distance between stationary microprojectiles and target tissue, as well as higher amount of particles could result in higher efficiency and might lead to stable transformed plants. A tissue culture approach to use bombarded hypocotyl tissue for callus induction followed by regeneration to viable and fertile plants can also be discussed to obtain germline-transformed *Aethionema* plants. Some success in tissue culture and shoot differentiation is provided in the following chapters.

VIGS - Virus induced gene silencing

With the virus-induced gene silencing (VIGS) method, genes of interest can be silenced within the entire plant, based on the phenomenon of RNA interference (RNAi).

The 3-compound system, previously described by Kerényi et al. (2008), was established for *N. benthamiana* and therefore, I performed the first experiment with 1-month-old *N. benthamiana* plants to show the effect of leaf whitening after infiltration with *Tobacco Rattle Virus* (TRV) and phytoene desaturase (PDS).

Ten days after infiltration, newly appearing leaves started to bleach, and a month after infiltration, all fresh leaves were completely white (Fig. 13, A). The silencing spread within the entire plant, indicating siRNAs triggering silencing of the PDS transcript in every single cell. Leaves were largely without any chlorophyll although a slight mosaic effect was observed, when chlorophyll remained in smaller leaf areas. This effect was observed particularly when the plants became older (Fig. 13, C). Also stems and flower calyces showed the whitening effect; nevertheless the plants managed to survive and produced viable seeds. However, the silencing effect was not transmitted to the filial generation, as seedlings showed no whitening and were not distinguishable from wildtype (Fig. 13, D).

Infiltration with the myc-p14 silencing suppressor (supposed to suppress the silencing response of the plant in an early stage of infection) in addition to the 2-component TRV showed no difference compared to the experiments without myc-p14, as it is shown in Fig. 13, B. Both approaches led to

clearly observable bleaching. This indicated that the viral RNA could even spread without the help of an additional silencing suppressor.

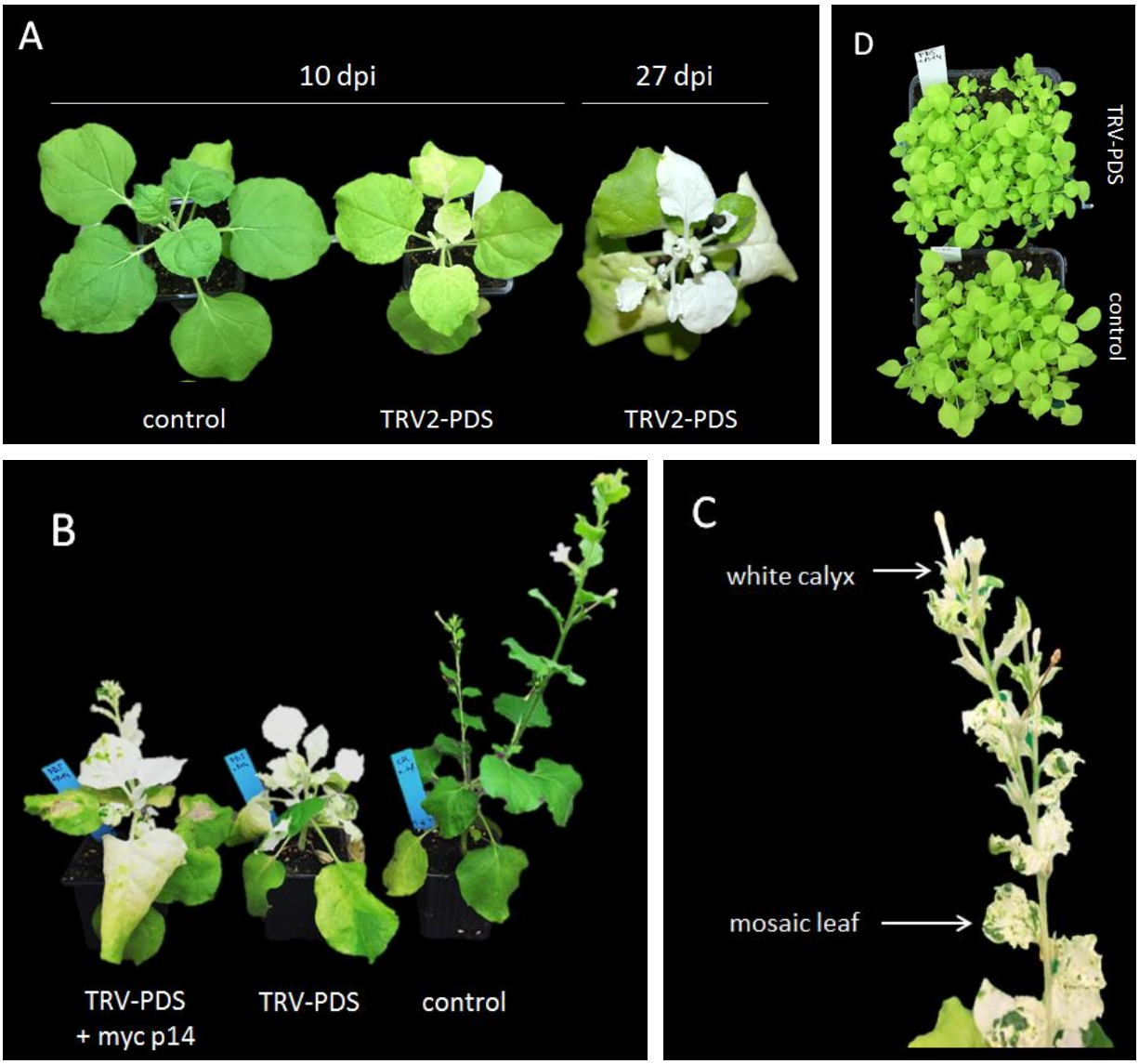

Fig. 13. Photobleaching after application of the VIGS system with TRV-PDS in *N. benthamiana*. A, TRV2-PDS infected plants 10 dpi and 27 dpi display whitening of leaves. B, comparison of whitening effect with and without silencing suppressor myc p14; plants 35 dpi. C, upper part of mature *N. benthamiana* plants indicating the mosaic effect in leaves and the whitening of floral tissue developed long after infiltration. D, *N. benthamiana* progeny of VIGS-exposed plants show no whitening and no difference to wildtype.

The cloned *Aethionema*-specific inserts (Fig. 6, B) were used for infiltration of *N. benthamiana* and *Ae. arabicum*, to prove the presence of the virus on RNA extracted from leaves 5 days post infiltration (dpi). The two RNA parts of the virus could be detected in *N. benthamiana* for both constructs (IND and PDS), which indicates the activity of the virus within the plant (Fig. 14). The AeIND virus construct showed clearer bands on the gel than the AePDS insert version. The whitening

effect in *N. benthamiana* was observed 10 dpi, because the constructs still carried also the NbPDS, also proving the activity of the virus within the plants. In *Aethionema* leaves however, neither virus RNA could be detected 5 dpi nor was a whitening effect visible.

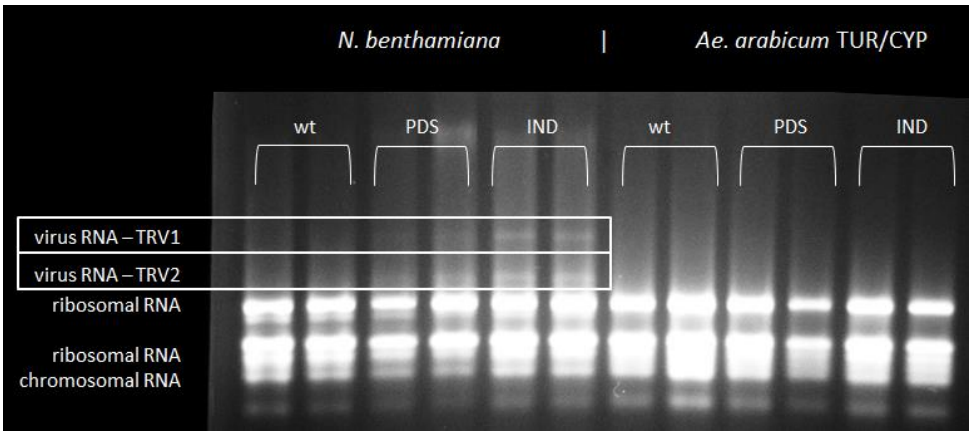

Fig. 14. RNA assay of infiltrated leaf material of *N. benthamiana* and *Ae. arabicum*; containing virus RNA of *Tobacco Rattle Virus* with the *Aethionema*-specific inserts PDS and IND. wt, wildtype. PDS, phytoene desaturase gene insert. IND, indehiscence gene insert. Two replicates per sample *in N. benthamiana*; *Aethionema* material from TUR and CYP (first line per sample TUR).

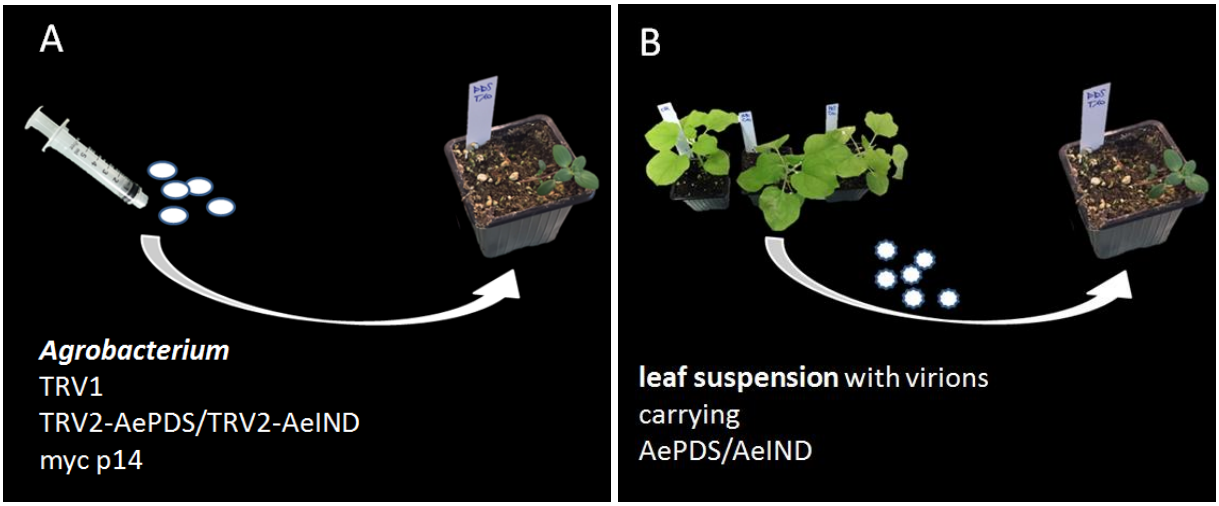

Fig. 15. Infiltration approaches used for VIGS. A, direct infiltration of the three-component system via mixed *Agrobacterium* suspension carrying three different plasmids/inserts. TRV1, *Tobacco Rattle Virus* part 1; TRV2-AePDS, *Tobacco Rattle Virus* part 2 + *Aethionema* phytoene desaturase insert; TRV2-AeIND, *Tobacco Rattle Virus* part 2 + *Aethionema* indehiscence gene insert; myc p14, silencing suppressor. B, infiltration of *N. benthamiana* extract carrying viral RNA (virions) with either PDS or IND insert.

Table 1 (see Materials and Methods) indicates the RNA concentrations measured by nanodrop and the calculated volume for loading on the gel.

These results led to another infiltration approach: to provide a higher density of virus in the initial stage, I prepared a leaf suspension of *N. benthamiana* from infiltrated leaves (5 dpi) carrying viral

RNA of the *Aethionema* specific constructs, which was supposed to contain amplified virions. I used this suspension to infiltrate *Ae. arabicum* leaves. Fig. 15 illustrates the two infiltration approaches. The TRV-VIGS system in *N. benthamiana* works well at 21°C and short day light conditions (8h light/16 h dark). For *Ae. arabicum* however, the temperature for the optimal performance of the virus had to be adjusted. Therefore I tested three different conditions:

- long day (16 h light/8 h dark), 19°C daytime, 16°C at night, direct and virion infiltration.
- short day (8 h light/16 h dark), 21°C, direct and virion infiltration.
- long day (16 h light/8 h dark), 21°C, direct infiltration.

No whitening effect could be observed for *Ae. arabicum* infiltrated with TRV-AePDS, neither in CYP nor in TUR, and regardless of the different conditions and infiltration approaches. This was confirmed by the RNA assay described above (Fig. 14).

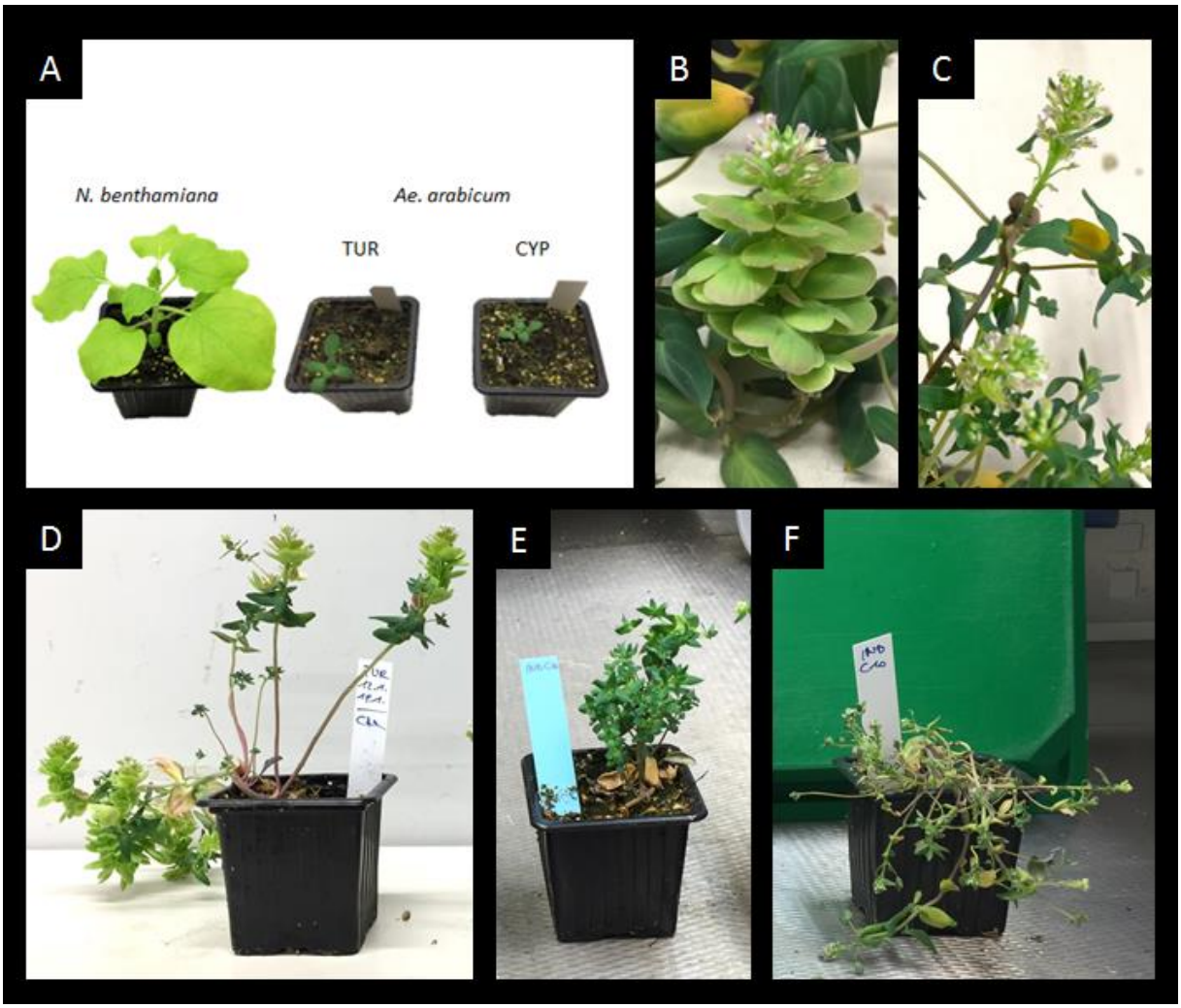

Fig. 16. Developmental plant stage for infiltration and resulting phenotype after TRV-IND infection in *Ae. arabicum* TUR. A, 1-month-old plants of *N.benthamiana*, *Ae. arabicum* TUR and CYP used for infiltration. B, inflorescence of a 3-month-old TUR plant. C, inflorescence of a 3-month-old TUR plant upon TRV-IND infection, 54 dpi. D, TUR control plant; 3.5 months old. E, phenotype of a TRV-IND infected TUR ecotype; 3 months old, 51 dpi. F, phenotype of a TRV-IND infected TUR ecotype, 3.5 months old, 65 dpi. B and C, grown in long day conditions at 21°C. D – F, grown in long day conditions at

The TRV-IND-infiltrated TUR plants clearly showed an altered phenotype 2 months after infiltration compared to non-infiltrated control plants, whereas for CYP no distinct phenotype was observed. TRV-IND-infiltrated TUR plants showed a brush phenotype with more side branches, smaller leaves and abnormal flower/fruit development (Fig. 16, B–F). The flowers or early fruits died before ripening and rarely only a few indehiscent fruits developed per plant (Fig. 16, B and C). Later, the plant died after 2 – 3 months, shown in Fig. F. Whether this indicates a response to the silencing of the indehiscent gene (IND) or just to the overall viral attack cannot be determined without RT-qPCR analysis. In the long day condition at 21°C, a similar, but weaker phenotype was observed for TRV-AePDS, which indicates rather a non-specific response of the plants to the *Tobacco Rattle Virus* infection or the manual process of infiltration.

Tissue culture

Co-cultivation of *Agrobacterium* and seedling tissue

The callus induction on the GMCI medium worked well for tissue explants from 10 d-old *Ae. arabicum* seedlings (Fig. 17). From the four parts tested, the best callus formation was observed for cotyledons and hypocotyls, particularly for the upper greenish part of the cotyledon-hypocotyl explant (Fig. 17, A–C). By further sub-cultivation, the callus could be maintained over time, by changing the medium every 2 weeks. However, the callus growth rate for *Ae. arabicum* was much lower than for *A. thaliana*, as it is indicated in Fig. 17, G–I. The GMCI medium had been adapted for *A. thaliana* and needs to be adjusted for *Ae. arabicum,* to increase the efficiency.

Since the callus induction worked, I performed a co-cultivation approach to introduce *Agrobacterium*, carrying the construct of interest, into the plant tissue. Before placing the explants on GMCI, the seedlings were dipped into the bacteria suspension. After 3 days of co-cultivation, bacteria were removed, by rinsing explants in sterile water and placing them on GMCI supplemented with 100 mg/l timentin. The selection against *Agrobacterium* worked well. Fig. 17, D shows co-cultivated explants immediately before the transfer to plates supplemented with 100 mg/l timentin. Once the plant tissue was on timentin plates, no more bacterial growth was observed.

Subsequently, callus expressing the GFP marker gene was formed and could be sub-cultivated over time, indicating that transformed cells contributed to the callus. I could maintain fluorescent callus, generated from five different constructs in CYP and four constructs in TUR. Although the marker genes may still be expressed only transiently, GFP was in some cases still visible several months after induction and co-cultivation (Fig. 19 and Fig. 20). Construct #1 (GFP-4t, Kan^R) and construct #3 (YFP-nost, Hyg^R), both introduced with the *Agrobacterium* GV3101 strain, showed the best efficiency.

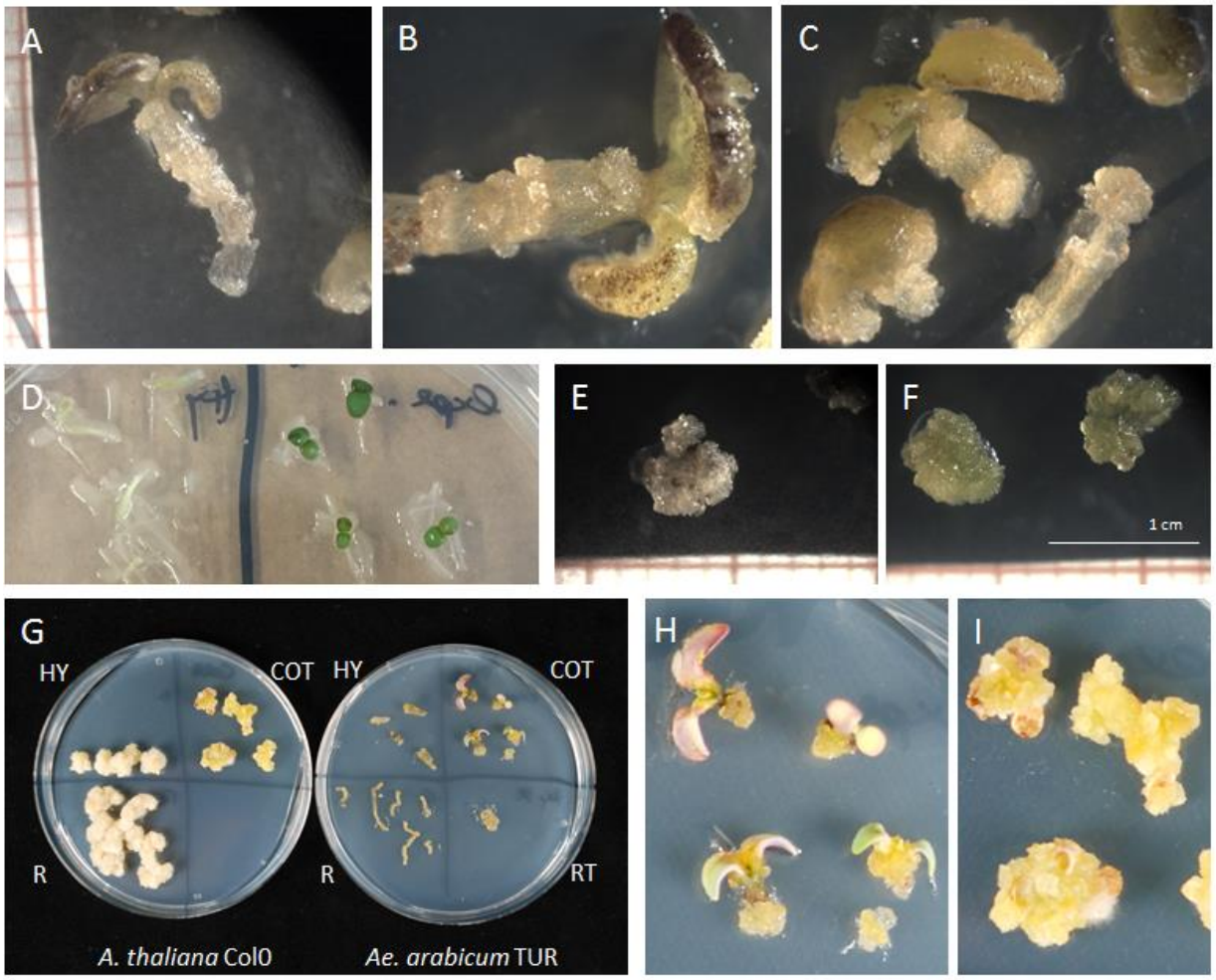

Fig. 17. **Callus induction and cocultivation of *Ae. arabicum* seedlings.** A – C, *Ae. arabicum* CYP explants 38 d after growth on callus-inducing medium. D, cocultivation of *Ae. arabicum* CYP hypocotyl parts (left) and explants (right) with *Agrobacterium* at the third day. E and F, subcultivated CYP callus (E) and TUR callus (F) 97 dpi. G, comparison of callus growth rate in *A. thaliana* and *Ae. arabicum* 48 dpi; COT, cotyledon explant; HY, hypocotyl; R, root; RT, root tip. H and I, explants of *Ae. arabicum* TUR (H) and *A. thaliana* Col-0 (I).

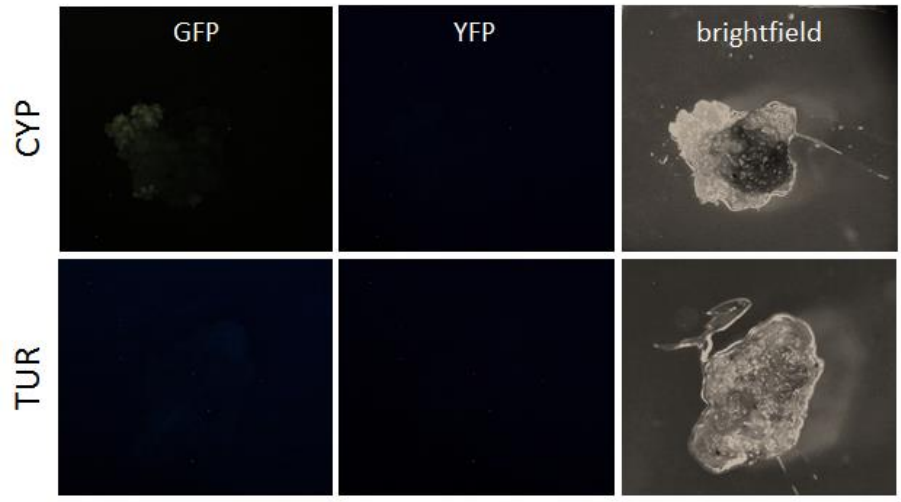

Fig. 18. **Autofluorescence of CYP and TUR control callus for GFP and YFP excitation.**

Ae. arabicum responded well to hygromycin selection (see results – selection system). As construct #3 carried the hygromycin B phosphotransferase (HPT) gene, callus transformed with this construct was maintained also on GMCI supplemented with 20 mg/l hygromycin. The combination of visible marker gene selection and hygromycin selection lead to a faster establishment of overall fluorescence in the callus during sub-cultivation. Moreover, construct #3 seemed to show the most stable expression over time, and there was no autofluorescence at all for YFP in seedling tissue and callus (Fig. 18). This YFP-Hyg^{R} construct is therefore the construct of choice for further transformation of *Ae. arabicum* using a tissue culture approach.

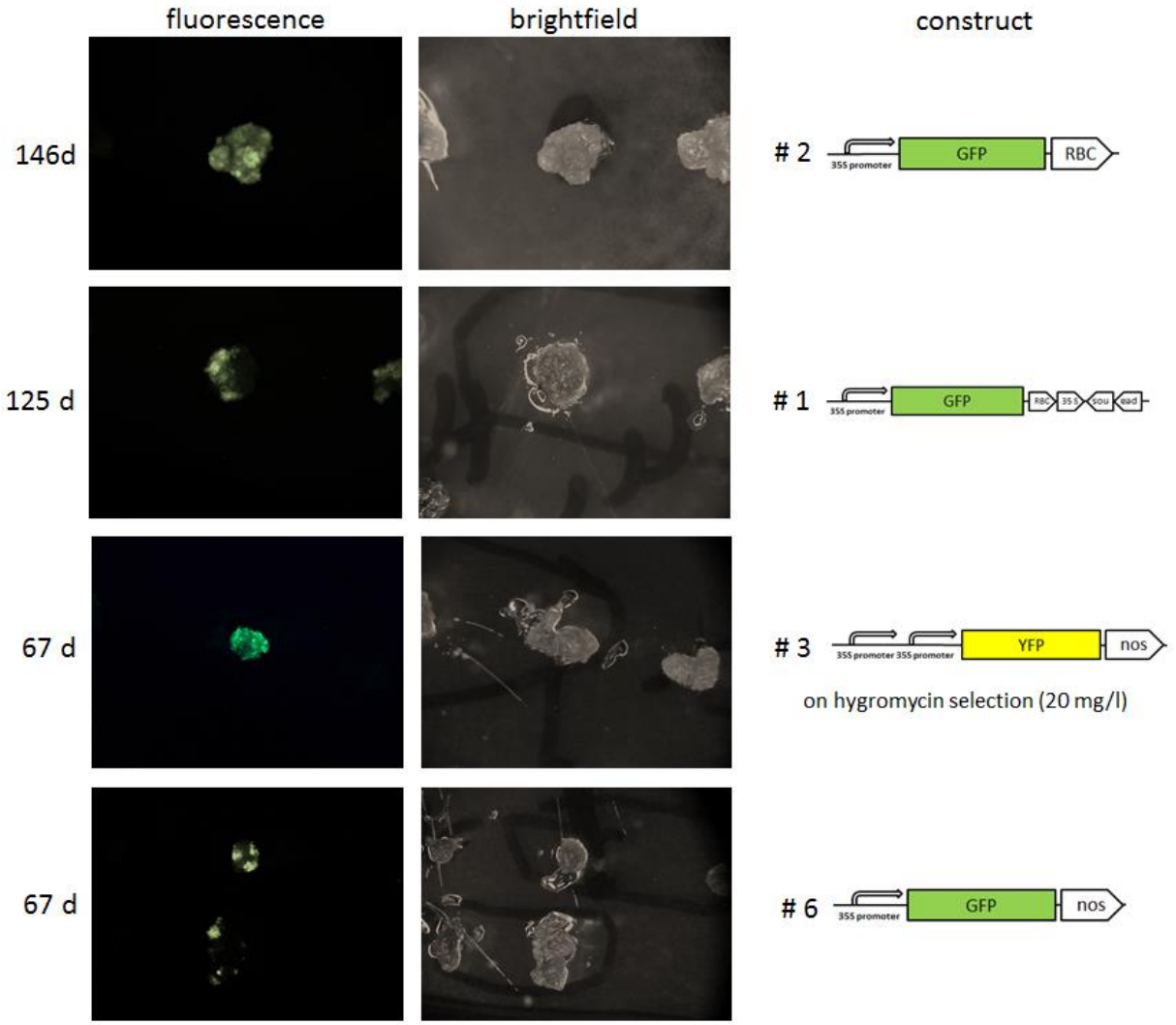

Fig. 19. Subcultivated fluorescent TUR callus after cocultivation with different constructs. Time indicates days after callus induction. Constructs are described in detail in the method part.

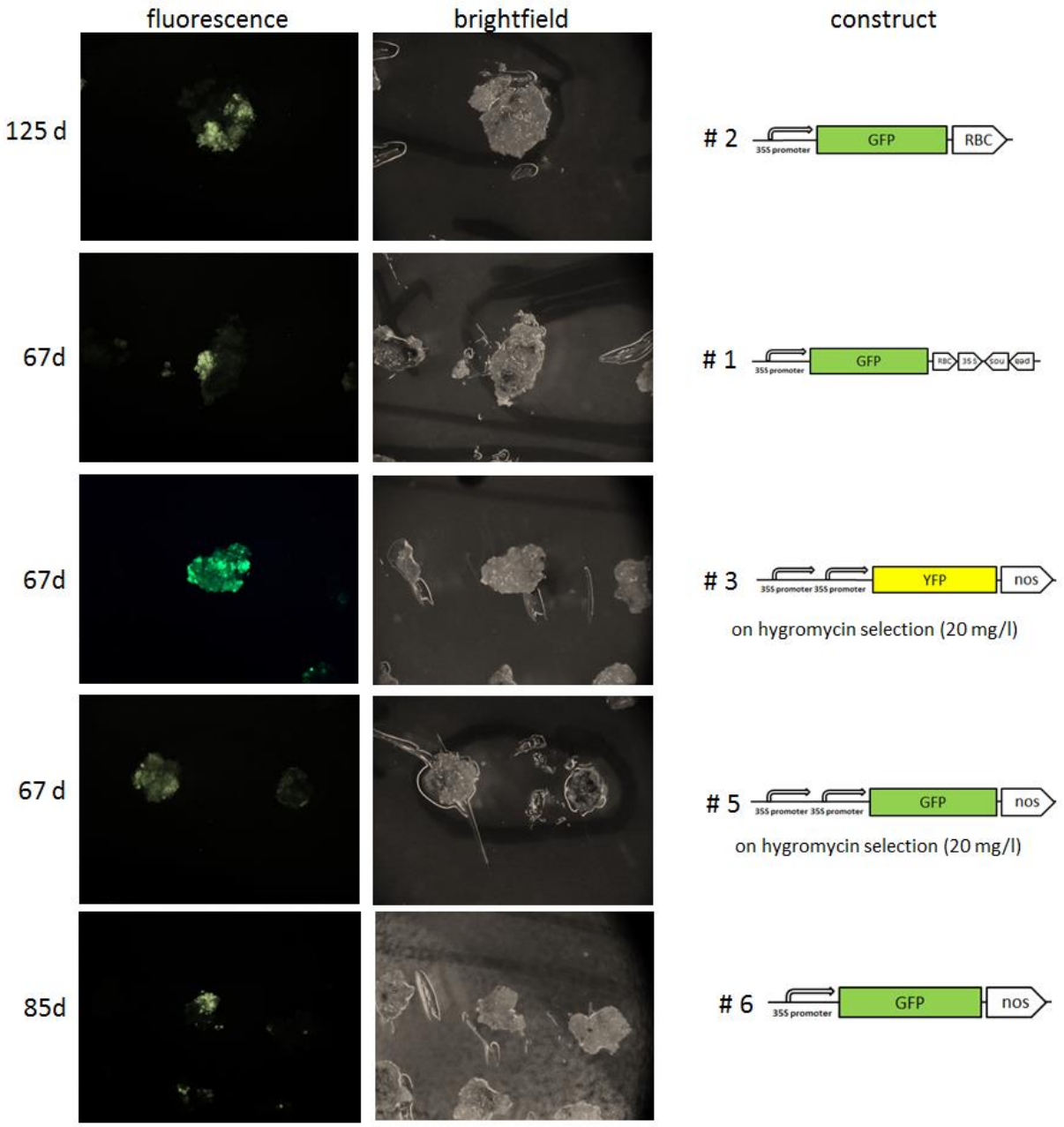

Fig. 20. Subcultivated fluorescent CYP callus after cocultivation with different constructs. Time indicates days after callus induction. Constructs are described in detail in the method part.

Fig. 21 and Fig. 22 show the fluorescent signal for the two most efficient constructs (#1 – GFP-4t, Kan^R and #3 – YFP-nost, Hyg^R) during callus formation for both accessions over a certain time period. To demonstrate stable integration of the T-DNA into the plant genome, it would require analysis by Southern blots. For this, more fluorescent callus material needs to be cultured to get a sufficient amount of DNA (min. 10 µg/sample).

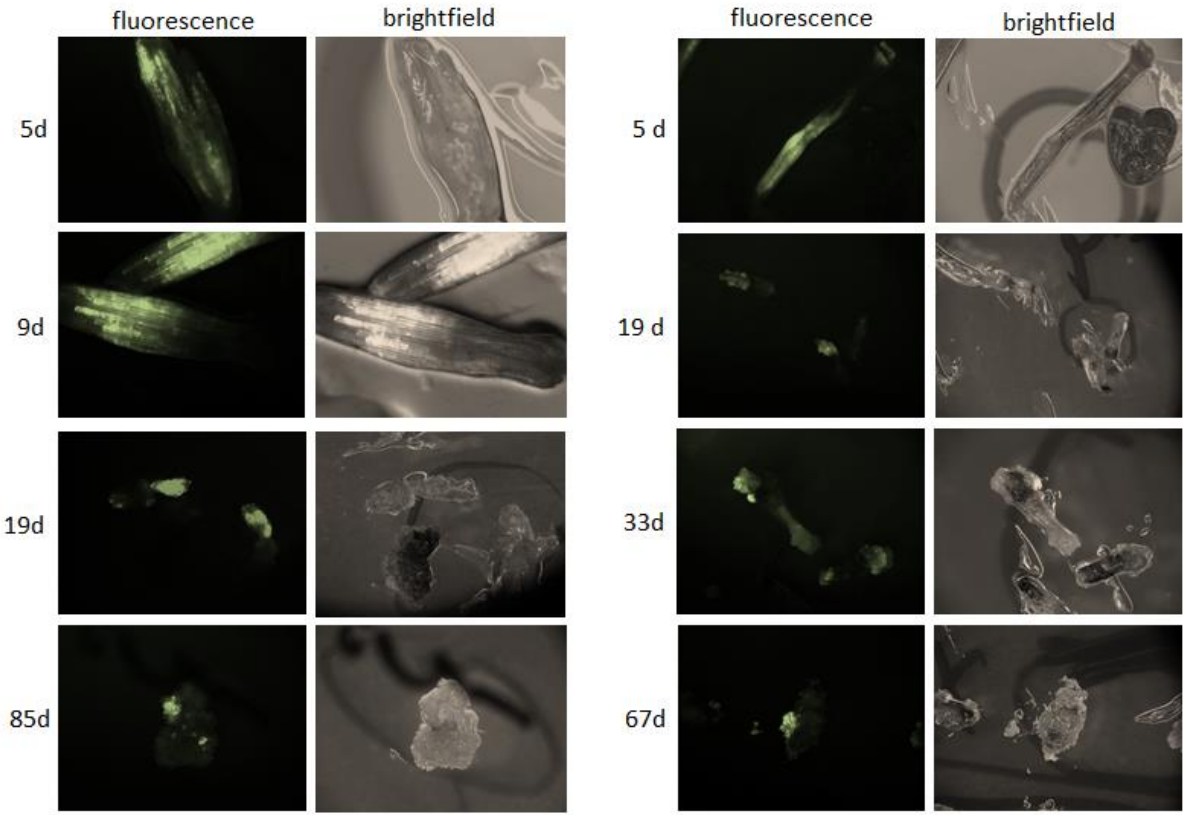

Fig. 21. GFP fluorescence of construct # 1 in plant tissue of TUR (left) and CYP (right) over time. The first two pictures per panel show hypocotyl tissue, the last two pictures newly formed callus.

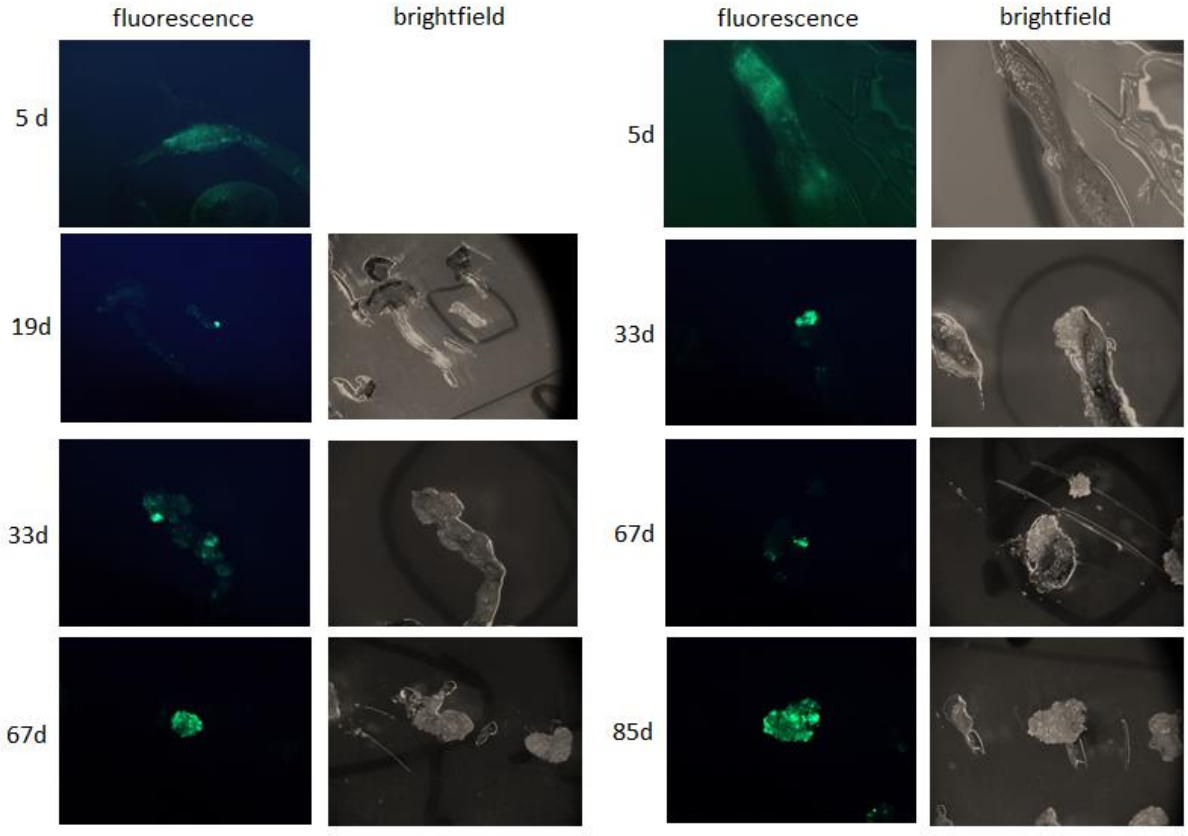

Fig. 22. YFP fluorescence of construct # 5 in plant tissue of TUR (left) and CYP (right) over time. The first two pictures per panel show hypocotyl tissue, the last two pictures newly formed callus.

Shoot differentiation out of mature leaves

Generation of stable transformants requires obtaining differentiated cells, formation of organs and growth into, fertile plants. This can be a limiting step after co-cultivation. Therefore, in parallel to the experiments with *Agrobacterium*, I tried different protocols for shoot differentiation. I was successful with a protocol using mature *Ae. arabicum* leaves and cultivating cut segments on medium with a 40:1 – cytokinin to auxin ratio (SI Jan medium). The high amount of cytokinin forced shoot induction in leaf tissue of both accessions. CYP seems to work better in this purpose. After one month of induction small leaflets are appearing, while for TUR only vertical shoot like callus can be observed (Fig. 23, A). Shoots could be sub-cultivated after 47 d and maintained on ½ MS medium (Fig. 23, B). A promising further investigation might be a cocultivation approach with cut mature leaves or even a cultivation of infiltrated leaves already transiently expressing the gene of interest.

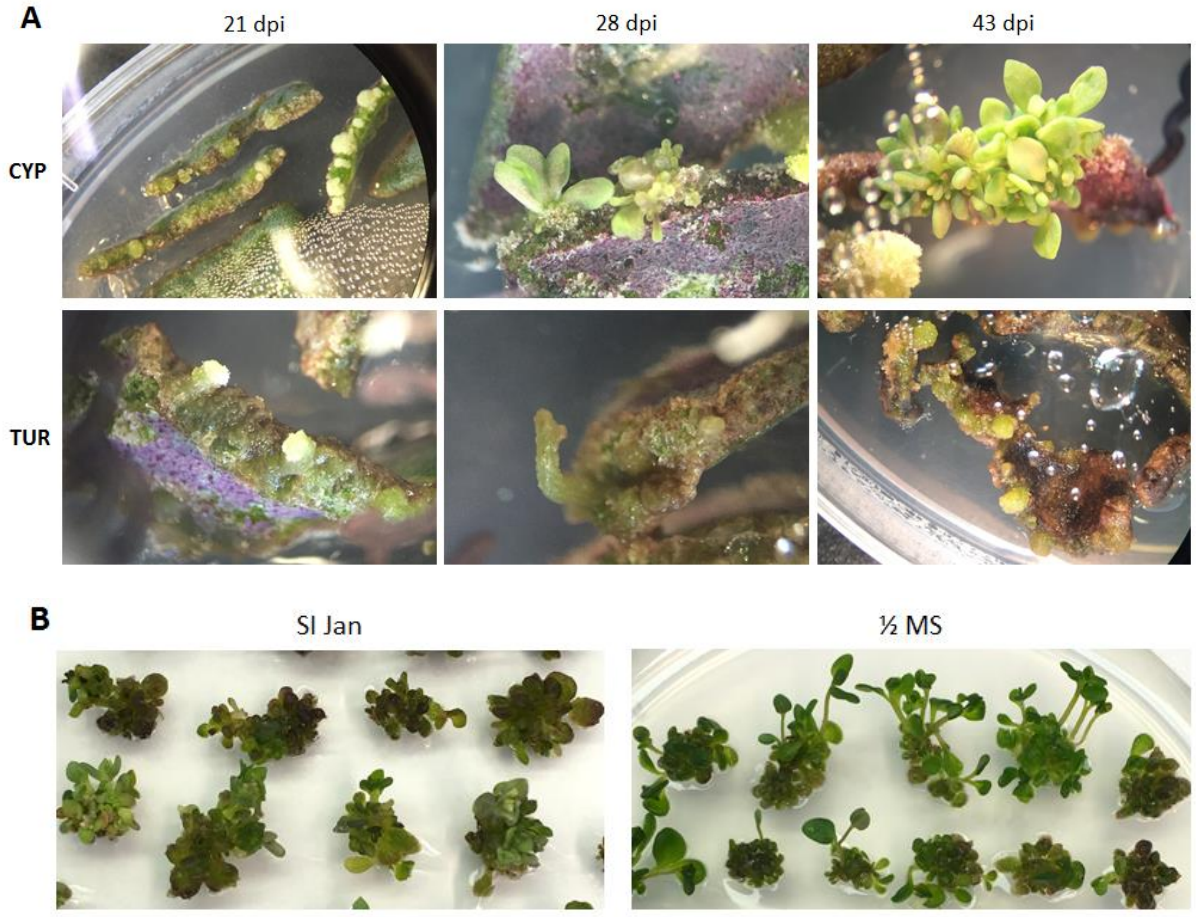

Fig. 23. Shoot differentiation out of mature *Ae. arabicum* leaves. A, shoot differentiation of CYP (upper line) and TUR (lower line) 21, 28, and 43 dpi on SI Jan medium. B, CYP shoots 70 dpi on SI Jan and on ½ MS (since 23 d).

The Supplementary Fig. 1 shows a comparative work flow scheme for the used techniques as an overview and indicates the time needed for each method.

Discussion

Since the first report in 1983 on transgenic plants about successful co-cultivation of protoplasts with *Agrobacterium tumefaciens* and subsequent plant regeneration from single protoplasts, many transformation techniques for various plant species were established for basic research and biotechnological applications (Wang, 2015). Plant transformation was probably one of the major paradigm shifts in modern molecular and genetic plant science that allowed approaching questions concerning gene function by reverse genetic methodologies. Also for plant breeding and high yield crop production, various transformation methods remain an important tool for genetic engineering. Although genetically modified plants and their extensive use in a few countries (but with remarkable extend) are controversially discussed in politics and society, it represents probably the second "green revolution" in industrial agriculture, which led to higher yields per area of land. The monoculture of genetically modified pants combined with the exploitative use of land, the application of high amounts of herbicides and fertilizer, and the huge economic benefit for several single companies, is strongly criticized. On the other hand, the threat of global starvation caused (among other factors) by unpredictable climatic changes and social crises could be reduced by using genetically modified crops that are highly adapted to a certain environment and purpose (Kush, 2001; Nature Publishing Group, 2013).

Nevertheless, the transformation approaches tested in this study do not touch on social or ethical issues but should provide a tool for basic molecular and genetic research investigating the heterocarpic plant *Aethionema arabicum*, in order to study gene function and understand the underlying molecular pathways of heteromorphism, as well as reaction and adaptation to environmental stresses.

Floral dip

GFP autofluorescence in seeds

The straightforward transformation by the ´floral dip method` is one of many advantages that established *Arabidopsis thaliana* as a commonly used model plant for genetic research in plant science. However, this method was restricted for many years to this species, and only recently could it be applied successfully in some other species, such as the crops tomato, wheat and maize (Bastaki & Cullis, 2014).

The close relationship between *A. thaliana* to *Ae. arabicum* as members of the Brassicaceae made it worth to try using the floral dip method also in this study. Additionally, the availability of an *Arabidopsis*-specific oleosin-GFP construct, which allows identifying transformed seeds without cultivating the T1 generation, was promoting this easy and fast approach to be tested in *Ae.*

arabicum. However, the strong GFP autofluorescence of the seeds did not allow any conclusion whether GFP was expressed at all, or whether the signal was just too weak to distinguish it from autofluorescence. For further attempt to transform *Aethionema* by floral dipping, non-GFP marker genes are recommended to overcome this issue.

Also the oleosin construct might need improvement. Blasting the protein sequences of this seed protein from the two Brassicaceae species indicated high similarity , but differences may be encoded in the promoter regions. A comparison between the two species concerning the regulatory regions of the oleosin gene might be important to decide, whether the *Arabidopsis*-derived construct can be used or needs to be adapted.

The construct used in this study included a sulfadiazine resistance gene, to select in T1 by spraying seedlings with this chemical (Thomson et al., 2011). This principle could also not be applied, as wildtype plants showed no effect when sprayed even with high concentration. This indicates insensitivity of *Ae. arabicum* and the need for distinct selectable markers. *Ae. arabicum* showed high sensitivity to hygromycin *in vitro* (see results – selection system), therefore, future attempts of transformation based on selection should be based on this antibiotic.

In summary, cloning of the *Aethionema* oleosin sequence with an endogenous promoter that drives the protein fused to a non-GFP tag and including a hygromycin B phosphotransferase gene might result in better chances to achieve successful floral dip transformation, or could at least conclusively exclude that this technique is working for *Ae. arabicum*.

Reasons for the latter case might lie in anatomical differences: the structure of the gynoecium of *A. thaliana* enables the access of *Agrobacterium* into the reproductive tissue of the plant during the floral dip procedure, and it is not clear yet if this is similar in *Aethionema*, where this gateway into the gynoecium at a certain stage of flower development has not yet been described (Desfeux et al., 2000). Nevertheless, when all these resources are available, the straightforward approach of floral dip transformation for *Aethionema* can easily be tested again. Additional adaptations of bacteria and surfactant concentration, or prolonged vacuum treatment, could further be tested.

Leaf infiltration

GFP expression in single cells with different constructs and efficiency

The leaf infiltration is a commonly used method to transiently express genes of interest in *N. benthamiana* leaves, to test their efficiency and to perform interaction studies *in vivo* (Wydro et al., 2006). Since protocols for other species are well established and different marker gene constructs were available, I have tested it in *Ae. arabicum* leaves for both accessions for the first time. Expression in *Ae. arabicum* cells was weaker and less abundant than in *N. benthamiana*, but

nonetheless fluorescent signal could be detected 3 dpi – 8 dpi, for both GFP constructs, regardless of the *Agrobacterium* strain used as vector. The strong 35S promoter (2x) already triggered high GFP expression, but it is likely that virulence and availability of the bacteria could be improved for higher expression efficiency by increasing the density of the bacterial suspension or infiltrating a higher volume; or a more efficient vir gene induction by a higher acetosyringone concentration. Klee et al. (1987) mention that a nurse culture of tobacco cells increases the transformation frequency due to better induction of vir genes. This could also be considered for further experimental design.

Cross sections are recommended to screen fluorescence of infiltrated leaf tissue, because they represent the infiltrated leaf better than surface peels of the lower epidermis. Additionally, preparation of surface peels is more difficult than cross sections: the latter result more often in uniform preparations that are suitable for microscopy.

For *N. benthamiana*, it was possible to generate stable transformed plants out of infiltrated leaves via *in vitro* tissue culture (Sparkes et al., 2006). As I achieved shoot differentiation out of mature leaf material with high amounts of cytokinin, this would be also a possible approach to test for *Ae. arabicum* infiltrated leaves, although the expression efficiency was low.

Particle bombardment

Expression in single cells in hypocotyl of etiolated seedlings

The biolistic approach using the particle gun to shoot DNA-coated gold particles into plant cells is a very fast technique suitable for many species or tissues. However, it requires a special device and quite high amounts of plasmid DNA to perform the bombardment.

When I introduced a 35S-GFP construct into hypocotyl cells of 10 d-old etiolated seedlings of both *Ae. arabicum* accessions, I could observe transient expression in 1-3 cells per entire hypocotyl. One limitation is likely the need to target the DNA with the microprojectiles specifically into the nucleus to attain gene expression. Potential for improved targeting of more nuclei and overall higher efficiency would come from adapting the pressure settings, the spread of the particles and the use of more particles per shot, with the same or higher amounts of DNA. High amount of DNA however, could lead to multicopy insertions. An optimal amount for bombardment of immature maize embryos (with subsequent callus induction and regeneration) was suggested to be 2.5 ng DNA/shot, in order to gain stable integration of single copies (Lowe et al., 2009).

When the technique of particle bombardment was first introduced bombarding suspension culture cells and mature leaves of *N. benthamiana* (Klein et al., 1988c), only a small fraction of the cells that transiently expressed the foreign gene incorporated it into the genomic DNA. However, by many adjustments of the protocol several years later, particle bombardment resulted in stable gene

expression in transgenic progenies in major monocot crops, such as rice, maize, wheat, sorghum and barley (Henry & Furtado, 2013). Monocots were inaccessible over many years to Agrobacterium-mediated transformation, and thus stable integration using the particle gun implicated a breakthrough for monocot transformation (Christou, 1993).

Once higher numbers of cells expressing the gene of interest are achieved, a tissue culture approach with bombarded hypocotyl tissue can be considered, to induce callus and subsequently generate transgenic plants. As shown, the callus induction on the GMCI medium worked well for hypocotyl tissue. Therefore, by sub-cultivating the GFP-positive callus cells, it might be possible to generate uniform callus for further regeneration. Bombarding callus tissue, further selection and regeneration can also be considered to establish a stable plant transformation protocol by using this biolistic approach coupled with tissue culture.

Targeting germ line cells or totipotent tissue such as pollen, embryos and meristems offers another future prospective to create transgenic plants, benefiting of the programmed potential of totipotency (McCabe et al., 1988b; Cao et al., 1992). While it may lead to chimeric plants, it seems to be a promising method, as it worked for many plant species (Sanford, 1990).

Virus-induced gene silencing

Proof of concept in *N. benthamiana*; no clear effect in *Ae. arabicum*

The knock-down method using the virus-induced gene silencing (VIGS) system leads to degradation of specific transcripts within the entire plant. A version of the *Tobacco Rattle Virus* sequence including a phytoene desaturase (PDS) insert was described previously for *N. benthamiana* gene silencing and is used frequently for establishing VIGS in other plant species (Kumagai et al., 1995). In my case, I applied the system in *N. benthamiana* as a proof of concept, using a TRV-PDS system modified by Kerényi et al. (2008). The whitening effect was clearly visible after 10 days, and an RNA assay has proven the presence of the viral RNA within the leaf. Senthil-Kumar & Mysore (2014) have recently published that a small percentage of PDS-VIGS progeny seedlings of *N. benthamiana* showed photobleaching, hence indicating transmittance of gene silencing to the next generation. I could not confirm such heritability in my case.

For *Ae. arabicum,* no effect was observed in early stages after infection, neither for the PDS insert nor for the IND insert. This was also confirmed by the RNA assay, failing to detect viral RNA. However, one month after infection, I observed an irregular phenotype in AeIND-infected TUR plants, indicating either activity of the virus or late consequences of the mechanical treatment during infection. It also could indicate indirect effects after silencing of the IND gene per se. In any case it is clear that the expected assumption of more indehiscent fruits by triggering successful silencing of the

IND gene was not confirmed. Detailed analysis of the RNA level by RT-qPCR for the IND gene might provide more convincing data and is highly recommended to draw a conclusion.

Concerning the two different infiltration approaches used in this study, I did not observe any difference in infection efficiency. The *N. benthamiana* leaf suspension infiltration was described previously for VIGS in *A. thaliana* (Lu et al., 2003). It was assumed to provide better infection of the plants for more successful silencing.

For CYP, no VIGS phenotype was ever detected. This could mean that either no or only a slight down-regulation of the transcript occurred, or that this accession is less sensitive to infiltration or virus infection. The latter would be interesting, as the accessions are genetically closely related. TRV has different temperature optima for distinct species, and temperature is claimed as one of the most important factors for effective silencing (Burch-Smith et al., 2004). Therefore, the temperature during the infection experiments might need to be adapted for each accession to reach desirable success.

TRV-derived vectors are known to spread throughout the plant and silence genes in most plant parts, even in meristem tissue and fruits (Bruch-Smith et al., 2004; Senthil-Kumar & Mysore, 2014). Thus, using TRV-derived vectors for targeting flower- respectively fruit-specific genes (e.g. the IND gene) remains a reasonable approach to attempt interfering with heterocarpic fruit and seed development regulation in *Ae. arabicum*.

Tissue culture

Agrobacterium-seedling co-cultivation leads to fluorescent callus

The co-cultivation of hypocotyl sections of 10 d-old seedlings with *Agrobacterium* on callus-inducing medium led to fluorescent callus, which transiently expressed the marker gene even several months after induction. By testing a couple of constructs for both accessions, I could identify one construct with the highest potential to establish an appropriate transformation protocol for *Ae. arabicum* using tissue culture in future experiments. The so called construct #3 (YFP-nost, Hyg^R; precise map in Fig. 5) gave the most stable fluorescent signal over time, clearly distinguishable from non-transformed callus cells. The wavelength for YFP excitation also does not result in autofluorescence of the callus. Additionally, the construct carries a hygromycin B resistance gene (hygromycin B phosphotransferase – HPT), which allows positive selection of transformed cells, because of cell death of non-transformed cells in the presence of hygromycin. Wildtype callus and seedlings on germination medium show high sensitivity against low concentrations of hygromycin: selection experiments have shown growth inhibition already on medium supplemented with 7.5 mg/l hygromycin, culminating in

cell death when 20 mg/l were added. Consequently, the construct with this YFP-Hyg^R combination is recommended for further co-cultivation studies in *Ae. arabicum.*

Transiently transformed callus cells represent only half of the story towards the aim to generate stable transgenic plants using tissue culture. Further regeneration of differentiated organs via somatic embryogenesis has to be achieved to reproducibly obtain transgenic plants. Many regeneration protocols are available for different plant species, but each species or even each variety of a species requires adaptation of the protocol. By the use of plant hormones in the right concentration, ratio and timely application in *in vitro* culture, shoots can be generated out of callus tissue, followed by root induction. Further cultivation can finally result in mature, fertile plants, as it was shown for some banana varieties, to name only one example (Tripathi et al., 2015).

However, the long cultivation period under the quite artificial *in vitro* conditions can lead to the phenomenon of somaclonal variation, one of the major difficulties during regeneration from callus that is often derived from multicellular origin. Somaclonal variation leads to diversity in genotype and/or phenotype between transformants that are not directly linked to T-DNA insertions. It can originate from single base-pair changes, chromosome deletions, translocations, changes in ploidy, or epigenetic modifications, and it represents a big problem in plant regeneration especially when identical material is wanted. Recently it was shown that a loss of methylation on a certain locus in a variant of oil palm predicts somaclonal variation in terms of abnormal fruit morphs (Ong-Abdullah et al., 2015). The often applied synthetic hormones for shoot/root induction have a strong role for somaclonal variation. The synthetic auxin 2,4-dichlorophenosyacetic acid (2,4 D) for example, seems to enhance the phenomenon in *A. thaliana*. Additionally, the multicellular origin may lead to chimeric plants with multiple and different T-DNA insertion events. This can be avoided during co-cultivation or direct gene transfer to protoplasts, as this guarantees plants out of single cells, which enhances uniformity (Martiínez-Zapater & Salinas, 1998; Skirvin et al., 1994).

Due to slow growth, not enough material could be obtained to analyze the fluorescent callus for stable integration by performing a Southern blot. I would have needed a high amount of DNA (min. 10 µg/sample) for conclusive results, which was not possible to reach so far. Further material cultivation is necessary and currently grown to perform the experiment. Since a Southern blot provides the only unambiguous verification of stable integration of the T-DNA into the plant genome, until now, the callus tissue can only provide evidence for transient expression of the marker genes. However, the stable fluorescence in callus over several months, partly selected on hygromycin medium, gives promising indication for a stable integration.

Shoot differentiation out of mature leaves

A very promising differentiation was achieved with mature leaf material on so called SI Jan medium (containing an auxin:cytokinin ratio of 1:40) to induce shoots at the cut sides. A next step would be to

figure out appropriate hormone concentrations to generate roots and subsequently cultivate plantlets until maturity.

By using *Agrobacterium*-infiltrated leaves for shoot induction on the SI Jan medium, single transgenic shoots could possibly be selected. This technique was already described previously for *N. benthamiana* (Sparkes et al., 2006) and is also mentioned above while discussing the leaf infiltration experiments.

For *Medicago ssp.,* a co-cultivation approach of mature leaf discs in an *Agrobacterium* suspension with applied vacuum and further cultivation on solid medium led to transformation success, and with subsequent regeneration, to fertile transgenic plants (Trinh et al., 1998). A comparable co-cultivation, combined with the shoot inducing SI Jan medium, can be considered as a promising straightforward technique to finally master the challenge of generating transformed *Ae. arabicum*. Another suggestion for future experiments is to use the SI Jan medium for co-cultivation of young hypocotyl tissue. SI Jan medium is supposed to rapidly induce shoot growth out of any plant material, and the *Agrobacterium* infection and callus induction was shown to work well for hypocotyl tissue.

As an overall conclusion of my experiments, I established marker genes, selection conditions, partide bombardment and leaf infiltration as straightforward methods for testing transient expression of any construct of interest *in vivo* in the model plant *Ae. arabicum* for the first time. The VIGS system, once proven to work for *Ae. arabicum*, might be considered as possible addition for functional gene studies in this species. For stable and heritable integration of transferred DNA, the tissue culture approach seems to be the most promising technique, although the single pieces of the big jigsaw puzzle have still to be rearranged. Fortunately, the work can be handed over to other lab members of the Mittelsten Scheid group (GMI) to accomplish the challenge hopefully soon.

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Summary

A major tool in plant reverse genetics is transformation, stably introducing foreign genes of inte rest into the genome. Many techniques are available to do so, and over the last decades, plenty of plant species were successfully transformed, for basic research as well as for crop design by genetic engineering.

The plant *Aethionema arabicum*, like the well-established model plant Arabidopsis thaliana a member of the genus Brassicaceae, shows two distinct fruit and seed morphs on the same individual plant, described as heterocarpy. To understand the molecular mechanism of heterocarpy and its role in adaptation to unpredictable environment, transformation of this species is an essential tool to study the underlying molecular pathways.

In this context, several well-known transformation techniques, already applied for many different plant species, were tested in order to generate transgenic *Ae. arabicum*. The techniques are based on either an *Agrobacterium*-mediated transformation approach or on a biolistic method to introduce foreign DNA into the plant cells.

The floral dip protocol, which is commonly used in *Arabidopsis* genetics, was tried with a marker gene that would express GFP in transgenic seeds. This attempt was not successful due to high autofluorescence in seeds even in the absence of the GFP marker gene. However, leaf infiltration and particle bombardment with constructs designed for constitutive expression of GFP allowed detection of transiently expressed GFP in different plant tissues, with different efficiencies dependent on the constructs and *Agrobacterium* strains.

Virus-induced gene silencing (VIGS) with the *Tobacco Rattle Virus* (TRV) and targeting phytoene desaturase (PDS) was successfully tested in *N. benthamiana* as a proof of concept, resulting in photobleaching in the entire plant due to PDS transcript degradation. When the *Aethionema* PDS was inserted into the viral constructs and tested for VIGS in *Aethionema* plants, no bleaching was observed. However, corresponding silencing of the indehiscent gene (IND) as target led to a modified phenotype in some plants. Presence of the viral RNA and down-regulation of the IND transcript remain to be confirmed.

The most promising method turned out to be the co-cultivation of young hypocotyl explants with *Agrobacterium* and subsequent plant regeneration. Transgene-expressing hypocotyl callus could be propagated over a long time and grown on selective medium. However, stable integration of the marker gene into the plant genome has yet to be confirmed by performing a Southern blot, while also a regeneration protocol for fertile plants has still to be established. Nevertheless, successful shoot differentiation from non-transgenic mature leaves could be achieved on medium with high amounts of cytokinin.

Altogether, the establishment of suitable marker genes and constructs, protocols for transient transgene expression after co-cultivation with *Agrobacterium* and regeneration of shoots from leaf explants provide a promising basis to combine these different elements on the way towards transgenic *Aethionema*.

Zusammenfassung

Transformation, der Einbau zusätzlicher DNA Abschnitte, ist eines der wichtigsten Werkzeuge in der modernen Molekularbiologie and Genetik, um Pflanzengenome modifizieren und folglich die Funktion und Regulation ausgewählter Gene studieren zu können. Verschiedene Techniken wurden entwickelt, mit denen unzählige Pflanzenarten für die Grundlagenforschung, aber auch für die moderne Landwirtschaft, transformiert werden konnten.

Die annuelle Pflanze *Aethionema arabicum*, wie die Modellpflanze *Arabidopsis thaliana* eine Vertreterin der Familie der Brassicaceae, bildet zwei verschiedene Frucht- und Samentypen auf demselben Individuum aus; bekannt als Heterokarpie. Um die zugrundeliegenden molekularen Zusammenhänge dieser Heteromorphie in Verbindung mit der Anpassung an stark schwankende Umweltbedingungen verstehen zu können, stellt die Transformation ein wichtiges Werkzeug dar, diese molekularen Prozesse zu studieren.

Im Zuge dieser Arbeit wurden verschiedene Methoden angewendet, die bereits in anderen Pflanzenarten zu einer erfolgreichen Tranformation geführt haben. Grundsätzlich kann zwischen einer *Agrobacterium*-vermittelten Transformation und einer biolistischen Transformation unterschieden werden, um Fremd-DNA in das Pflanzengenom zu integrieren.

Das für *A. thaliana* entwickelte Protokoll der „Floral Dip" Methode wurde für *Aethionema* mit einem Markergen getestet, welches GFP direkt im Samen exprimieren sollte. Aufgrund hoher Autofluoreszenz der Samen im GFP Absorptionsspektrum war dieser Ansatz nicht erfolgreich. Jedoch konnte eine transiente GFP Expression in einzelnen Zellen des jeweiligen Gewebeverbands mit zwei anderen Methoden erreicht werden: zum einen durch eine *Agrobacterium*-Infiltration von Blattgewebe (leaf infiltration) und zum anderen über einen biolistischen Ansatz, indem Plasmid-DNA verbunden mit Goldpartikeln auf Hypokotylgewebe von Keimlingen geschossen wurde (particle bombardment). Die Expression hielt in der Regel einige Tage an, und der Wirkungsgrad war vom verwendeten Konstrukt sowie vom *Agrobacterium* Stamm abhängig.

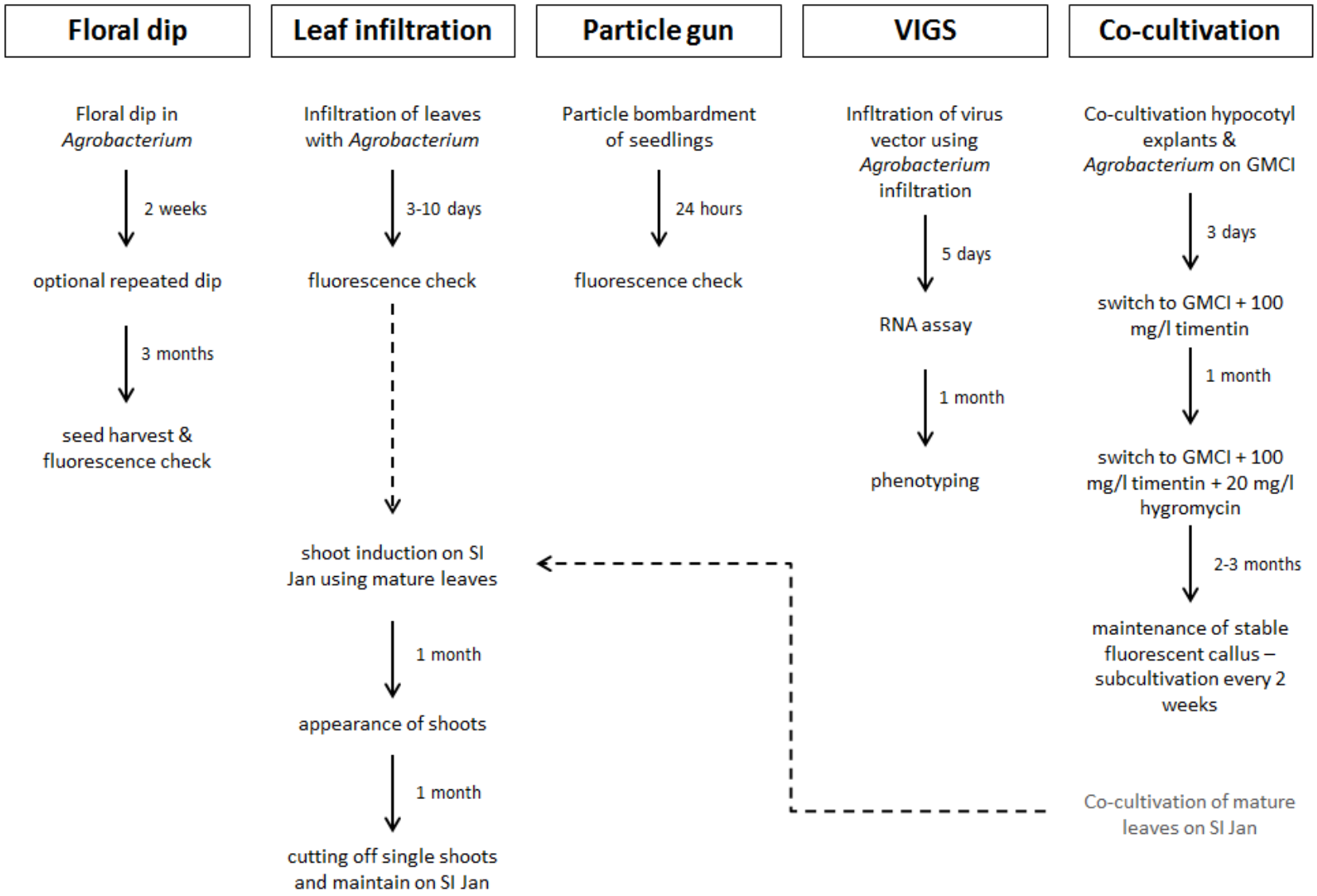
Virus-induced gene silencing (VIGS) ist ein System, um gezielt Transkripte in Zellen herunterzuregulieren. Das verwendete *Tobacco Rattle Virus* (TRV) mit einem eingebauten Phytoenedesaturase Insert bewirkt eine Ausbleichung der Blätter in *Nicotiana benthamiana*. Diese Kombination wurde in dieser Arbeit erfolgreich als Kontrolle verwendet. Für *Ae. arabicum* konnte ein solcher Bleichungseffekt nicht nachgewiesen werden, jedoch wurde in einem der beiden Ökotypen eine veränderte Morphologie des Hauptsprosses beobachtet, wenn das Gen Indehiscent (IND) als potentielles Target für die Inaktivierung bereitgestellt wurde. Die Präsenz der viralen RNA in der Pflanze sowie die Reduktion des IND Transkripts bleiben zu bestätigen, bevor Schlussfolgerungen gezogen werden können.

Der vielversprechendste Ansatz jedoch scheint über eine Gewebekultur von jungem Hypokotylgewebe zu funktionieren. Mittels einer Co-Kultivierung mit *Agrobacterium* wurde eine Expression von diversen Markergenen in induziertem Kallus erreicht, welche über Monate hinweg beibehalten werden konnte. Zusätzlich wuchs der Kallus auf einem Hygromycinmedium, was die Expression des entsprechenden Resistenzgens vermuten ließ. Eine stabile Integration in das Pflanzengenom muss jedoch erst mit einem Southern Blot bestätigt werden. Außerdem muss ein Regenerationsprotokoll entwickelt werden, um aus dem Kallus schließlich fruchtbare Pflanzen zu erhalten. Nichtsdestoweniger konnten in einer *in vitro* Kultur mit entsprechend hohen Mengen des Pflanzenhormons Zytokinin Sprosse aus Blattschnitten generiert werden.

Die Etablierung von möglichen Markergenen und Konstrukten sowie Protokolle für die transiente Expression nach Co-Kultivierung mit *Agrobacterium* und für die Regeneration von Sprossen aus Blattschnitten bilden eine vielversprechende Basis, diese Ansätze zu verbinden, stellen wichtige Schritte auf dem Weg zu transgenen *Aethionema* Pflanzen dar.

Supplementary Table 1. Comparison of different plant transformation techniques

Method	Implementation	Time of result after application	Stable/transient transformation	Species specificity	Transgene vector	Transformation uniformity	Limits	Worked out for *Ae. arabicum*
Floral dip	straightforward; no special equipment needed	in next generation; dependent on life cycle of the plant	stable	over long time restricted to *A. thaliana*; recently reported for other species	plasmid in *A. tumefaciens*	clonal in progeny, because targeting natural reproductive plant parts	anatomy of reproductive plant parts as limit for bacterial access	no
Co-cultivation	laborious; need of labor expertise and equipment	several months up to years	stable	no restriction, but establishment of adapted protocols necessary	plasmid in *A. tumefaciens*	chimeric tissue possible, but uniformity in next generation possible	time, labor expertise and equipment for tissue culture	transiently transformed callus
Leaf infiltration	straightforward; no special equipment needed	3-5 days	transient	*N. benthamiana* as model; many other species tested	plasmid in *A. tumefaciens*	only single cells in infected plant tissue transformed	species-specific; single cells transformed or overexpression/saturation	transient expression in leaf cells
Particle bombardment	straightforward; special equipment needed	24 hours	transient, except targeting meristematic tissue	effective regardless of species and tissue type	plasmid attached to gold/tungsten particles	single cells, which integrated particle in nucleus	special instrumentation; transformation of single cells	transient expression in single hypocotyl cells
Virus-induced gene silencing	straightforward leaf infiltration	1-4 weeks; same generation	transient	broad species range; dependent on virus vector	plasmid in *A. tumefaciens*, which encodes for virus	silencing throughout whole plant, but mosaic patterns possible	gene "knock-down", eventually no phenotype difference due to remaining functional protein	observation of abnormal phenotypes



Supplementary Fig. 1. Comparative work flow diagram for the used methods. Time between the working steps is indicated. Suggested experiment in grey. Dashed arrows indicate conceivable connection for future prospects.

Curriculum Vitae

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Personal data

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School & academic studies

2013 – 2016	Master Botany, University of Vienna
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2010 – 2013	Bachelor Biology with focus on Botany, University of Vienna
2004 – 2009	Höhere Lehranstalt für Umwelt und Wirtschaft (Yspertal)
2000 – 2004	Hauptschule (Taufkirchen/Pram, Upper Austria)
1996 – 2000	Volksschule (Taufkirchen/Pram, Upper Austria)

Job experience

Oct. 15 – June 2016	Gregor Mendel Institute of Molecular Plant Biology (GMI), Ortrun Mittelsten Scheid group
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