VIGS, HIGS and INTACT to analyse function and translocalizion of arbuscular mycorrhizal effectors

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Abstract

Microorganisms have a key role in plant development and evolution. One of the most successful beneficial plant-fungus interactions is the endosymbiosis between arbuscular mycorrhizal fungi and a broad range of host plants. Our current hypothesis is that besides the well-studied common SYM pathway, AM fungi use secreted effectors to induce transcriptional change in the plant that enables mycorrhization. Although expression of ~550 putative (small) secreted proteins that could be effectors is confirmed, the function and translocalization of these proteins is not known yet. In this study, we investigate several research methods that could be used to study the function or translocalization of these effector candidates. We investigated the reliability and efficiency of *virus-induced gene silencing* (VIGS) and *host-induced gene silencing* (HIGS) for functionality studies and *isolation of nuclei tagged in specific cell types* (INTACT) for translocalization studies. In VIGS and HIGS, dsRNA is introduced by either the plant host or a virus and cut into siRNA by a dicer protein. siRNA is loaded on a RISC complex, which degrades complementary mRNA to silence the corresponding gene. When attempting to use VIGS to silence *Rhizophagus irregularis* AQP and effectors with a pTRV system in *Nicotiana benthamiana*, no silencing was detected, leading us to believe that VIGS and HIGS is not suitable for the study of effector function. For INTACT, nuclear-envelope targeting NTF was expressed in mycorrhized *Medicago truncatula* cells and biotinylated by expressing BirA. Successful isolation of specific NTF-tagged nuclei was achieved by adding magnetic streptavidin beads with an affinity for biotinylated NTF. Although the INTACT method was not very efficient, it is promising for future translocation studies when optimized.

Introduction

Microorganisms have a key role in the history of plant development and evolution (Sedzielewska-Toro et al. 2016). Studies suggest that the first land plants were able to cope with their new environment because of symbiotic interactions with fungi and bacteria (Selosse et al. 1998). Mutualism between plants and fungi is still prominent today and is beneficial for both species involved. One of the most successful beneficial plant-fungus interactions is the endosymbiosis between plants and arbuscular mycorrhizal fungi, belonging to the Glomeromycota. This symbiosis is thought have originated ~450 million years ago and still occurs in the vast majority of all land plants (Smith and Read 2010). Over the course of time, the AM fungi and his many host species have co-evolved and developed a complex mechanism of interaction. To establish a successful symbiosis there needs to be a continuous communication between the two partners. This communication already starts before the fungus and the plant have made physical contact. In this pre-contact phase, both the fungus and the plant already begin expressing a subset of genes involved in induction of the symbiosis. This phase is characterized by hyphal branching of the fungus, in response to strigolactones (SLs) excreted by the plant (Akiyama et al. 2010). On the other hand, the fungus releases so called Myc factors (lipochito-oligosaccharides (LCOs) and/or short chito-oligosaccharides (COs)), that trigger a symbiotic response in the roots, in part through the activation of a well characterised symbiotic signalling pathway, called the common SYM pathway (Parniske 2008, Maillet et al. 2011, Genre et al. 2013). As the fungi and the plant roots make contact, the nucleus moves closer to the surface of contact (Gutjahr and Parniske 2013) and hypopodia are formed by the fungus on the root surface, a process which is depended on cutin monomers produced by the host (Wang et al. 2012). The hyphopodia induce formation of a prepenetration apparatus (PPA) in the plant, which enables the fungi to pass deeper into the cell layer (Genre et al. 2005). This is accompanied by high-frequency calcium spiking in the plant cells (Sieberer et al. 2012). This combination of calcium spiking and subsequent PPA formation guides the fungus until it reaches the cortex. There, the fungus grows mostly through the apoplast and finally branches into the inner cortical cells to starts the formation of arbuscules, tree-shaped structures that enable nutrient exchange with the host (Gutjahr et al. 2013). This requires a drastic morphological change in the plant cell, for which several plant genes are needed (Kistner et al. 2005). Although the arbuscles are formed intracellulary, the hyphae and arbuscle are not in direct contact with the cytoplasm of the plant (Parniske 2008). Instead, the fungal

structures are surrounded by a peri-arbuscular membrane (PAM) formed by the plant. The space in between the PAM and the fungal membrane is called the per-arbuscular space (PAS) (Parniske 2008).

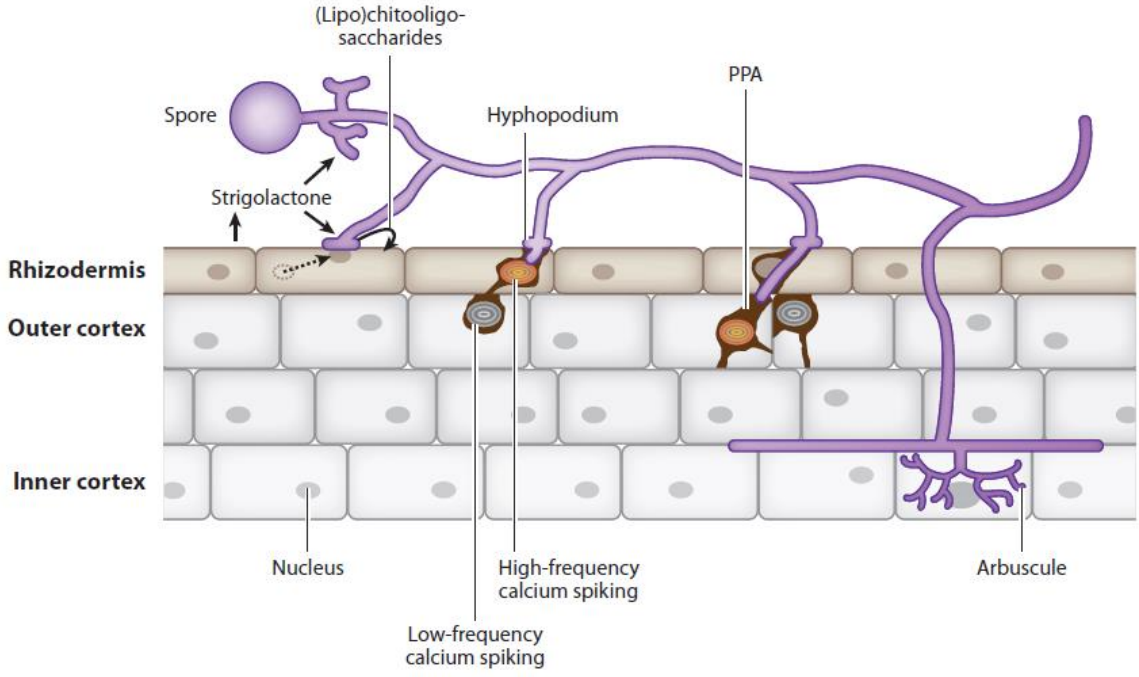

Figure 1: Infiltration of the AM fungi into the host plant. In the precontract phase, the plant excretes strigolactones and the fungus myc factors. The plant nucleus moves towards the surface of contact and the fungus forms a hypopodium. (chitooligosaccharides). The PPA guides the hyphae further into the plant, which is accompanied by calcium spiking. In the inner cortex, the fungus starts to form arbuscles for nutrient exchange (picture from Gutjahr and Parniske 2013).

In addition to the LCOs/COs, it has recently become clear that AM fungi may also use small secreted proteins to communicate with the host plant. It is very plausible that these small secreted proteins are in fact effectors. Effectors are well studied in pathogenic fungi, where they are used by the fungus to modulate MAMP-triggered immune response and enable infection (Kamoun 2017). However, recently it was discovered that effectors are also used by biotrophic AM fungi, when one effector, Secreted Protein 7 (SP7), was characterized in *Rhizophagus irregularis* (previously known also *Glomus intraradices*) (Kloppholz et al. 2011). SP7 suppresses the pathogenesis-related plant transcription factor MtERF19 in *Medicago truncatula*, counteracting the ethylene pathway that mediates the immune response of the plant host in which ERF19 is involved. Another effector secreted by *R. irregularis* is SIS1, which is induced by plant strigolactones (Tsuzuki et al. 2016). Knock down of SIS1 resulted in significant suppression of colonization as well as the formation of stunted arbuscles, suggesting an important function in mycorrhization. More examples of effectors in symbiotic fungi are studied, such as MiSSP7 from the ectomycorrhizal fungus *Laccaria bicolor* (Plett et al. 2014).
Recently the first genome sequence of the AM fungus *Rhizophagus irregularis* was revealed. Bioinformatics mining of this genome identified ~550 putative (small) secreted proteins, many of which may be acting as AM effectors. Transcriptome analysis at the lab of Molecular Biology, have shown that many of these secreted proteins are induced at specific stages during the interaction with diverse plants (Tian Zeng, personal communication). The discovery of these effectors in AM fungi leads us to believe that not only the common SYM pathway, but also second pathway involving effectors is important for establishing mycorrhization and that understanding its mechanisms is crucial to understanding how AM fungi can engage it beneficial symbiosis with such a broad range of species, without triggering immune

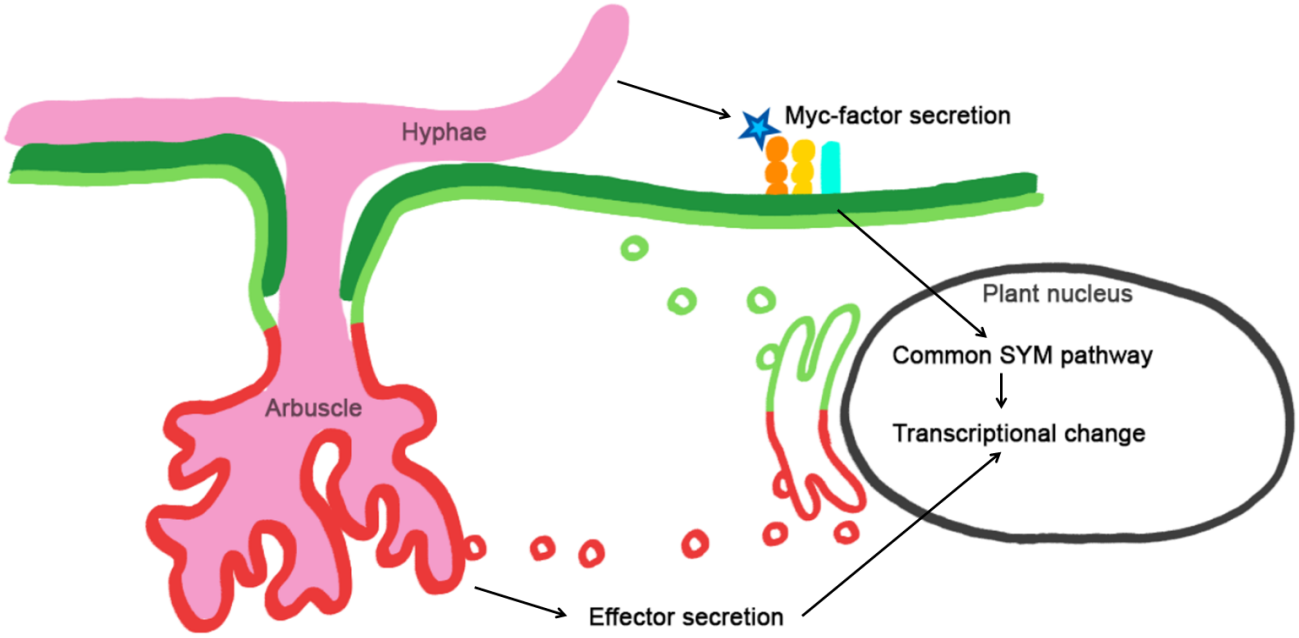

Figure 2: Our current hypothesis: Transcriptional change required for mycorrhization is induced by the Common SYM pathway, as well as secreted fungal effectors that translocate towards the nucleus.

response. Effectors could function to contribute the extremely broad host range or to influence symbiotic efficiency. Our current hypothesis is, that some of these effectors are secreted by the fungus and translocate to the plant nucleus to induce transcriptional change that is required for successful mycorrhization.

To study our hypothesis, a selection of effector candidates was made from the small proteins secreted by the fungus. These candidates are characterized by high expression in arbuscles, internal hyphae or both during specific stages of mycorrhization. In addition, the expression of these candidates is conserved among interaction with *Medicago, Nicotiana* and *Allium*. Although it is known that these candidates are expressed, their function in establishing mycorrhization is still unknown. Furthermore, it has not been confirmed that these candidates actually translocate to the plant nucleus after secretion, even though some of them have domains that predict this localization (Tian Zeng, personal communication).

Aim and Approach

The most straightforward method to learn more about the function of a gene is usually to generate a mutant that lacks this gene and observe any change in phenotype or expression levels. However, as our species of interest, *Rhizophagus irregularis,* has many nuclei in a single cell at all stages (Marleau et al. 2011), it

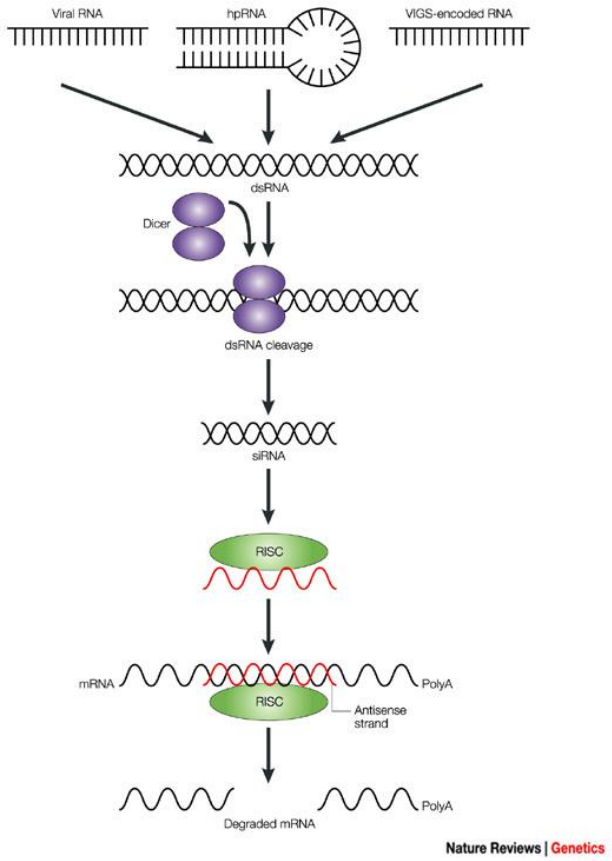

Figure 3: Mechanism of gene silencing by introducing dsRNA. The dsRNA is cut by dicer into siRNA. siRNA forms a complex with RISC, which degrades mRNA with a sequence complementary to the siRNA, thus silencing the corresponding gene (picture from Waterhouse and Helliwell 2003)

is currently very hard to generate a stable transgenic line. This is why a different method is required to study the function of effector candidates. To confirm effector candidate translocation, a method that separates the nuclei of mycorrhized host cells and non-mycorrhized host cells is needed. The focus of this research will be to test the efficiency and reliability of a few techniques, which could potentially be used to study the functionality and localization of our effector candidates. The research questions we tried to answer in this paper are:

Can "*Virus induced gene silencing*" (VIGS) and "*host induced gene silencing*" (HIGS) be used as reliable and efficient methods to study the function of arbuscular mycorrhizal effectors?

Can "*isolation of nuclei tagged in specific cell types*" (INTACT) be used as a reliable and efficient method to study translocation of arbuscular mycorrhizal effectors to the plant nucleus?

VIGS and HIGS

Silencing of genes with VIGS as well as HIGS is based on the same principle of introducing double stranded RNA (dsRNA). In VIGS, this dsRNA is transcribed from a viral vector (Lange et al. 2013). In HIGS, a transgene is introduced in the plant host that forms a so called hair pin construct when transcribed to RNA (Kumagai and Kouchi 2003). In both methods, dsRNA is cut by a dicer protein in smaller double stranded pieces, known as small interference RNA (siRNA). The siRNA is loaded into the RISC complex, which will degrade mRNA with a sequence, complementary to the loaded siRNA, thus silencing the corresponding gene. (Waterhouse and Helliwell 2003) Although the presence of this mechanism in *R. irregularis* is mostly unknown, attempts to silence fungal genes were successful in several previous studies. In Kikuchi et al. 2016, VIGS was used to successfully silence the water transporter aquaporin in *Rhizophagus clarus* (RcAQP3) by using a cucumber mosaic virus system. This resulted in a measurable decrease in expression levels and phosphate transport (Kikuchi et al. 2016). In an earlier study, by Helber et al. 2011, the MST2 transporter was silenced in *R. irregularis* with the use of HIGS, resulting in reduction of mycorrhization, as well as impaired arbuscles (Helber et al. 2011). A fivefold decrease in MST2 expression and almost completely abolished PT4 expression of the *M. truncatula* host was observed. In another example based on Helber's study, HIGS was used to silence aforementioned SIS1 effector, also in *R. irregularis*. Again, silencing resulted in a decrease in expression levels and mycorrhization (Tsuzuki et al. 2016). VIGS and especially HIGS have also been used to successfully silence and study fungal genes, including effectors, in (hemi)biotrophic pathogenic fungi (Nowara et al. 2010, Tinoco et al. 2010, Koch et al. 2013, Panwar et al. 2013, Hu et al. 2015, Cheng et al. 2015, Govindarajulu et al. 2015).

This leads to the assumption that dsRNA introduced in VIGS or HIGS may be able to travel to the fungus and induce silencing through a similar mechanism as described above once inside. It is also possible that the dsRNA is first cut inside the plant host and that the siRNA travels to the fungus instead. (Baulcombe 2015) In this study, we attempted to silence several *R. irregularis* effector candidates in *Nicotiana benthamiana* using VIGS. As silencing of AQP has been done successfully in a related fungal species, but didn't result in a decrease of mycorrhization (Kikuchi et al. 2016), the homolog gene in *R. irregularis* will be used as a positive control to see if silencing can be observed in our setup, which has to be confirmed before conclusion can be drawn about other apparent silencing effects that we may find. To test HIGS, we tried to silence a similar group of effector candidates in hairy root cultures of *Medicago truncatula* and used the known effector SIS1 as a positive control, as it was successfully silenced with HIGS before (Tsuzuki et al. 2016).

INTACT

For the third experiment in this study, we attempted to isolate nuclei of mycorrhized *Medicago truncatula* cells with the INTACT method to confirm translocation of our effector candidates to the plant nucleus of arbuscule containing cells. To perform INTACT, two transgene constructs have to be introduced into the host plant. The first (1) is a nuclear target fusion protein (NTF), which consists of a nuclear-envelope targeting domain, a green fluorescent protein (GFP) and biotin ligase recognition peptide (BLRP). When NTF is expressed in a cell, it will translocate to the outer shell of the nucleus of that cell. The second (2) construct, actin biotin ligase (BirA) will biotinylate the BLRP part of NTF. Magnetic streptavidin beads, which have an affinity for biotin, will attach to nuclei tagged with NTF after addition. With the use of a magnet, the beads and attached nuclei can then be isolated from the rest of the plant material (Deal and Henikoff 2015, Wang and Deal 2015). To use INTACT specifically for isolation of nuclei from arbuscule containing cells, we expressed NTF and BirA with PT4 and BCP1 promoters, both specific for arbuscule cells (Harrison et al. 2002, Hohnjec et al. 2005). Successful NTF expression with pPT4 has already been

observed by previous research in our lab (Erik Limpens, personal communication). However, expression of BirA with pBCP1 was not. To confirm specificity, a third construct, consisting of a detectable mCherry and HA-tag was expressed from a 35S promotor and targeted to the apoplast through the presence of an N-terminal BCP1 signal peptide (Ivanov and Harrison 2014). If INTACT is successful, this secreted mCherry protein should not be detected in the INTACT isolated nuclei.

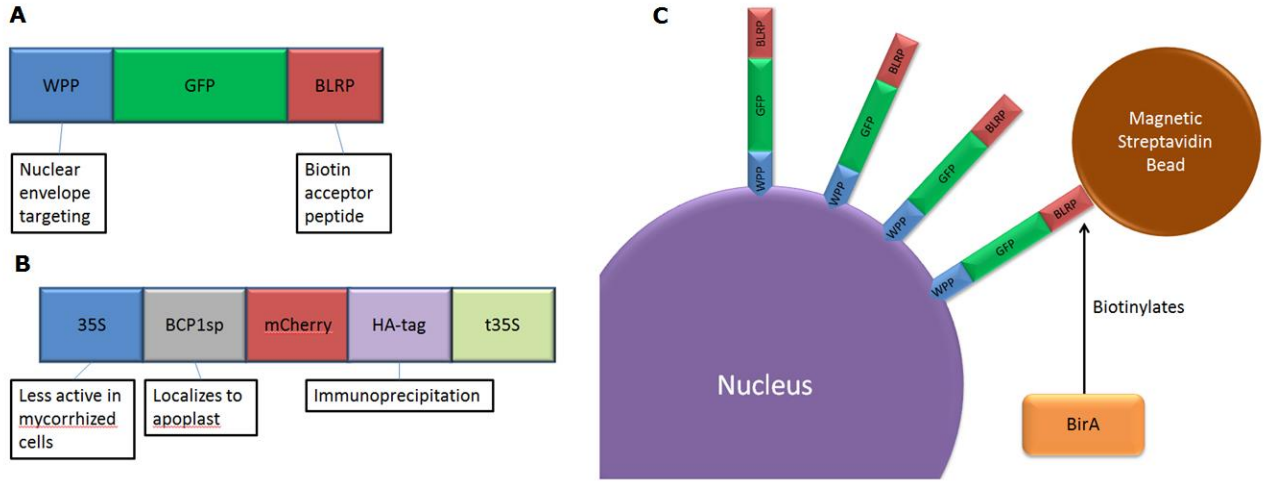

Figure 4: The INTACT mechanism. (A) The NTF construct, consisting of nuclear envelope targeting WPP, GFP and biotin acceptor peptide BLRP. (B) The complete mCherry construct, consisting of 35S promotor, BCP1 signal peptide, mCherry, HA tag and t35S stop codon. (C) NTF constructs attach to the outer shell of the nucleus in which they were expressed. The BLRP part is biotinylated by BirA, increasing its affinity for magnetic streptavidin beads.

Results

Over the course of 7 months, VIGS and HIGS experiments were performed with several effector candidates (Table 1), as well as INTACT, to investigate their efficiency and reliability.

Table 1: A selection of effector candidates that was used in the HIGS and VIGS experiments.

Effector candidate	Experiment	Highly expressed in	Plant hosts
Ri245	VIGS/HIGS	Arbuscles/hyphae	*Medicago, Nicotiana, Allium*
Ri172	VIGS/HIGS	Arbuscles/hyphae	*Medicago, Nicotiana, Allium*
Ri128	VIGS	Arbuscles	*Medicago, Nicotiana, Allium*
Ri167	VIGS/HIGS	Arbuscles	*Medicago, Nicotiana, Allium*
Ri238	VIGS/HIGS	Arbuscles	*Medicago, Nicotiana, Allium*
Ri123	VIGS/HIGS	Hyphae/(Arbuscles)	*Medicago, Nicotiana, Allium*
Ri118	HIGS	Arbuscles	*Medicago*

VIGS

Insertion of effector fragments in pTRV2 vector

From the effector candidates, a selection was made of effectors that we would try to silence with VIGS, using the Tobacco Rattle Virus system (Ratcliff 2001), which has very successfully been used for silencing experiments in *Nicotiana benthamiana* (Velásquez et. 2009). Chosen effectors were : Ri245 and Ri172, which was highly expressed in both arbuscles and hyphae, Ri238, Ri167 and Ri128, which were highly expressed in arbuscles and Ri123, which was highly expressed in hyphae (although more recent data suggests it may also be expressed in arbuscles). For these effectors, as well as the positive control RiAQP, primers were designed to create short DNA fragments. These fragments were amplified and purified, after which they were inserted into a pTRV2 vector by digestion and ligation (Figure 5). The vectors were sequenced, which confirmed that RiAQP and all effectors, except for Ri172 were successfully inserted into pTRV2. Because of limited time, Ri172 was left out for the remaining VIGS experiments. After successful insertion of effector fragments, as well as RiAQP, into pTRV2, the vectors were transformed into *Agrobacteria*. An already available *Agrobacteria* stock containing pTRV2-GUS was also included in the experiment as a negative control and an *Agrobacteria* stock containing pTRV2-PDS, was included as a positive control for virus infection, as it leads to white colouring of the leaves (Busch et al. 2002).

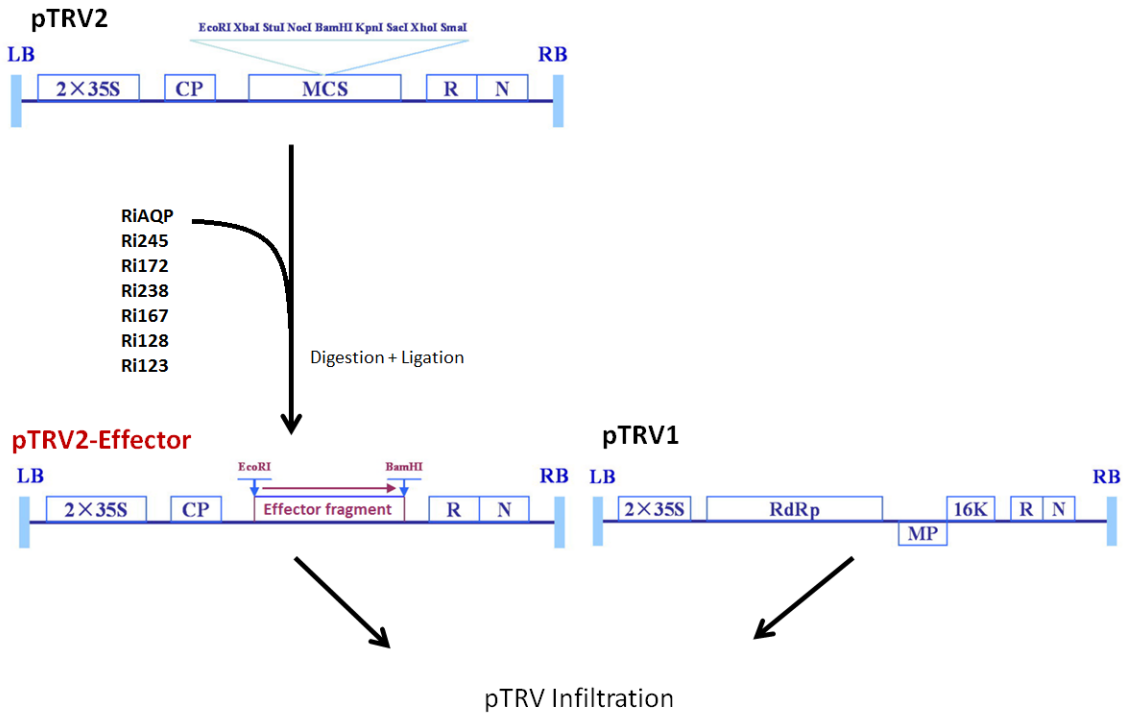

Figure 5: Effector fragments were inserted into pTRV2 by digestion and ligation with EcoRI and BamHI and transformed into *Agrobacteria*. pTRV1 was was transformed into *Agrobacteria* as well and combined with pTRV2 to obtain the complete pTRV infiltration product (picture modified after Wang et al 2013).

Efficiency of leaf infiltration and agrodrench of *Nicotiana benthamiana* with PDS

Before infiltration, *Agrobacteria* with pTRV2 were mixed *Agrobacteria* containing pTRV1, to form the complete pTRV virus. To infiltrate *Nicotiana bethamiana* plants, two methods were used. The first is leaf infiltration, in which the *Agrobacteria* with virus are directly injected into the leaf and the second is agrodrench, in which they are pipetted on top of the plants. Both methods were tested with a PDS control, which causes leaves to become pale. 5 plants were treated with leaf infiltration of PDS and 18 plants with agrodrench of PDS. Of those 18 plants, 6 were only drenched once, 6 were drenches a second time one day after the first treatment and 6 were treated three times. After 5 weeks, the 3 surviving plants that had been treated with leaf infiltration all showed the pale leaf phenotype. Of the 4 surviving plants that got one agrodrench treatment, none showed this phenotype, of the 3 surviving plants that got two treatments, 1 showed this phenotype and of the 4 surviving plants that got three treatments, 2 showed this phenotype. This was already an indication that leaf infiltration is more efficient than agrodrench, although several consecutive agrodrench treatments seem to improve its success. As PDS control was used, which gives a phenotype in the leaves (Busch et al. 2002); it is still possible that agrodrench treatment had only caused viral spread in the roots, which is the location of interest of this study. To check this, we used qPCR to monitor the spread of the fungus to the roots, see below.

Harvesting *Nicotiana* plants and qPCR setup

The first batch of plants was only infiltrated with leaf infiltration. The constructs that were used are GUS, as a negative control, RiAQP, as a positive control, Ri245, Ri238, Ri167, Ri128 and Ri123. For the second batch, plants were sorted based on size. Big plants were infiltrated with leaf infiltration and small plants with agrodrench. Samples in the second batch were labelled based on treatment: L for leaf infiltration and D for agrodrench. In this second batch, only GUS, RiAQP and Ri245 were used, to be able to focus on efficiency of silencing. After 5 weeks, all plants were harvested. In both batches, plants that received the same treatment and were similar sizes at the time of infiltration, already showed variation in final size. Final biomass ranged from 0.07g to 0.69g in the first batch, from 0.19g to 2.65g in the leaf infiltrated plants of the second batch and from 0.10g to 0.56g in the agrodrench plants of the second batch. It is possible that this variation is caused by different amounts of stress caused by transfer from perlite to pots or injecting the plants during leaf infiltration. Such a big difference in plant-fungus ratio between different plants may cause unwanted variation in the results.

Some roots were stained with trypan blue and screen under the microscope to see if mycorrhization could be observed. Both infecting hyphae and arbuscles could be found (Figure 6), although total amount of fungus seemed low compared to the amount of root. The amount of arbuscles was not quantified, due to low amount of root material and suboptimal staining.

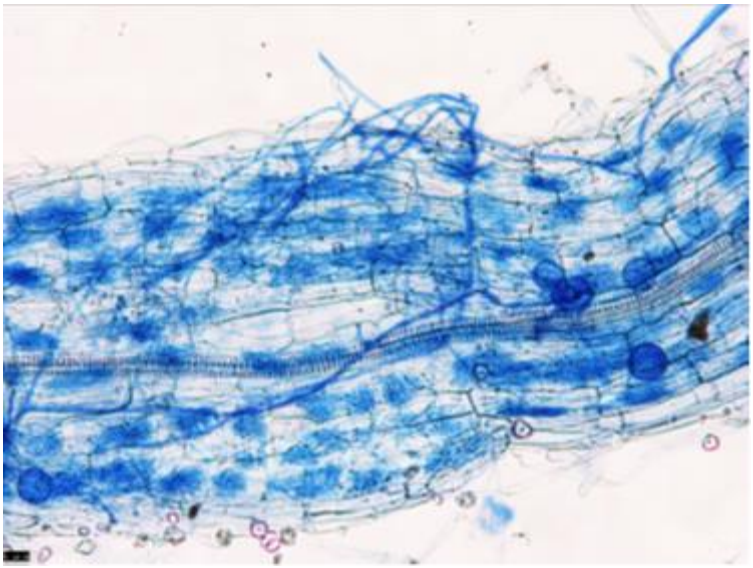

Figure 6: A root piece of sample L GUS-5.1, stained with trypan blue. Both hyphae and arbuscles can be observed.

RNA was isolated from roots that showed a green signal, cDNA was generated and qPCR was performed on cDNA to quantify the expression levels of effector genes and RiAQP. For plant genes, two primers were used: NbEF which is a housekeeping gene (Dean et al. 2002) and NbPT4, which is a marker for arbuscles (Javot et al. 2007) For the fungus, primers for housekeeping gene RiEF, as well as genes expressed in arbuscles Ri128, Ri167 and Ri238 (Tian Zeng, personal communication) were used, as well as primers for the respective effector candidate that was attempted to silence in each plant. One pTRV2 primer was used, to detect the level of virus. From the output of the qPCR, relative expression levels of each gene were calculated by comparing average Ct values of the gene of interest with the average Ct value of a housekeeping gene (Haimes and Kelley 2010)

Table 2: List of primers that were used in qPCR and their function in the analyzation. For sequences of primers, see Appendix A.

Primer	**Function**
NbEF	Housekeeping gene of plant as reference for plant biomass
NbPT4	Plant gene active in mycorrhized cells
RiEF	Housekeeping gene of fungus as reference for fungal mass
Ri167	Fungal gene highly expressed in arbuscles
Ri128	Fungal gene highly expressed in arbuscles
Ri238	Fungal gene highly expressed in arbuscles
RiAQP, Ri245, Ri123, Ri167, Ri128, Ri238	Effector candidates that were attempted to silence
pTRV2	Viral vector as reference for virus abundance

Efficiency of leaf infiltration and agrodrench of *Nicotiana benthamiana* analysed by qPCR
First, the relative pTRV2 expression in each plant compared to NbEF was determined. When comparing the leaf infiltrated plants from the second batch to the agrodrench plants (Figure 7), the results show that a large amount of virus is expressed in the majority of leaf infiltrated plants, but hardly any virus is expressed in agrodrench plants. Together with the lack of PDS phenotype in PDS control plants, it can be concluded that agrodrench is less effective than leaf infiltration. Agrodrench plants were not used for further analysis, as no silencing effect is expected in plants that do not contain virus.

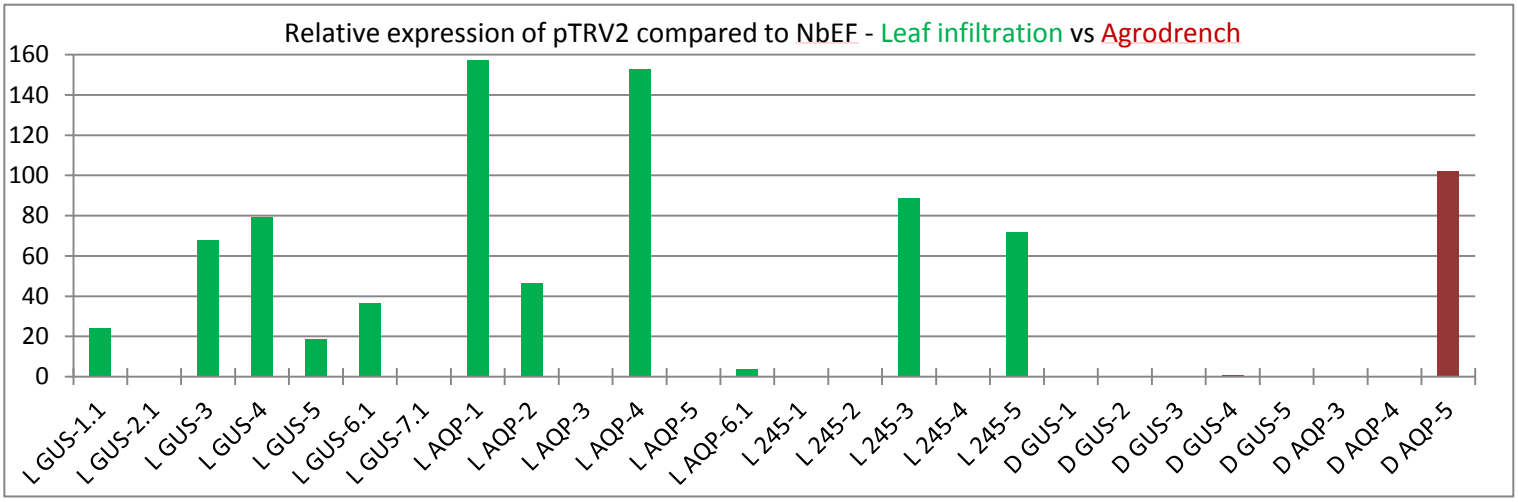

Figure 7: The relative expression of pTRV2 when compared to NbEF for leaf infiltrated (L) and agrodrenched (D) *N. benthamiana* of the second batch.

Mycorrhization levels of harvested *Nictotiana benthamiana*

After confirming virus abundance, the expression of arbuscular marker gene NbPT4 relative to NbEF was investigated (Figure 8, Figure 10), as well as the total mass of the fungus compared to the plant by looking at RiEF relative to NbEF (Figure 9, Figure 11). The results show that there is plenty of variation in mycorrhization levels and fungal biomass, even in plants that received the same treatment. When looking at the first batch, another interesting observation is the low mycorrhization and low fungal biomass in Ri128 plants. This could be an effect of silencing Ri128 or simply coincidence, as similar low levels are also observed in other plants. Before conclusions can be drawn, silencing has to be confirmed first. The second batch of leaf infiltrated plants show a significantly lower expression level of NbPT4 in all plants, when compared to the first batch. Absolute Ct values of NbPT4 are approximately the same for the first and second batch (Ct of 27-28), but total plant biomass is larger in the second batch (NbEF of 25-27 instead of 26-29) resulting in this huge difference in relative expression. The same difference can be seen for fungal biomass when comparing RiEF relative to NbEF for the first and second batch. This indicates that there was less total root colonization in the second batch.

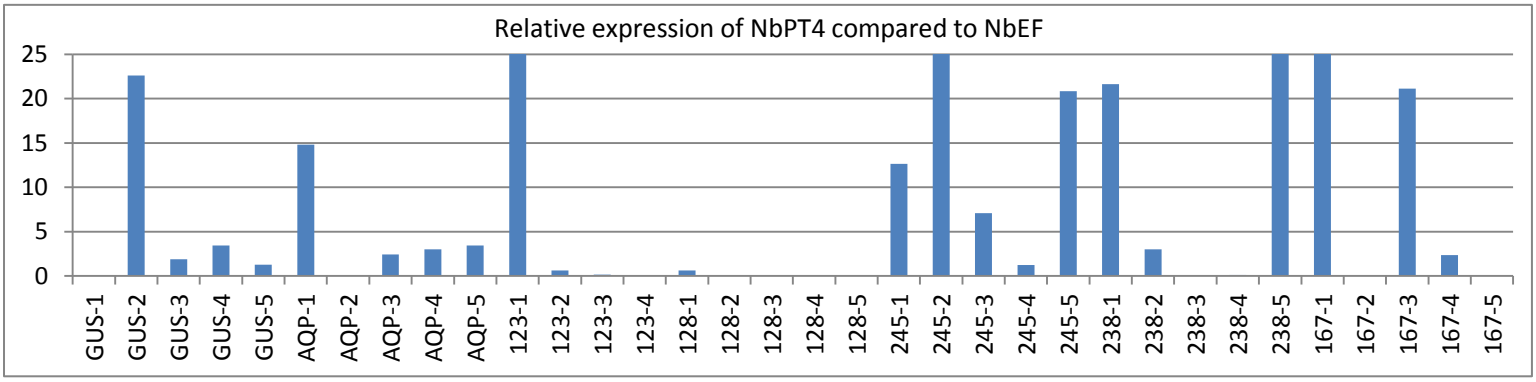

Figure 8: Expression of NbPT4, relative to NbEF in the first batch of plants. This is an indicator for the amount of arbuscles in the total root

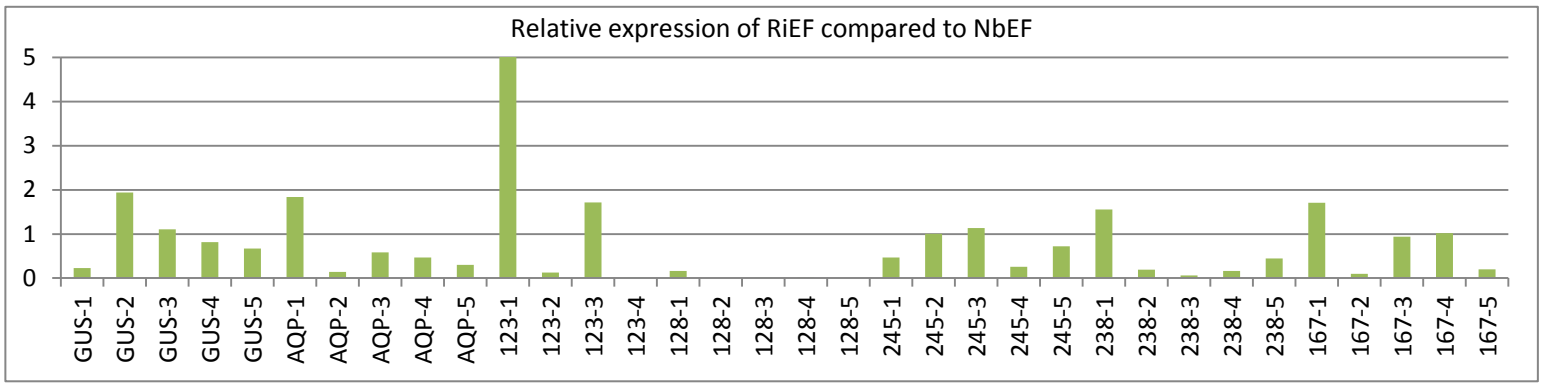

Figure 9: Expression of RiEF, relative to NbEF in the first batch of plants. This is an indication of the total amount of fungus in the total root.

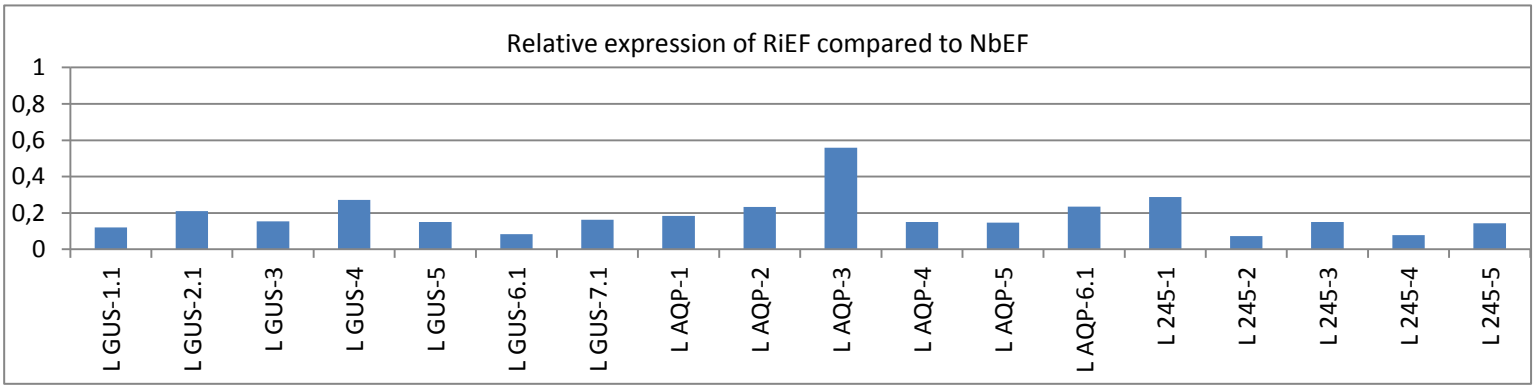

Figure 10: Expression of NbPT4, relative to NbEF in the second batch of plants. This is an indicator for the amount of arbuscles in the total root

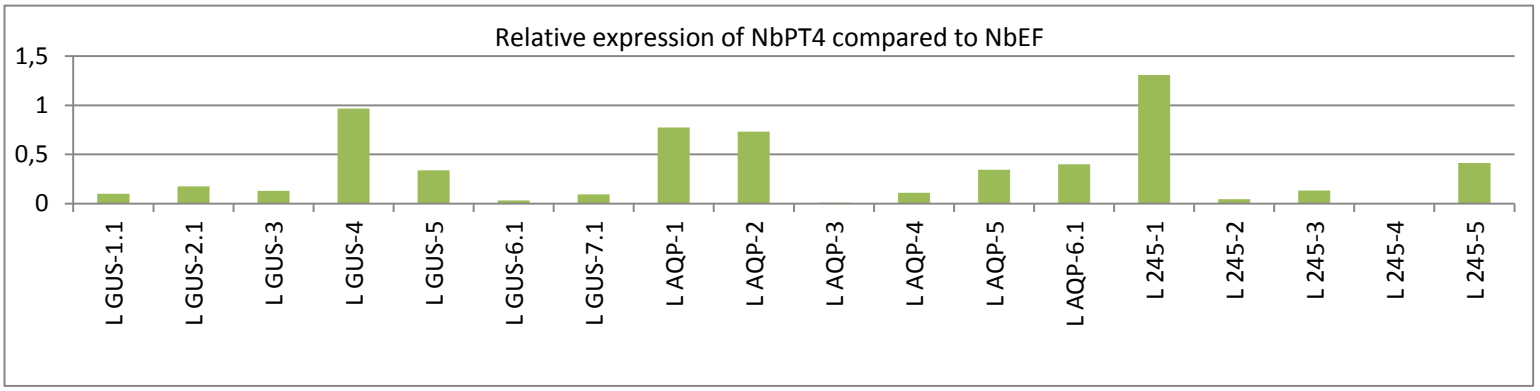

Figure 11: Expression of RiEF, relative to NbEF in the second batch of plants. This is an indication of the total amount of fungus in the total root.

Silencing of RiAQP and correlation with virus abundance

Expression of the RiAQP positive control relative to RiEF was compared in GUS (negative) control plants and plants in where an attempt was made to silence RiAQP (Figure 12, Figure 14). In both the first and second batch of leaf infiltrated plants, levels of RiAQP are not significantly lower in RiAQP plants compared to GUS plants. Between all plants similar variation in RiAQP can be observed. Surprisingly, in the second batch, detected RiAQP levels appeared to be higher in some RiAQP plants than in GUS plants. Since the qPCR primers are outside of the fragment targeted in the pTRV2 vector, these higher levels are not caused by the VIGS construct. When comparing the RiAQP levels to the abundance of virus in each plant, there appears to be no correlation in the first batch of plants (Figure 13, Figure 15). However, in the second batch, it appears that the level of RiAQP expression is high when the virus abundance is low and that RiAQP is low when the virus abundance is high. However, when comparing the lowest levels of RiAQP that are observed in the RiAQP VIGS plants to the levels of expression in GUS control plants, they are not significantly lower. Therefore, apparent decrease in RiAQP levels in RiAQP VIGS plants is no proof for silencing. It should be noted that RiAQP in both the GUS and the RiAQP plants is very low compared to expression of all other genes that were investigated. Because of this, variation in expression because of silencing could be hard to detect. It is more likely that variation in mycorrhization levels is causing the observed differences.

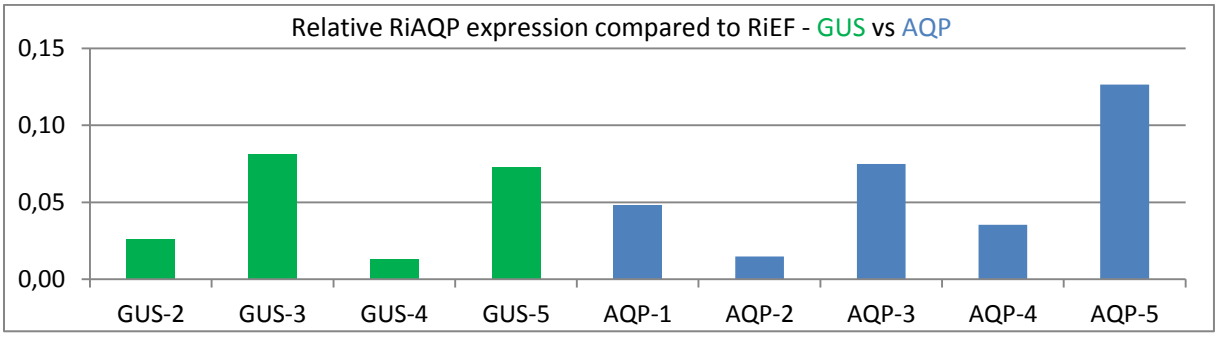

Figure 12: RiAQP expression relative to RiEF in GUS and RiAQP plants of the first batch. GUS-1 was left out because of bad mycorrhization.

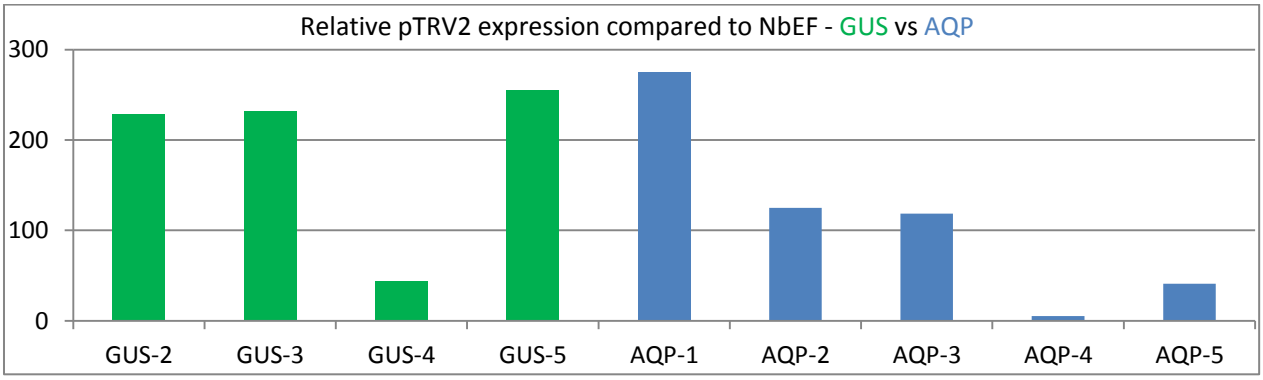

Figure 13: pTRV2 expression relative to NbEF in GUS and RiAQP plants of the first batch. GUS-1 was left out because of bad mycorrhization.

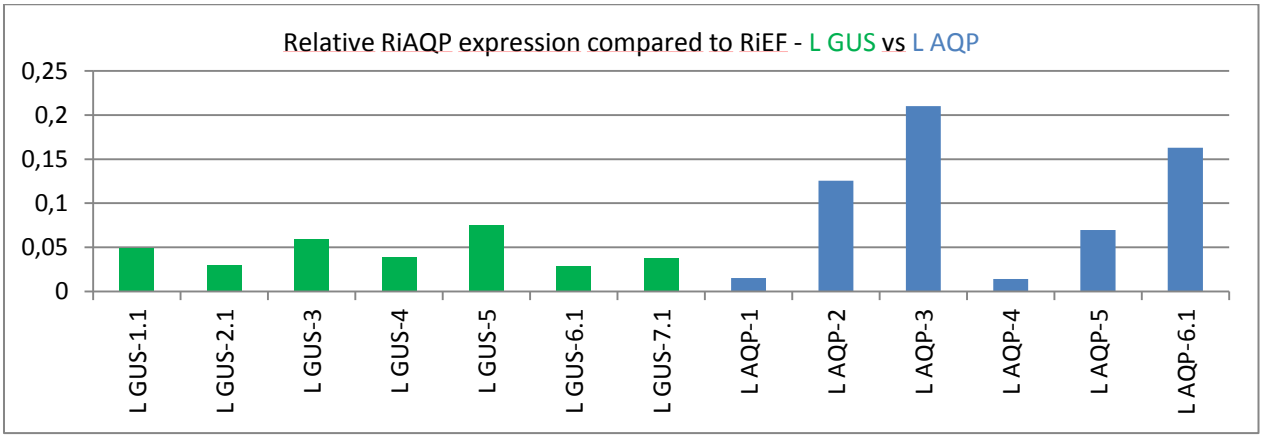

Figure 14: RiAQP expression relative to RiEF in GUS and RiAQP plants of the second batch.

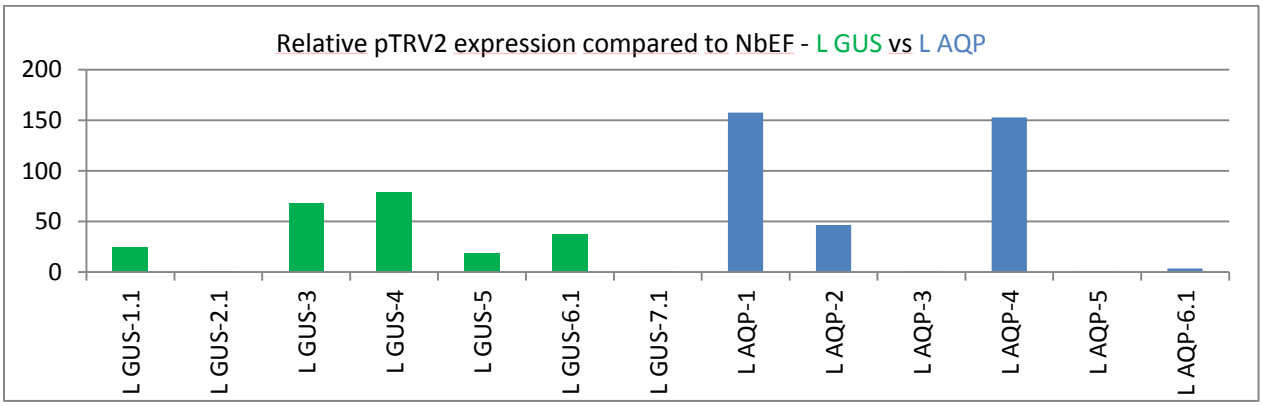

Figure 15: pTRV2 expression relative to NbEF in GUS and RiAQP plants of the second batch.

Silencing of effector candidates

Even though no silencing is observed for RiAQP, the levels of effector candidates relative to RiEF were compared between GUS plants and plants in which an attempt was made to silence them (Figure 16). Similar to AQP, Ri167 expression does not seem to change in Ri167 VIGS plants. Variation between GUS and Ri167 VIGS plants, as well as the highest and lowest expression levels of Ri167, appears to be similar. This indicates that there is no proof of silencing.

When looking at Ri128 expression in GUS and Ri128 plants, it appears that Ri128 is not expressed in Ri128 VIGS plants. However, it is important to note that all of these Ri128 plants also had almost absent mycorrhization levels and total fungal biomass, which would also cause absence of Ri128 expression. It could be argued that mycorrhization and biomass is low because Ri128 has an important function in establishing the interaction, which is now silenced. However, it is not possible to confirm this with the available data as no silencing is observed in any of the other plants.

Surprisingly, expression levels of Ri238 and Ri245, relative to RiEF, are elevated in plants where and attempt was made to silence them compared to GUS control. Again, qPCR primers for both constructs are not inside the silencing sequence, so this is not the cause of this increase. An apparent increase in expression could also be caused by normalization with decreased RiEF expression, which would change the ratio between RiEF and effector. Especially if the amount of arbuscles would stay the same and amount of hyphae, which also expresses RiEF, decreases because of a silencing effect. However, no significant difference in RiEF expression, NbPT4 expression and elevated expression of Ri238 and Ri245 is clear.

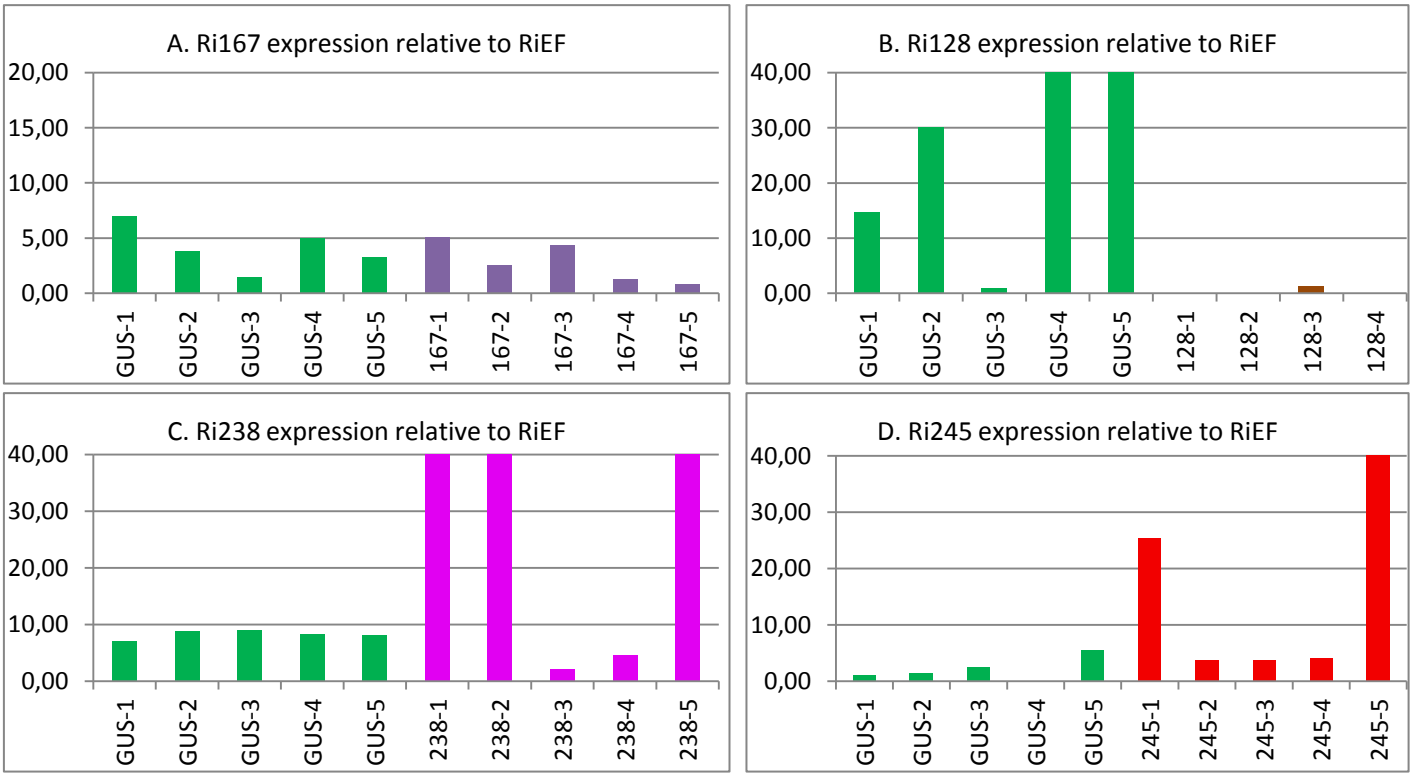

Figure 16: Expression of effector candidates relative to RiEF, compared in GUS and the plants in which an attempt was made to silence the effector. (A) Ri167, (B) Ri128, (C) Ri238 and (D) Ri245 are shown here.

Correlation between Ri128 and Ri167

Even though silencing was not confirmed for Ri128 and Ri167, their expression levels relative to RiEF were compared in GUS and AQP VIGS plants, to see if their expression is correlated with respect to arbuscule abundance. Both Ri128 and Ri167 were identified as arbuscule specific effector proteins from previous RNAseq data. As shown in Figure 17, the difference in expression level between Ri128 and Ri167 is the same in almost all samples, except for AQP-5. This indicates that both are relatively stable and that their expression is indeed correlated. As both are specifically expressed in arbuscles, it is safe to assume that Ri128 and Ri167 can indeed be used as arbuscles markers. However, as the direct correlation between these two effectors and the abundance of arbuscles is still not clear, they cannot be used yet as reliable reference genes for future qPCR experiments.

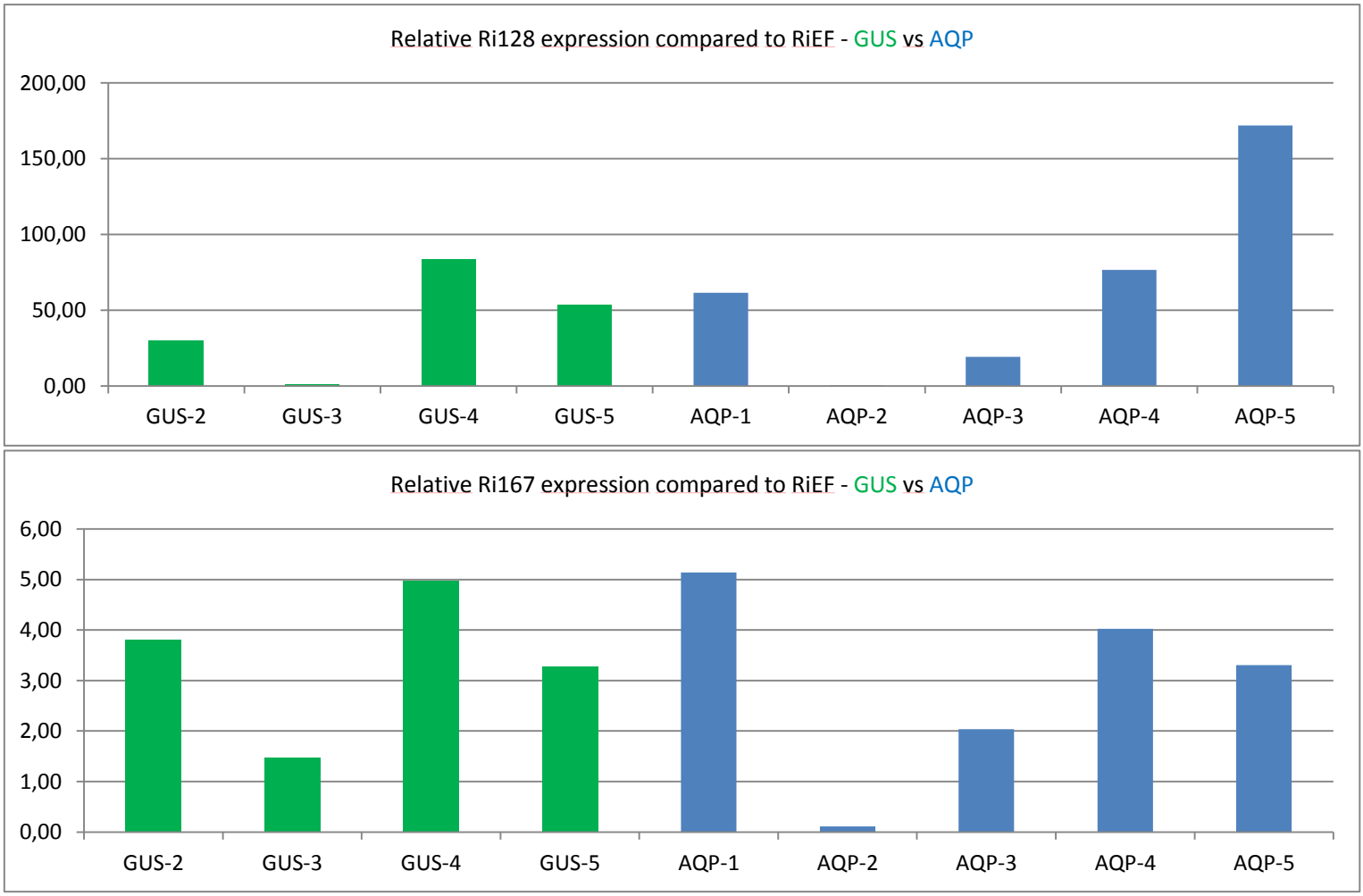

Figure 17: Expression of Ri128 and Ri167, relative to RiEF in GUS and AQP plants of the first batch. In these plants, myccorhization shouldn't be effected by the viral treatment thus Ri128 and Ri167 levels should only depend on the arbuscle abundance. GUS-1 was left out because of bad mycorrhization.

HIGS

Generation of double hairpin transgene with Gateway cloning

From the effector candidates, a slightly different selection was made for HIGS. Again, arbuscles and hyphae specific effectors Ri245 and Ri172, arbuscles specific Ri238 and Ri167 and hyphae (and possibly arbuscles) specific Ri123 were used. In addition, SIS1 was included as a positive control, as well as Ri118, an effector candidate that was also highly expressed in arbuscles but specifically in interaction with *Medicago* and not with *Nicotiana* and Allium. Primers were designed to amplify short fragments from these genes and the DNA was purified. The fragments were assembled in a double hairpin formation by Gateway cloning. First, all fragments except Ri172, were successfully inserted into a pDONR vector using a BP-reaction. Next, the first copy in pDONR and a second copy in the opposite direction were successfully inserted into the final destination vector 277 which allows the expression of the hairpin fragment from a constitutive EF1alpha promotor. The vectors were sequence and aligned with reference sequences, to confirm that insertion was successful. The vectors were transformed into agrobacteria cells and used for transformation of *Medicago* hairy root cultures.

Transformation of *Medicago truncatula* and hairy root cultures

To generate transgenic host plant roots expressing the desired hairpin construct, 8 day old *M. truncatula* plants were transformed using *Agrobacterium rhizogenes* (Limpens et al. 2004). The effectors for which plants were transformed were Ri245, Ri238, Ri118, Ri123 and Ri167. GUS plants were generated as a negative control and SIS1 plants as a positive control. Two weeks later, the plants were screened for red roots, which is an indication of successful transformation. However, SIS1 plants were really small and shrivelled, possibly due to stress caused to the seeds during sterilization. Although new plants were grown and transformed with SIS1, this created a delay in the experiment as the positive control plants were behind on schedule. For the other effector constructs, as well as GUS, red roots were observed. Individual red root pieces were cut off and grown on separate plates to create axenic root cultures (Boisson-Dernier et al. 2001), which turned out to take a long time. Even after 4 months, Ri245, Ri238 and Ri118 plates were not completely covered yet. GUS, Ri123 and Ri167 plates, which were made earlier, did show complete coverage after 3 months. Three slices of root material were cut from each plate and transferred to separate plates, where they were inoculated with approximately 50 spores to start mycorrhization. This turned out to take longer than expected as well. After two months, roots had spread out of the slices. All 3 GUS and Ri123 plates showed spread of hyphae and only 1 out of 3 Ri167. However, hyphae spread and infiltration levels were very variable between plates, even between plates with the same construct. This led us to believe that the method that was used was too susceptible for variation. Because of this shortcoming of our method, as well as the missing SIS1 positive control and limited time, the generated HIGS root cultures will need to be further analysed in the future. Therefore, the inoculation method for these root cultures will first need to be optimized, and the variation among control lines needs to be thoroughly investigated.

INTACT

Generation of INTACT constructs with Golden Gate

To investigate if INTACT would be suitable to study translocalization of effectors, three constructs had to be expressed in *Medicago* plants. The first two constructs, pPT4-NTF and pBCP1-BirA, were already available in our lab (Erik Limpens, personal communication). To generate the third construct, p35S-BCP1sp-mCherry-HA, and combine it with the other two constructs, Golden Gate cloning was used. This method is based on the use of standardized vectors for each part of the desired gene and combining them in a single reaction with a single restriction enzyme. First, level 0 vectors for p35S, BCP1sp-mCherry, HA and a stopcodon were successfully combined into a level 1 vector. Next, level 1 vectors of all three constructs were combined into a single level 2 construct. After sequencing the construct with forward primers for pPT4, pBCP1 and BCP1sp-mCherry, it was confirmed that the level 2 vector was correctly assembled. The level 2 vector was transformed into *Medicago* plants using *A. rhizogenes* mediated root transformation.

Expression of NTF, BirA and BCP1sp-mCherry-HA

Before transferring transformed *Medicago* plants to cones for mycorrhization, they were screened for red expression in the roots, as indication of mCherry expression. Only a faint red signal was observed in roots of about half of the plants. After transfer to cones with spores and growing for 5 weeks, plants were harvested. When observed under fluorescence microscope, all plants showed patches of GFP signal in some of their roots, as expected. However, in none of the plants a clear mCherry signal could be observed, even though it was observed before in some of the plants. This indicates that the mCherry construct is either not functional anymore, expressed in low amounts or rapidly degraded.

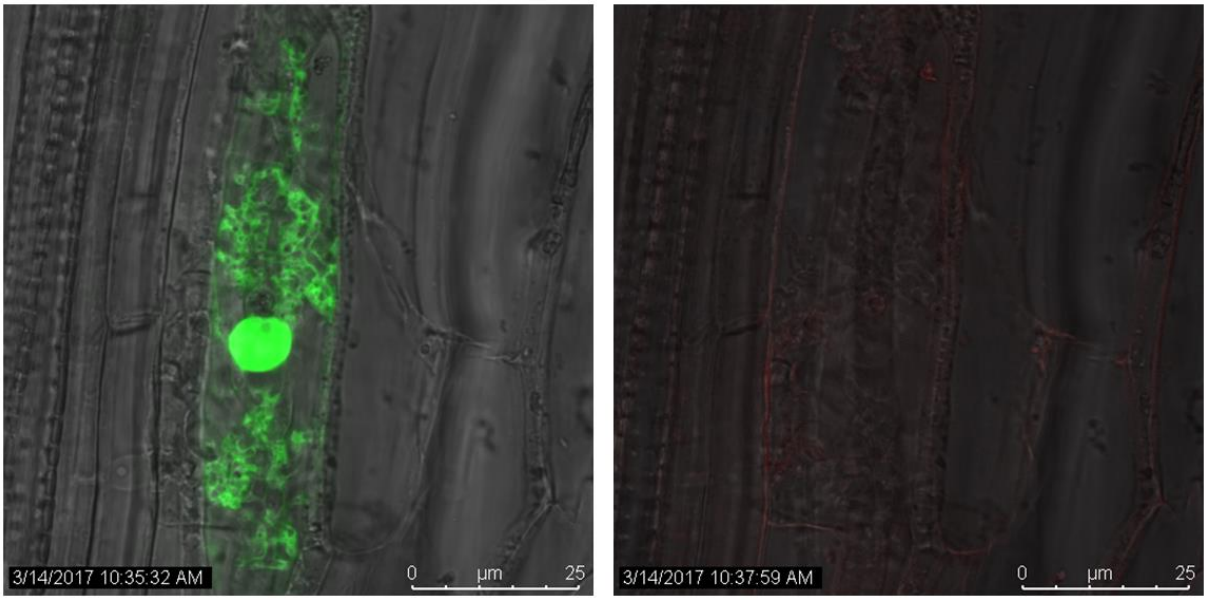

Figure 18: A mycorrhized *Medicago* cell that expresses NTF (left), but only shows a very faint red signal in the apoplast that could be mCherry (right). Green signal is not only observed on the outer shell of the nucleus, but in the whole nucleus, as well as arbuscles in the cell.

To obtain a better picture of the localization of NTF and possibly mCherry, some roots where sliced in half and studied with a confocal microscope (Figure 18). In arbuscule containing cells, a clear GFP signal was observed, not only on the nuclear envelope as expected, but also inside the nucleus and inside the cytoplasm, where is can be seen accumulating around the hyphal branches inside the cell . A red signal, possibly from mCherry, could only be observed in the apoplast when turning up the gain very high (image was made at 580).

To obtain more inside about NTF and mCherry expression, as well as BirA, RNA isolation and Western Blot were done on frozen green root samples. cDNA was generated from isolated RNA of 5 plants and cDNA was amplified with PCR. After running on agarose gel, all 5 samples show bands for NTF (1150 bp), BirA (1056 bp) and BCP1sp-mCherry (850 bp) (Figure 19(A)). Plasmid DNA of corresponding level 0 constructs was used as a positive control; however, this didn't show any clear bands. Possibly, the PCR reaction was inhibited by an excessive amount of plasmid.

Western blot was done with anti-GFP, streptavidin (anti-biotin) and anti-HA antibodies (Figure 19(B).). The GFP blot showed two clear bands in 3 of the 5 samples, one at 42 kDa and one at 26.9 kDa. These sizes correspond with the size of NTF and free GFP. This indicates that a lot of the NTF constructs are degraded and GFP is released. This could also explain why GFP signal was observed not only on the nuclear envelope, but also in the cytoplasm. Free GFP is not expected to have a negative effect on nuclear isolation. The streptavidin blot shows a clear band for 4 of the samples at 42 kDa, which corresponds with the size of NTF. This indicates that NTF is successfully biotinylated by BirA. A lot of faint bands of other sizes can also be observed, showing that more proteins in the plant roots can be biotinylated. Unfortunately, no bands were observed in the HA blot. Since the RNA isolation showed that BCP1sp-mCherry is expressed, these results suggest that the fusion protein is rapidly degraded. The sequence of the mCherry construct was analysed, and one base pair mismatch was found, however this did not result in a different amino acid or a frame shift. Despite the absence of measurable mCherry, root samples of these plants were used for nuclear isolation and INTACT.

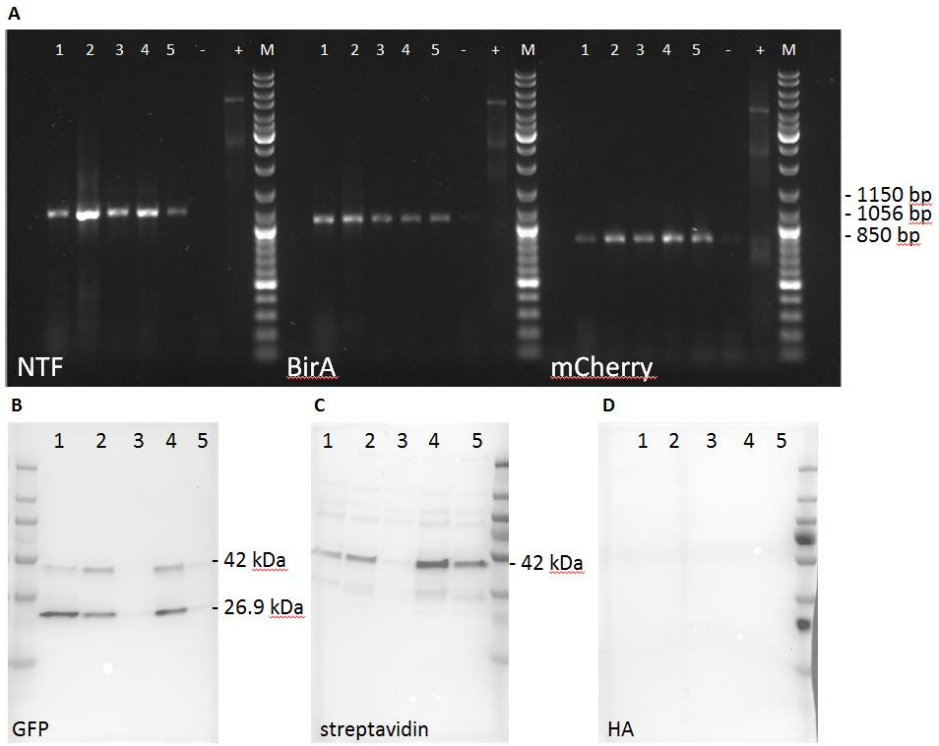

Figure 19: (A) PCR of cDNAs, generated of RNA isolated from green INTACT roots. A bands can be seen for NTF (1150 bp), BirA (1056 bp) and BCP1sp-mCherry (850 bp) in all 5 plants that were sampled. In the Western Blot with anti-GFP antibodies (B), two bands can be seen, one corresponding with NTF (42 kDa) and one with free GFP (26.9 kDa). In the streptavidin (anti-biotin) blot (C), an NTF band can also be seen (42 kDa), as well as many other vague bands. No bands were observed for anti-HA (D).

Specific isolation of nuclei with nuclear purification and INTACT

After determining successful NTF and BirA expression in the *Medicago* roots, root tissue was powdered in liquid nitrogen, filtered and centrifuged to isolate nuclei from other cell material. In each 10 uL sample, around 20 isolated round structures of about 5-10 um could be found that emitted a faint green signal (Figure 20). Presumably, these structures are isolated nuclei tagged with NTF. In control samples stained with SYTOX green, a total amount of about 100 nuclei per 10 uL could be observed. Besides isolated nuclei, each sample that was observed still contained a lot of cell debris, despite previous filtration steps. These results show that the isolation method that was used wasn't very efficient.

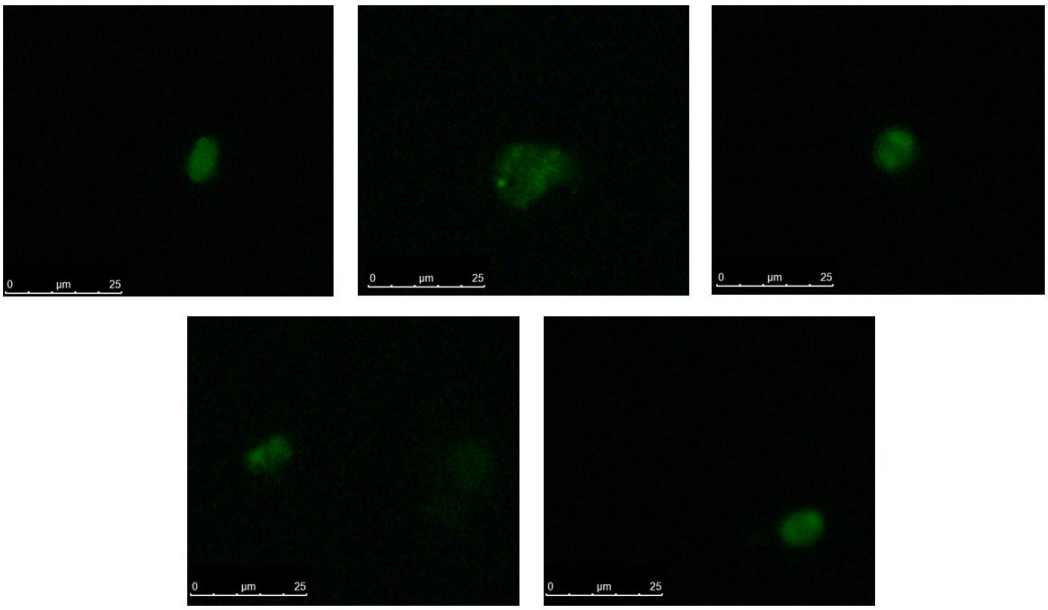

Figure 20: *Medicago* nuclei isolated from other plant material. Green signal indicates expression of NTF in these nuclei.

After purification of the nuclei, magnetic streptavidin beads were added to the sample and incubated to enable attachment of the beads to the specific nuclei with NTF on their outer shell. The sample was inserted into a magnetic clamp to pull magnetic beads to the side and liquid with cell debris was removed. Magnetic beads were resuspened and observed under the microscope (Figure 21). Magnetic beads could be seen as small green circles of approximately 3 um diameter. Rare instances of beads clustered around plant material could be found as well. However, no NTF signal distinguishable from the green beads could be observed in these clusters. DAPI staining revealed that beads were clustered around nuclear material after all, among other unidentified plant debris. This leads us to believe that isolation of nuclei with INTACT was successful, although the method as applied in this study was highly inefficient and specificity of the isolation was not confirmed.

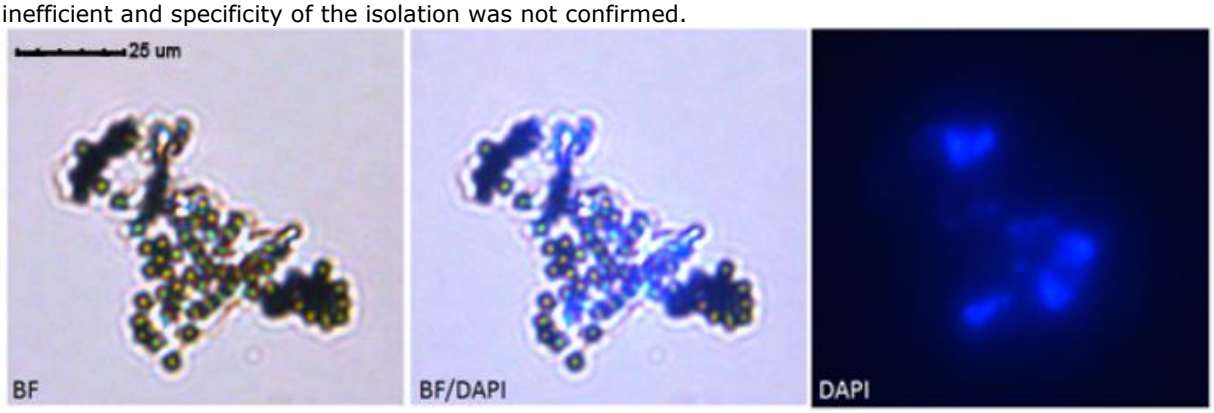

Figure 21: Beads (green in bright field) clustering around nuclear plant material (blue with DAPI).

Discussion and Conclusion

The focus of this research was to test the efficiency and reliability of a few techniques, which could potentially be used to study the functionality and localization of our effector candidates. The research questions we tried to answer in this paper were:

Can "*Virus induced gene silencing*" (VIGS) and "*host induced gene silencing*" (HIGS) be used as reliable and efficient methods to study the function of arbuscular mycorrhizal effectors?

Can "*isolation of nuclei tagged in specific cell types*" (INTACT) be used as a reliable and efficient method to study translocation of arbuscular mycorrhizal effectors to the plant nucleus?

VIGS and HIGS

In the VIGS experiment, no silencing was confirmed of the RiAQP or any of the effector candidates. For RiAQP, no observation of silencing could be caused by the low expression of RiAQP in the wildtype plant. There are more examples, such as Groten et al. 2015, in which an attempt was made to silence *R. irregularis* genes without success. In this study, VIGS was successfully applied on to silence host plant genes of *Nicotiana attenuate*. However, when the same technique was used in an attempt to silence fungal gene MST2, which was previously silenced in hairy root cultures by HIGS by Helber et al 2011, this was unsuccessful. This leads us to the question whether or not VIGS and HIGS can be applied to silence fungal genes, as different results are observed in different studies. In Helber et al. 2011, a significant decrease in fungal MST2 expression and mycorrhization, as well as almost completely abolished PT4 expression was observed in plants were an attempt was made to silence MST2 with HIGS. This is similar to what we observe when VIGS is used to silence Ri128. However, in both cases, these observations could be caused by lack of mycorrhization, as in both cases mycorrhization levels vary, even the control. Helber et al. do not show a positive control that does not have an effect on mycorrhization levels when silenced to proof that HIGS has an effect on fungal genes. Although MST2 in their study and Ri128 in our study both show a significant decrease in mycorrhization and expression levels, the difficulty to replicate a silencing effect in a positive control and large differences in mycorrhization levels in negative controls makes it difficult to proof silencing in both cases, although the observed decrease would still implicate that these genes may be interesting for future studies. In Kikuchi et al. 2016 they claim that they did silence RcAQP, for which they do not observe a change in mycorrhization level which would make it a proper positive control for silencing. It is important to note that this was done in *Rhizophagus clarus*, instead of *Rhizophagus irregularis* which we used, so despite the close relationship between the two fungi, the silencing mechanisms of dsRNA, as well as the homolog AQP genes, may be different in these species. However, in both species the expression levels in control plants are already very low. The sample size used by Kikuchi et al. for each experimental group is only 3 plants and here as well a variation in mycorrhization levels and fungal biomass is observed, which could also be the cause of an apparent difference in expression levels of RcAQP. Based on the doubtful data in

these experiments and the lack of proof for successful silencing in our own data, we conclude that the VIGS and HIGS approach is likely not a reliable and efficient method to silence fungal genes.

Furthermore, several factors in our own experimental setup were causing variation, even in control plants, making it difficult to draw significant conclusion from the final data. Such variation includes different plant sizes, mycorrhization levels and virus abundance. From the final data, it is difficult to conclude if an observation is caused by one of these variations or an actual effect from the treatment. If VIGS is given another try, it will be critical to synchronize these factors to rule out variation in the results caused by variation in the setup. It may be difficult or even impossible to completely get rid of all variation, but one thing that would help to realize this is increasing the number of plants and select for equal plant sizes both before inoculation and before harvesting. The same is true for HIGS, as we were unable to continue the HIGS experiment in this study because our hairy root cultures were not synchronized at all. When analysing data, only control plants with similar NbPT4 and RiEF levels should be compared at first, as this would indicate similar mycorrhization. Of course, if mycorrhization is decreased in plants that received VIGS treatment of some of the effector genes, NbPT4 and RiEF may also be decreased, making it difficult to compare. That's why it is important to first confirm a silencing effect with one or possibly many positive controls. It is necessary that the positive control gene has no effect on mycorrhization levels when silenced, to make sure any decrease in expression level that is observed is not caused by a decrease in mycorrhization or arbuscles abundance. As RiAQP may not be the best choice as a positive control due to its low expression, it would be better to use genes that known to be highly expressed in the fungus, making any effects of VIGS treatment stand out more. Another thing that could have caused variation in the data is the setup of the qPCR plates (See Appendix B.) cDNA from each biological sample was divided over a single plate with different primer combination. A small difference in treatment between plates when applying the qPCR program could make samples on different plates incomparable when different primers do not have the same primer efficiency. Instead, putting a single primer in each plate in combination with all samples would make comparing the samples for expression of the gene corresponding to that primer more reliable.

Instead of attempting to optimize VIGS and HIGS, the use of different techniques to silence fungal genes may be a preferred option. One of the main uncertainties of the technique is whether or not dsRNA or siRNA reaches the fungus to induce an effect. In some studies with pathogenic fungi, fungal genes were silenced by directly spraying the fungus or the host surface with dsRNA, so called SIGS (spray-induced gene silencing) (Koch et al. 2016, Wang et al. 2016, Wang and Hailing 2017). Apparently these fungi are able to take up the dsRNA, both directly and via the plant. Possibly, silencing effect will be maintained because of siRNA amplification, a mechanism known in other species in which secondary siRNA are generated by RNA-dependent RNA polymerase after contact with degraded mRNA. (Sijen et al. 2001) It may be possible to apply this in *R. irregularis*, by adding dsRNA directly to the spores or to the root surface.

Besides silencing of fungal genes, other methods to assess functionality of effectors candidates may also be useable. For example, interaction studies can be performed, between the effectors and plant proteins, especially transcription factors that show different levels during mycorrhization compared to unmycorrhized cells. Disabling interacting domains or knocking down the gene of plant proteins that show interaction with fungal effector candidates may reveal an effect on mycorrhization.

INTACT

Out of the three experiments, INTACT appears to be the only one for which desired results were obtained. Nuclei were isolated with magnetic beads. However, it wasn't confirmed if these nuclei were specifically from mycorrhized cells, as NTF wasn't detected in the final sample and the mCherry construct wasn't functional. To confirm this, a follow up experiment could be performed in which a functional mCherry construct is expressed, or possibly a different construct that is less prone to degradation. Next, the final sample should be analysed for the new mCherry construct as well as NTF, for example with Western Blot.
If the experiment turns out to be successful, its efficiency still needs to be improved. After nuclear purification, a lot of cell debris was still in the sample and after INTACT with magnetic beads, clusters surrounding nuclei were rarely found. If the nuclei are to be used for further analyzation of effector localization, it is necessary to scale up the process and efficiently get more specific nuclei isolated in the final sample. A different protocol (Otto 1990, Dolezel and Godhe 1995) has been used before for nuclear purification in our lab and higher amount of nuclei was observed with this protocol compared to the protocol that was used in our study (Erik Limpens, personal communication), so this may also be a viable option for future research. When isolation of nuclei can be done successfully, the research on effector localization can continue by screening the nuclei with mass spectrometry.

Acknowledgements

I would like to show my gratitude to Erik Limpens and Tian Zeng for being my supervisors during the course of this study and for all the help they offered. Of course I would also like to thank all other members of the Molecular Biology group, who assisted me in the lab or supported me in general.

Materials and Method

Preparation of agar plates
1% agar was added to LB medium and autoclaved for 1.5 hour. As soon as the medium was cooled down to around 60 °C, 1% of 1000x antibiotic stock (100mg/mL) was added when necessary. Agar plates were poured with 25 mL medium each and left at room temperature for 1 hour to solidify.

LB medium
5g/L yeast extract
10g/L NaCL
10g/L tryptone

LB medium without salt
5g/L yeast extract
10g/L tryptone

Preparation of competent *E. coli* cells for transformation
On day 1, one LB agar plate without antibiotics was inoculated with E. coli. On day 2, one 50 mL Falcon tube with 5 mL LB medium without salt was inoculated with the E. coli cells at 21°C. On day 3, the 5 mL LB medium was transferred to 500 mL LB medium without salt. The 500 mL LB medium was distributed over 3 PVC buckets and centrifuged at 4000 rpm at 4°C. The pellets were centrifuged at 4000 rpm at 4°C in demi water, twice. The resulting pellets are centrifuged again in a total volume of 40 mL 8.5% glycerol at 4000 rpm at 4°C. The final pellets are resuspended in 2,5 mL 8.5% glycerol, which is then aliquoted in 50 uL volumes to 1,5 mL Eppendorf tubes and transferred to –80°C storage freezer in liquid nitrogen.

Electroshock transformation of *E. coli* and *Agrobacteria rhizogenes*
The plasmid containing the construct was diluted to approximately 25 ng/uL. Competent cells were taken from the -80°C and transferred to ice, making sure they stayed cold at all time. 1 uL of diluted plasmid was added to the cells. The cells with plasmid were transferred to electroshock cuvettes and a shock of 2500V was applied to induce take up of the plasmid, after making sure the cuvette was dry on the outside. 150 mL LB medium was added to the cells and the mixture is transferred to an 1.5 mL Eppendorf tube. E. coli cells were then incubated at 37°C for 30 minutes, *Agrobacteria* (MSU440 or C58) cells at 28°C for 90 minutes. Next, 50 uL of incubated cells was spread with sterile glass beads on an LB plate with appropriate antibiotics. The cells were left to grow overnight at 37°C for E. coli and two days at 28°C for *Agrobacteria* (MSU440 or C58).

Colony PCR
For colony PCR, a single colony was briefly touched with a clean pipet tip and transferred to 50 uL mQ. For each plate, three colonies were collected. 5 uL of this dilution was transferred to a PCR tube and 11 uL mQ, 2 uL PCR Buffer, 0.5 uL TAQ polymerase, 0.5 uL forward primer and 0.5 uL reverse primer was added. The PCR tube was transferred to a BioRad T100 Thermal Cycler and the following program was applied:

94 °C		5 min
35 cycles	94 °C	1 min
	55 °C	1:30 min
	72 °C	1:30 min
72 °C		5 min

Gel electrophoresis
Agarose gel was made by adding 1% agarose to 50 mL or 200 mL TAE buffer, depending on the number of samples. The TAE with agarose was heated in the microwave for approximately 2 minutes and cooled down in cold water. 5 uL or 20 uL ethidium bromide was added and after mixing, the gel was poured into a mold. After half an hour, the gel is transferred to a horizontal electrophoresis cell filled with TAE buffer. To 20 uL PCR product, 4 uL 6x loading buffer was added, after which 4 uL PCR product was loaded on the gel, as well as a GeneRuler marker. The gel had to run for half an hour at 100 V and was visualized under UV light in a BioRad Gel Doc machine with Image Lab Software

Effector candidate fragment amplification for VIGS and HIGS

To generate DNA fragments for VIGS and HIGS, *Rhizophagus irregularis* DNA samples were used as a template. To 125 ng DNA template in 5 uL mQ, 9.25 uL mQ, 4 uL 5x PCR Buffer, 0.5 uL dNTPs, 0.25 uL Phusion protein, 0.5 uL forward primer and 0.5 uL reverse primer was added in a PCR tube for a total volume of 15 uL, with a different primer pair for each effector fragment. The PCR tube was transferred to a BioRad T100 Thermal Cycler and the following program was applied:

98 °C		30 s
30 cycles	98 °C	10 s
	60 °C	30 s
	72 °C	30 s
72 °C		10 min

Afterwards, 3 uL 6x loading buffer was added to the PCR product and 5 uL of PCR product with loading gel was used for gel electrophoresis, to check if the correct fragment has been amplified. 2 uL of PCR product of each sample was used for a second PCR amplification, by adding 33.635 mQ, 10 uL 5x PCR Phusion Buffer, 1.25 uL dNTPs, 0.675 uL Phusion DNA polymerase, 1.25 uL forward primer and 1.25 uL reverse primer in a PCR tube for a total volume of 50 uL. For VIGS fragments, the same primers were used as in the first amplification step, for HIGS fragments, adapter primers were used instead to add attB1F and attB2R sites to the sequences. The PCR tube was transferred to the BioRad T100 Thermal Cycler and the same program as above was applied. Afterwards, 10 uL loading buffer was added and 5 uL PCR product was used for gel electrophoresis. Samples that showed a correct and specific band were used for DNA purification.

DNA purification
For DNA purification, the Thermo Scientific GeneJET Gel Extraction Kit was used, according to the manufacturer's instructions. Before starting, Wash Buffer was diluted 6x with 100% ethanol. An equal volume of Binding Buffer was added to the amplified PCR product. Up to 800 uL of the solution was transferred to a GeneJET purification column placed in a 2 mL collection tube. After centrifugation for 1 minute at full speed (15000 rpm), the flow-through was discarded. 100 uL Binding Buffer was added to the column and the column was centrifuged for another 1 minute at full speed. After discarding the flow-through, 700 uL Wash Buffer was added to the column and the column was centrifuged again for 1 minute at full speed. The flow-through was discarded and the column was centrifuged again for 1 minute at full speed to completely dry the column. The column was transferred to a clean 1,5 mL collection tube. 50 uL Elution Buffer was applied directly to the membrane of the column and the column was centrifuged one last time for 1 minute at full speed to elute the purified DNA. Purified DNA was stored at -20°C or directly cloned into the appropriate vector.

Miniprep
For isolation of plasmid DNA from *E. coli* cells, The E.Z.N.A Plasmid DNA Mini Kit was used, according to the manufacturer's instructions. Before starting, buffers were prepared by adding RNase A to Solution I, and storing it at 4°C, diluting DNA Wash Buffer 5 times with 100% ethanol and diluting HBC Buffer 1.4 times with isopropanol. First, a single E. coli colony containing the desired construct was briefly touched with a clean pipet tip and transferred to a 50 mL Falcon tube containing LB medium with the appropriate antibiotics. The culture was incubated overnight at 37°C while shaking. The next day, the tube was centrifuged at 10000 g for 1 minute at room temperature. The medium was aspirated and the pellet was resuspended in 250 uL Solution I containing RNase A by pipetting. The suspension was transferred to a 1.5 mL Eppendorf tube and 250 uL Solution II was added. The tube was gently inverted to obtain a clear lysate and incubated at room temperature for 3 minutes. Next, 350 uL Solution III was added and the tube was immediately inverted several times until a white precipitate had formed. The tube was centrifuged at full speed (15000 rpm) for 10 minutes and the cleared supernatant was transferred to a HiBind DNA Mini Column placed in a 2 mL collection tube without disturbing the white pellet. The column was centrifuged at full speed for 1 minute and after discarding the flow-through, 500 uL HBC Buffer was added. Again, the column was centrifuged at full speed for 1 minute and the flow-through was discarded. 700 uL DNA Wash Buffer was added to the column and the column was centrifuged at full speed for 1 minute, after which flow-through was discarded. The column was centrifuged for 2 minutes at full speed to make sure the column is completely dry. Then the column is transferred to a clean 1.5 mL collection tube and 50 uL Elution Buffer is added directly to the column membrane. After waiting 1 minute, the column is centrifuged at full speed for 1 minute to elude the plasmid DNA. The plasmid DNA is stored at -20°C or directly used for transformation or inserting fragments.

Digestion and ligation of VIGS fragments
To generate the VIGS constructs, each effector fragment was inserted into a pTRV2 vector. This was done by first digesting both 10 uL pTRV2 an 10 uL VIGS fragments separately by addition of 2 uL digestion buffer, 6 uL RNAse-free water, 1 uL EcoRI and 1 uL BamHI. After incubation for 5 minutes at 37°C, the mixtures were left overnight at room temperature. The next day, 15 ng of the digested fragment in 2 uL was added to 500 ng digested pTRV2 in 7.7 uL, 7.3 uL RNAse-free water, 2 uL t4 ligase buffer and 1 uL t4 ligase and incubated at room temperature for 1 hour. After ligation, the construct was

transformed into E. coli, which was then grown on LB + kanamycin at 37°C overnight. Colony PCR and gel electrophoris was performed on these cells to confirm insertion of the correct construct. Miniprep was performed on correct samples and 2 uL of these constructs were sent to Ezseq for sequencing after addition of forward and backward primers for the pTRV2 vector. The sequence was checked for mismatches and transformed into C58 cells, which were then grown on LB with kanamycin, gentamycin and rifampicin at 28°C for two days. Colony PCR and gel electrophoresis was performed on the transformed C58 cells to confirm insertion of the correct construct, after which the cells could be used for infiltration of *Nicotiana* plants.

Gateway cloning of HIGS fragments

10 ng of 1 uL amplified HIGS fragments was inserted into a pDONR vector by adding 75 ng pDONR in 1 uL, 1 uL BP Clonase and 4 uL BP Clonase buffer. After incubation for at least 1 hour at room temperature, 1 uL proteinase K was added to stop the reaction. The resulting construct was transformed into competent E. coli cells, which were then grown on LB with kanamycin at 37°C overnight. Colony PCR was performed with three colonies for each construct and 4 uL PCR product was used for gel electrophoresis. For the colonies that showed the correct bands corresponding with the inserted construct, the pDONR plasmids were isolated with Miniprep. To form the double hairpin construct, Classis LR-Reaction II was performed on 25 ng pDONR plasmid in 1 uL, by addition of 50 ng destination vector 277+pEF, 1 uL mQ and 1 uL 5x LR-Clonase II. After incubation at room temperature for at 1 hour, 0.5 uL proteinase K was added and the sample was heated to 37°C for 10 minutes. The resulting construct was transformed into competent E. coli cells, which were then grown on LB with spectinomycin at 37°C overnight. Colony PCR and gel electrophoris was performed on these cells to confirm insertion of the correct construct. Miniprep was performed on correct samples and 4 uL of these constructs were sent to Ezseq for sequencing after addition of forward and backward primers for the 277+pEF vector. The sequence was checked for mismatches and transformed into *Agrobacteria rhizogenes* cells (MSU440), which were then grown on LB with spectinomycin at 21°C for two days. Colony PCR and gel electrophoresis was performed on the transformed *Agrobacteria* to confirm insertion of the correct construct, after which the cells could be used for hairy root transformation of *Medicago* plants.

Golden Gate cloning of INTACT constructs

To create the p35S-BCP1sp-mCherry-HA construct, level 0 vectors containing the different parts of the gene had to be combined into a level 1 vector with Golden Gate cloning (Engler et al. 2014). First, 5 uL of available level 0 constructs were sent to Ezseq for sequencing after addition of forward and backward primers, to check if the sequence was correct. Next, a mix was made in a PCR tube of 2 uL 10x t4 buffer, 2 uL 10x bovine serum (BSA), 4.5 uL RNAse-free water, 115 ng of p47751 backbone in 2 uL, 66 ng of 35S+UTR (pICSL13001) in 1 uL, 66 ng of BCP1sp-mCherry in 3.5 uL, 66 ng of HA-tag (no stop codon) in 2.5 uL, 66 ng of terimantor (pICH41414) in 1.5 uL, 0.5 uL t4 ligase (30u/uL) and 0.5 uL BsaI (10u/uL). The PCR tube was transferred to a BioRad T100 Thermal Cycler and the following program was applied:

37 °C		20 s
25 cycles	37 °C	3 min
	16 °C	4 min
50°C		10 min
80°C		10 min

The construct was transformed into competent E. coli cells, which were then grown on three LB plates with ampicillin and Xgal. A blue/white selection was done and white colonies were diluted and used for colony PCR and gel electrophoresis to check the bands. Samples showing the correct bands, were miniprepped and sent to Ezseq for sequencing.

After confirming that the sequence was correct, the level 1 p35S-BCP1sp-mCherry-HA construct had to be combined with already available level 1 constructs pPT4-NTF and pBCP1-BirA by a second Golden Gate reaction. A mix was made in a PCR tube of 2 uL 10x t4 buffer, 2 uL 10x bovine serum (BSA), 8.25 uL RNAse-free water, 100 ng pAGM47231 backbone in 05 uL, 150 ng pPT4-NTF in 3 uL, 150 ng pBCP1-BirA in 1.5 uL, 300 ng of p35S-BCP1sp-mCherry-HA in 0.75 uL, 100 ng endlinker (pICH41766) in 1 uL, 0.5 uL t4 ligase (30u/uL) and 0.5 uL BpiI (10u/uL). The PCR tube was transferred to a BioRad T100 Thermal Cycler and the same program as mentioned above was applied. After PCR, the construct was transformed into competent E. coli cells, which were then grown on three LB plates with kanamycin. A red/white selection was done and white colonies were diluted and used for colony PCR and gel electrophoresis to check the bands. Samples showing the correct bands were miniprepped and sent to Ezseq for sequencing. Constructs with the correct sequence were transformed into *Agrobacteria* (MSU440), which were later used for transformation of *Medicago* plants for INTACT.

Rhizophagus irregularis spore isolation

45 uL Commercial spore inoculum was added in a sterile 50 mL Falcon tube. The spores were poured into a stack of a 250um, 100um and 50um sieve and washed with autoclaved mQ. Plant material would stay in the first sieve and isolated spores would stay in the second and third sieve. 20 mL autoclaved mQ was then poured into the second and third sieve from the bottom to flush the isolated spores into another sterile 50 mL Falcon tube. A few 20 uL droplet of isolated spores in mQ were placed on a glass slide and used to count spore concentration.

***Nicotiana benthamiana* growth and inoculation**

Day 1: Sterilization and germination

About 200 *Nicotiana benthamiana* seeds were collected in a 50 mL Falcon tube. 1% bleach was added and the tube was rotated for 10 minutes to sterilize the seeds. Afterwards, the seeds were transferred to Färhaeus medium at -4°C in darkness to start germination.

Day 2: Moving seeds

The seeds from 3 plates were distributed over 7 plates and put in lines, to give the plants more space to grow.

Day 3: Exposure to light

Plates with seeds were moved to the 25°C growth chamber and exposed to light

Day 14: Moving small plants to pots/perlite

The first batch of small *Nicotiana* plants was moved to pots with a sand/clay mixture. However, as transferring adult plants to new pots showed to be damaging for the delicate roots of *Nicotiana*, the second batch of small *Nicotiana* plants was moved to perlite at this point instead.

Day 84: Transfer and inoculation of adult plants.

After growing in pots/perlite for 10 weeks, the adult *Nicotiana* plants were selected for plants of similar size and moved to individual pots filled with sand and clay, in which a hole was made with 500 isolated spores in 500 uL for inoculation. The next day, *Nicotiana* plants were infiltrated. After infiltration, plants were watered twice a week, once with 25 mL demi and once 25 mL with Hoagland solution.

Fähraeus medium (1 liter)

0.12 g	$MgSO_4.7H_2O$
0.10 g	KH_2PO_4
0.358 g	$Na_2HPO_4.\ 12H_2O$
1 mL	15 mM Fe-citraat (19%)
2.5 mL	Spore-elements β-

After adding all components, the pH was adjusted to 6.7 with 1M KOH, 0.9% Daichin agar was added and the medium was autoclaved for 1.5 hour at 120°C. After autoclaving and cooling the medium down to 60°C, 0.76 mL 1M $Ca(NO_3)_2$ and 0.70 mL $CaCl_2.\ 2H_2O$ was added.

Spore-elements β- (1 liter)

0.0354 g	$CuSO_4.5H_2O$
0.4620 g	$MnSO_4.1H_2O$
0.9740 g	$ZnSO_4.7H_2O$
1.2690 g	H_3BO_3
0.3980 g	$Na_2MoO_4.2\ H_2O$

½ Hoagland solution 1xN (1 liter) (Hoagland et al. 1950)

5 mL	Stock I
1 mL	Stock II
0.5 mL	Stock III
0.2 mL	Stock IV
1 mL	Stock V
1 mL	Stock VI

Adjust pH to 6.1

Stock I 400x, per liter:

236.2g	$Ca(NO_3)_2.4H_2O$ (1M)
101.11g	KNO_3 (1M)

Stock II 1000x, per liter:

246.48g	$MgSO_4.7H_2O$ (1M)

Stock III 2000x, per liter:

36.7g	NaFeEDTA (0.1M)

Store in darkness

Stock IV 5000x, per liter:

13.6g	KH_2PO_4 (0.1M)

Stock V 1000x, per liter:

618.4mg	H_3BO_3
48.4mg	$Na_2MoO_4.2H_2O$
287.6mg	$ZnSO_4.7H_2O$
395.8mg	$MnCl_2.4H_2O$
124.8mg	$CuSO_4.5H_2O$
47.6mg	$CoCl_2.6H_2O$
1.25mg	10M HCl

Stock VI 1000x, per liter:

97.5g	MES

Agroinfiltration of *Nicotiana* plants for VIGS

For each effector construct, two 50 mL tubes with 10 mL LB containing 10 ug/mL rifampicin and 50 ug/ml kanamycin were prepared. One tube was inoculated with a single colony of C58 containing pTRV1, the other with a single colony of C58 containing pTRV2 with the effector fragment inserted. The tubes were incubated at 28°C in a shaker overnight. The tubes were centrifuged at 3000g for 15 minutes at room temperature and the pelleted cells are resuspened in 10 mL MMA infiltration buffer for leaf infiltration or in 10 mL Agrodrench induction buffer for agrodrench. After incubating in a shaker for at least 1 hour at room temperature, the tubes for Agrodrench were centrifuged again at 3000g for 15 minutes at room temperature and the pelleted cells were resuspened in 5 mL Agrodrench infiltration buffer. For both leaf infiltration and agrodrench, 10 mL of pTRV1 and pTRV2 cultures had to be combined in a 1:1 ratio. For leaf infiltration, the pTRV mix was directly injected into the bottom of two leaves of each separate plant with a needleless syringe. For agrodrench, 5 mL of pTRV mix was slowly pipetted on top of each separate plant (Senthil-Kumar and Mysore 2014).

MMA Infiltration buffer

10 mM	MES
10 mM	MgCl2
100 µM	acetosyringone

Adjust to pH 5.5

Agrodrench Induction buffer (100 mL)

0.195g	MES
200uL	acetonesyringone (200 mM stock)

Adjust to pH 5.5

Agrodrench Infiltration buffer (100 mL)

0.0975 MES

Adjust to pH 5.5

***Medicago truncatula* growth and root transformation**

Day 1: Sterilization and cold shock

First, seeds were removed from their pods by softly tapping them with a hammer and grinding them with a trowel until the pods were completely open.

Seeds were collected for sterilization in a 50 mL Falcon tube, to which concentrated H_2SO_4 was added. After rotating for 8 minutes, the H_2SO_4 was immediately removed and the seeds were washed at least 6 times with demi-water. 4% bleach was added and the tube was left rotating for another 10 minutes, after which it was washed at least 7 times with sterile mQ. The tube was left for 1 hour in the dark for inhibition.

After inhibition, the seeds were moved to filter paper on Fähraeus medium and left at 4°C in the dark overnight to induce a cold shock.

Day 2: Germination

The next day, the plate was turned upside down and left at 25°C in the dark to induce germination.

Day 3: Exposure to light

A little water was added to the plate to enable careful removal of the seed coat. The naked seeds were transferred to new plates with Fähraeus medium and half a filter paper, with 5 seeds per plate in a straight line. The plate was closed for 2/3 with parafilm and put vertically in a 21°C growth chamber.

Day 8: Transformation

Any roots that are formed are cut off. *Agrobacteria* (MSU440) cells containing the required construct are streaked on the wound, after which the plate was sealed again for 2/3 with parafilm and moved back to a 21°C growth chamber.

Day 12: Transfer plants to Emergence medium
The plants are transferred to new plates with Emergence medium and the wound surface was covered with half of another filter paper. The plates are closed for 2/3 with parafilm and put vertically in a 21°C growth chamber.

Day 30:
The plants for HIGS were used to generate hairy root cultures. The plants for INTACT were moved to cones, containing a mixture of sand and clay with chopped up mycorrhized chive roots. An extra 750 uL of isolated spores, containing about 500 spores, was added to each cone before planting the plants to improve mycorrhization levels. Plants with roots that showed red fluorescence, an indication of successful transformation, were marked. The plants were watered with 15 mL Hoagland solution twice a week and used for nuclear purification after growing for 6 weeks.

Fähraeus medium (1 liter)

0.12 g	$MgSO_4.7H_2O$
0.10 g	KH_2PO_4
0.358 g	$Na_2HPO_4.\ 12H_2O$
1 mL	15 mM Fe-citraat (19%)
2.5 mL	Spore-elements β-

After adding all components, the pH was adjusted to 6.7 with 1M KOH, 0.9% Daichin agar was added and the medium was autoclaved for 1.5 hour at 120°C. After autoclaving and cooling the medium down to 60°C, 0.76 mL 1M $Ca(NO_3)_2$ and 0.70 mL $CaCl_2.\ 2H_2O$ was added.

Spore-elements β- (1 liter)

0.0354 g	$CuSO_4.5H_2O$
0.4620 g	$MnSO_4.1H_2O$
0.9740 g	$ZnSO_4.7H_2O$
1.2690 g	H_3BO_3
0.3980 g	$Na_2MoO_4.2\ H_2O$

Emergence medium (1 liter)

50 mL	20x concentrated SH-A
50 mL	20x concentrated UM-C
10 g	1% sucrose
3 mL	1M MES pH 5.8

After adding all components, pH was adjusted to 5.8 with 1M KOH, 0.9% Daichin agar was added and the medium was autoclaved for 1.5 hour at 120°C. After autoclaving and cooling the medium down to 60°C, 300 ug/mL Cefotaxime was added.

20x Concentrated SH-A (1 liter)

50 g	KNO_3
8 g	$MgSO_4.7H_2O$
6 g	$NH_4H_2PO_4$
4 g	$CaCl_2.2H_2O$
0.2 g	$MnSO_4.7H_2O$
0.1 g	H_3BO_4
0.02 g	$ZnSO_4.7H_2O$
0.02 g	KI
0.004 g	$CuSO_4.5H_2O$
0.002 g	$NaMoO_4.2H_2O$
0.002 g	$CoCl_2.6H_2O$
0.3 g	$FeSO_4.7H_2O$
0.4 g	Na_2EDTA (TiTriplex III)

SH-A is stored at -20°C

20x Concentrated UM-C (1 liter)

2 g	Myoinositol
0.1 g	Nicotinic acid
0.2 g	Pyriodoxine HCl
0.2 g	Thiamine HCl
0.04 g	Glycine

UM-C is stored at -20°C

Hoagland solution
See above

Medicago truncatula hairy root cultures and inoculation for HIGS
Medicago plants transformed with HIGS constructs were selected for red fluorescent roots. Red roots were cut off and transferred to M-medium, 1 root piece of at least 1 cm a plate. The plates were left at 25°C in the dark for 6 weeks, until the plates were covered with freshly grown roots. Three slices of 1x1 cm root material were cut out and moved to new M-medium. About 50 isolated spores (in 40 uL) were pipetted on top of the slice and left for at least 6 weeks for mycorrhization. When sufficient roots are mycorrhized, the roots can be harvested for further research.

M-medium (1 liter)

50 mL	20x Macro-element stock solution
10 mL	100x Micro-element stock solution
10 mL	100x Vitamin stock solution
10 g	sucrose

After adding all components and mQ water, pH was adjusted to 5.5 with 0.1M KOH and the solution was poured into a bottle containing 4 g Phytagel. After autoclaving and cooling down to 60°C, 300 ug/mL Cefotaxime was added. All medium was poured into plates right away, as the medium cannot be reused after solidifying.

20x Macro-elements stock (1 liter)

14.62 g	$MgSO_4.7H_2O$
1.6 g	KNO_3
1.3 g	KCl
5.76 g	$Ca(NO_3)_2.4H_2O$

100x Micro-elements stock (1 liter)

480 mg	KH_2PO_4
800 mg	NaFeEDTA
75 mg	KI
600 mg	$MnCl_2.4H_2O$
265 mg	$ZnSO_4.7H_2O$
150 mg	H_3BO_3
13 mg	$CuSO_4.5H_2O$
0.24 mg/140 uL	$Na_2MoO_4.2H_2O$

100x Vitamins stock (1 liter)

300 mg	Glycin
10 mg	Thiamin HCl
10 mg	Pyridoxin HCl
50 mg	Nicotinic Acid
5 g	Myo-inositol

***Nicotiana* and *Medicago* plant root harvesting**
Both *Nicotiana* plants for VIGS and *Medicago* plants for INTACT were harvested after 5 weeks of inoculation with spores. Plant pots of *Nicotiana* plants were submerged in a vat with water and carefully cut open on four sides to release sand from the roots into the water, without damaging the delicate roots of the plant. When the plant roots had been released from the pot, remaining sand was cleaned of with water. The plant was dried with paper towels and total biomass was measured. The roots were cut off and the shoot biomass was measured as well. The roots were cut into 1 cm long pieces and mixed thoroughly. Half of the roots was transferred to a 1.5 mL Eppendorf tube to which 10% KOH is added immediately for staining, the other half was transferred to a 2 mL Eppendorf tube and frozen in liquid nitrogen for RNA isolation. If not enough root material was available, only roots for RNA isolation were collected. Plant cones of *Medicago*, which has more robust roots, were put upside down and the plant and sand clump was pushed out by a metal rod from the bottom. Roots were released from the sand by carefully kneading the sand clump until it crumbled. Remaining sand was washed off from the roots by water and the roots were screened under the microscope for green or red roots, which would indicate expression of the NTF and mCherry constructs. A few green or red roots were cut off and cut in the length for observation under the confocal microscope. Remaining green and red roots were distributed to 2 mL Eppendorf tubes and frozen in liquid nitrogen for RNA isolation, protein extraction and nuclear isolation.

***Nicotiana* root staining and slide preparation**
Roots of *Nicotiana* plants were cut off and transferred to a 1.5 mL Eppendorf tube with a hole in the cap. The tube was immediately filled with 10% KOH and put in a 90°C heat block for 20 minutes. The KOH

was poured out and trypan blue staining solution was added. The sample was heated again at 90°C for 3 minutes, after which the trypan blue staining was poured out and 40% glycerol was added. The stained roots in glycerol were stored in 4°C until they were used to make slides. When making slides, a droplet of 40% glycerol was spread out over a glass slide. Root pieces were put in rows to form 3 straight horizontal lines of 5 cm and covered by a 50x10mm coverslip. Three such slides were made for each sample, if enough root material was available, to enable quantification of arbuscle abundance. However, quantification was not performed for this study.

RNA isolation from *Nicotiana* plants for VIGS

For RNA isolation of the *Nicotiana* plant for VIGS, the Qiagen RNeasy Micro Kit was used according to the manufacturer's instructions. Before starting, buffers were prepared by addition of 4 volu,mes of 100% ethanol to Buffer RPE and 10 uL β-mercaptoethanol to 1 mL of Buffer RLT. A maximum of 100 mg plant material was frozen in 2 mL Eppendorf tubes in liquid nitrogen. If more material was available for one sample, that sample was split in two halves smaller than 100mg and treated as two separate samples. Frozen Eppendorf tubes were transferred to a Qiagen TissueLyser LT, precooled at -80°C and plant tissue was disrupted for 2 minutes at 50 Hz. Tubes with powdered tissue were transferred to ice, after which the total volume was adjusted to 350 uL with Buffer RLT. Tubes were vortexed vigorously for 30 seconds and 1 volume of 70% ethanol was added. The mix was homogenized by pipetting and transferred to a RNeasy MinElute spin column placed in a 2 mL collection tube. The column was centrifuged for 15s at full speed (15000 rpm) and the flow-through was discarded. 350 uL Buffer RW1 was added to the column and the column was centrifuged again for 15s at full speed. Flow-through was discarded and 70 uL RDD with 10 uL DNAse I was added to each column to start DNAse treatment. After 45 minutes, another 350 uL Buffer RW1 was added to the column and the column was centrifuged for 15s at full speed. 500 uL Buffer RPE was added to the column and the column was centrifuged for 15s at full speed. 500 uL 80% ethanol was added and the column was centrifuged for 2 minutes at full speed. The column was transferred to a dry 2 mL collection tube and centrifuged for 5 minute at full speed to make sure the column is completely dry. The column is then placed into a 1.5 mL collection tube and 15 uL RNAse-free water is directly added to the column membrane. The column is centrifuged a final time for 1 minute at full speed to elute the RNA. 2 uL of the 50 uL RNAse-free water, containing isolated RNA, was used on a NanoDropper to measure RNA concentration, after which the remaining RNA solution was stored at -80°C or directly used for RT-PCR.

RNA isolation from *Medicago* plants for INTACT

For RNA isolation of the *Medicago* plants for INTACT, the Qiagen RNeasy Plant Mini Kit was used, according to the manufacturer's instructions. Before starting, buffers were prepared by addition of 4 volumes of 100% ethanol to Buffer RPE and 10 uL β-mercaptoethanol to 1 mL of Buffer RLT. A maximum of 200 mg plant material was frozen in 2 mL Eppendorf tubes in liquid nitrogen. Frozen Eppendorf tubes were transferred to a Qiagen TissueLyser LT, precooled at -80°C and plant tissue was disrupted for 2 minutes at 50 Hz. Tubes with powdered tissue were transferred to ice, after which 450 uL of RLT was added to each tube. Tubes were vortexed vigorously for 30 seconds and the lysate was transferred to a lilac QIAshredder spin column, placed in a 2 mL collection tube. After centrifugation for 2 minutes at full speed (15000 rpm), the flow-through was transferred to a new 1.5 mL Eppendorf tube, containing 250 uL 100% ethanol. The flow-through and ethanol were mixed by pipetting and transferred to a pink RNeasy Mini spin column, placed in a 2 mL collection tube. The tube was centrifuged for 15s at full speed and the flow-through was discarded. 350 uL Buffer RW1 was added to the column and the column was centrifuged again for 15s at full speed. Flow-through was discarded and 70 uL RDD with 10 uL DNAse I was added to each column to start DNAse treatment. After 45 minutes, another 350 uL Buffer RW1 was added to the column and the column was centrifuged for 15s at full speed. 500 uL Buffer RPE was added to the column and the column was centrifuged for 15s at full speed. Another 500 uL Buffer RPE was added and this time the column was centrifuged for 2 minutes at full speed. The column was transferred to a dry 2 mL collection tube and centrifuged for 1 minute at full speed to make sure the column is completely dry. The column is then placed into a 1.5 mL collection tube and 50 uL RNAse-free water is directly added to the column membrane. The column is centrifuged a final time for 1 minute at full speed to elute the RNA. 2 uL of the 50 uL RNAse-free water, containing isolated RNA, was used on a NanoDropper to measure RNA concentration, after which the remaining RNA solution was stored at -80°C or directly used for RT-PCR.

RT-PCR

5 uL of isolated RNA sample from either *Nicotiana* plants for VIGS or *Medicago* plants for INTACT in RNAse free water (around 100 ng) was combined with 1 uL iScript Reverse Transcriptase and 4 uL 5x iScript reaction mix in a PCR tube. The total volume was adjusted to 20 uL by addition of RNAse-free water. The tube is inserted into a BioRad T100 Thermal Cycler and the following program is applied:

25°C	5 minutes
42°C	30 minutes
85°C	5 minutes

After RT-PCR, the generated cDNA was stored at -20°C or directly used for qPCR.

qPCR
To quantify gene expression in VIGS plants, qPCR was performed on cDNA generated from isolated RNA. First, cDNA was diluted 10 times. First, a mastermix is made for each sample with 5 uL BioRad Super mix, 1 uL diluted cDNA and 1 uL mQ for each primer pair that will be added, with an extra safety margin of 15%. This was divided in 24.2 portions over 1.5 mL Eppendorf tubes, one for each primer pair. Next, 5 uM of forward and reverse primer in 10.4 uL were added to each separate Eppendorf tube with mastermix and the tubes vortexed thoroughly. 3 times 10 uL of each combination of sample and primer was pipetted to a 96-wells plate. Samples from the same plant with different primers were placed on the same plate. The plate was sealed with BioRad Ahesive Sealer and centrifuged for 1 minute at 10000 rpm. The plate was inserted into a BioRad CFX Connect machine and the following qPCR program was applied:

95°C		3 minutes
40 cycles	95°C	15 seconds
	60°C	30 seconds
95°C		10 seconds
65-95°C (In 0.5°C increments)		5 seconds

Data output was analysed using Microsoft Excel (See Appendix B). The average Ct value of the reference gene (REF) was subtracted from the gene of interest (GOI). The relative expression level of the gene of interest was calculated with the following formula (Haimes and Kelley 2010):

$$2^{-(GOI-REF)}$$

Protein extraction from INTACT plants
Plant root tissue, frozen in liquid nitrogen, was ground to powder in an ice-cooled pestle and mortar. The powder was transferred to a potter tube and 2 mL RIPA buffer was added. The solution was pottered until the mix was homogenously disrupted and no visible particles were left. 1 mL of the liquid was transferred to a 1.5 mL Eppendorf tube and incubated on ice for 30 minutes. After incubation, the solution was centrifuged for 10 minutes at maximum rpm at 4°C. The supernatant, containing the proteins, was transferred to a precooled Eppendorf tube and stored at -80°C. This cell lysate would later be used for Western Blotting.

RIPA buffer
10 mM Tris/HCl pH 7.5
150 mM NaCl
0.1% SDS
1% Triton X-100
1% Sodium Deoxycholate (DOC)
5 mM EDTA pH 8
1 tablet/25 mL cOmplete Protease Inhibitor (stored at 4°C and added freshly for each isolation)

Western Blot
100 uL 4x SDS-sample buffer was added to 100 uL cell lysate from INTACT plants. Two premade agarose gels were placed in a vertical electrophoresis cell with two slots filled with electrophoresis buffer and loaded with 15 uL of each sample, as well as 10 uL PageRuler Protein Ladder. The gels ran at 200V for about 30 minutes, while a PVDF membrane was wetted in blotting buffer. A stack was made of a pile of wet filterpaper, membrane, a gel and another pile of wet filterpaper. The stacks stayed in the blotting machine for 10 minutes, after which the membranes with protein were moved to TBS with 0.2% Tween20 and stored at 4°C overnight.

The next day, the blots were blocked in 50 mL TBS with 2% BSA and 0.3% Tween20 for 1 hour while shaking. After this, the blots were cut and divided over separate tubes, each containing 15 mL TBS with 1% BSA and 0.3% Tween20, as well as the respective antibodies, for at least 1 hour. In this experiment, anti-GFP-HRP (1:5000), peroxidase conjugated streptavid (1:10000) and anti-HA-peroxidase (1:1000) were used as antibodies. The blots were washed three times 10 minutes with 50 mL TBS with 0.3% Tween20 and once with 50 mL TBS. After washing, the blots were moved to separate petridishes and a mix of 0.5 mL of SuperSignal West Peroxide Solution and 0,5 mL Enhancer Solution was added on top. Immediately after, reaction of the substrate with the antibodies is monitored using a BioRad Gel Doc machine with Image Lab Software for 10 minutes. In addition, an epi white image of the marker is made.

4x SDS-sample buffer
0.2 M Tris/HCl pH 6.8
8% SDS
40% glycerol
4% β-mercaptoethanol
50 mM EDTA pH 8
0.08% bromophenol blue

Electrophoresis buffer (1 liter)
100 mL 100x TAE
900 mL mQ

Blotting buffer (1 liter)
200 mL 5x Trans-Blot Turbo Transfer Buffer
600 mL mQ
200 mL 95% ethanol

TBS (Tris Buffered Saline)
50 mM Tris/HCl pH 7.5
150 mM NaCl

Nuclear Purification
Root tissue frozen with liquid nitrogen was ground to powder using a cooled pestle and mortar. Powdered tissue was transferred to a 50 mL Falcon tube on ice, containing 10 mL cold NPB and mixed carefully. The suspension was filtered with a 40 um cell strainer into another 50 mL Falcon tube on ice to remove most plant material from the sample. The sample was centrifuged at 1000g at 4°C for 10 minutes. The low speed is necessary to prevent damage to the nuclei. Pelleted material is resuspended in 1mL NPBt in a 1.5 mL Low-Bind Eppendorf tube on ice. Again, the sample is centrifuged at 1000g at 4°C for 10 minutes. The pellet was resuspended in 250 uL NPB. In control roots, 0.5 uL SYTOX green (0.5 mM stock) was added to visualize the nuclei with a microscope. After purification, nuclei were used for INTACT. (Deal and Henikoff 2010, Deal and Henikoff 2011)

NPB:
20 mM MOPS pH 7
40 mM NaCl
90 mM KCl
5 mM EGTA pH 8
2 mM EDTA pH 8
0.5 mM Spermidine (stored at -20°C and added freshly for each purification)
0,2 mM Spermine (stored at -20°C and added freshly for each purification)
1 tablet/50 mL cOmplete Protease Inhibitor (stored at 4°C and added freshly for each purification)

NPBt:
NPB with 0.1% Triton X-100

INTACT
20 uL magnetic Invitrogen M280 Streptavidin Dynabeads suspension was washed by adding 1 mL cold NPB and pelleting the beads at 1500 g. The beads were resuspended in 40 uL cold NPB, which was distributed to isolated nuclei samples, 10 uL bead suspension to 250 uL sample. The beads and nuclei mix was slowly rotated at 4°C for at least 30 minutes.

After 30 minutes, 50 uL beads and nuclei mixture was pipetted into individual PCR tubes, clamped in a Macs Multistand magnetic holder. The solution was washed three times by adding cold 250 uL NPBt and slowly pipetting out the liquid with cell debri from the bottom, while the beads with nuclei are held on the side of the tube by the magnet field.

After washing, the beads and nuclei were resuspended in 100 uL NPBt. To visualize the nuclei under the microscope, 10 uL 2mg/uL DAPI was added (Wang and Deal 2015, Deal and Henikoff 2011).

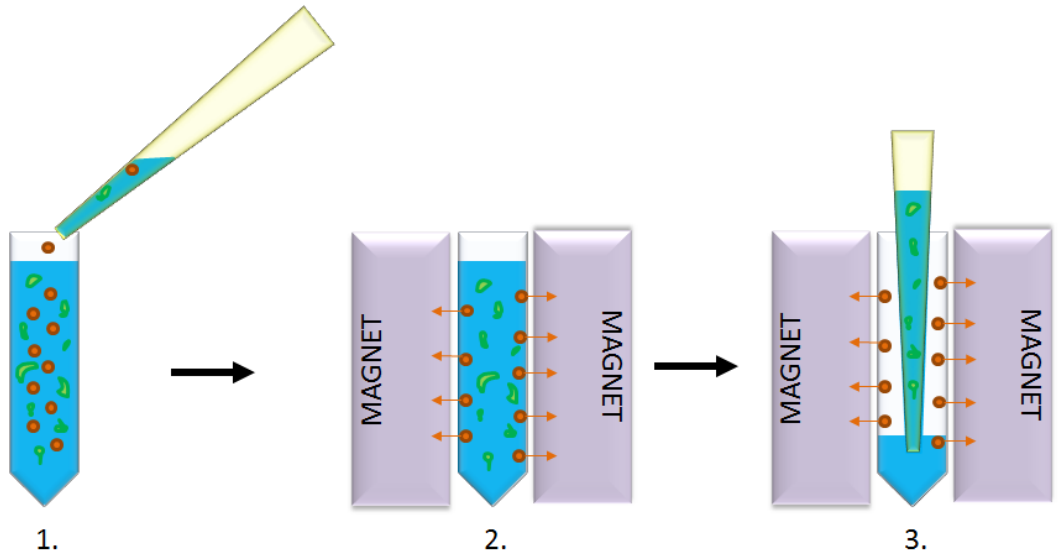

Figure 22: (1) Isolated nuclei and magnetic beads are mixed in a PCR tube and incubated. (2) The PCR tube is inserted into a magnetic clamp, pulling the beads to the side. (3) Liquid containing cell debris is removed, leaving only the beads with tagged nuclei attached behind.

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APPENDICES

Appendix A. Primers

VIGS Primers	**Sequences**
TZ-VIGS-167760-F	ACCGAATTCTTCTCCAGGTGCGAATGATT
TZ-VIGS-167760-R	ACCGGATCCGGTATTCCCACCCTCATT
TZ-VIGS-123350-F	ACCGAATTCGGTGCTATGGTAAGGGCTCT
TZ-VIGS-123350-R	ACCGGATCCTTACCGGTTGGATCTTGAGC
TZ-VIGS-245330-F	ACCGAATTCTAAACGTTGTGGTGGTTACA
TZ-VIGS-245330-R	ACCGGATCCTCACGCTTGTAACCATATGCT
TZ-VIGS-238820-F	ACCGAATTCAATTCGCATCAGTCTTAATC
TZ-VIGS-238820-R	ACCGGATCCCAGCTTCTTTTATTTCTTTAT
TZ-VIGS-128600-F	ACCGAATTCTCTTTCACATTTACGTTATT
TZ-VIGS-128600-R	ACCGGATCCTAACATGTGTATGACCTTCT
(AQP)Ri028370-VIGS-F	ACCGAATTCGGTGTAGGAGCTATCGCTCAAAC
(AQP)Ri028370-VIGS-R	ACCGGATCCACTAACTGCAATACCCAAAG
RirG245330-VIGS-F	ACCGAATTCATAAACGTTGTGGTGGTTACAAGCG
RirG245330-VIGS-R	ACCGGATCCTGCTTCGGCGTCGCGTTTGT
RirG172350-VIGS-F	ACCGAATTCAACGCGGCGCCGACTCC
RirG172350-VIGS-F	ACCGGATCCATAGCCATACGCTTCTGCG
HIGS Primers	
attB1-SIS-HIGS-F	AAAAAAGCAGGCTTCGGCATTAGATGGTTCTAA

attB2-SIS-HIGS-R	CAAGAAAGCTGGGTCTGACTCTCTAATGTCCACAAC
RirG245330-HIGS-F	AAAAAAGCAGGCTTCATAAACGTTGTGGTGGTTACAAGCG
RirG245330-HIGS-R	CAAGAAAGCTGGGTCTGCTTCGGCGTCGCGTTTGT
RirG172350-HIGS-F	AAAAAAGCAGGCTTCAACGCGGCGCCGACTCC
RirG172350-HIGS-R	CAAGAAAGCTGGGTCATAGCCATACGCTTCTGCG
RirG238820-HIGS-F	AAAAAAGCAGGCTTCATGAAATTCGCATCAGTCTTAATCA
RirG238820-HIGS-R	CAAGAAAGCTGGGTCGACCTTTTTCACCTTATTGTTAAGG
RirG118200-HIGS-F	AAAAAAGCAGGCTTCATGAAATCTTCCGTTTCTTTTACCA
RirG118200-HIGS-R	CAAGAAAGCTGGGTCATATGAACCACGAGCTACCTTGTTT
qPCR Primers	
(AQP)Ri028370-qPCR-F	CAGGAGGAACAAGAGGAGCACC
(AQP)Ri028370-qPCR-R	CGATAGCTCCTACACCGAATGCAAC
EL-Rir128-qF3	CACTTCCCCAAGGAATGAAG
EL-Rir128-Qr3	ACAAAATTTTCGGCATTTGG
LM-RirT238820-qF	GAAAAAGCGTACGGTGAAGC
LM-RirT238820-qR	GCCCATGGCATTAGAGATTG
LM-RirT167760-qF	TTTCAATCTTTGCACGGTCA
LM-RirT167760-qR	CCTGCGGTGACTTGTTCTTT
RiEF-qF2	AACCCCTTCGTCTTCCACTT
RiEF-qR2	ATTGTTCGTGGTGCATTTCA
ELNbTEF-qF	CTCTCAGGCTCCCACTTCAG
ELNbTEF-qR	AAGAGCTTCGTGGTGCATCT
RikH_qNbPT4F	GAGACAAACTTGGCCGAAAG
RikH_qNbPT4R	ACCCGAATGACAAACCAGAG
Ri245330-qF	GACGCCGACCCAGTAAAT
Ri245330-qR	CGTCACGCTTCTCCTCTTGT
RirT123350-qPCR-F1.2	GTTGCAGAGACAGATCCTGTTG
TZ-RirT123350-qPCR-R	ACATTGACGGCTATGTGCAG
Golden Gate Primers	
BCP1-GG-F	TTGAAGACATCTCAGGAGAGAGAGGGAGATGTGTTTTTAAG
BCP1-GG-R	TTGAAGACAACTCGCATTGATCAAAATTTGATTTTGGAA
Bcp1-sp-mCherry GG-F	TTGAAGACAAAATGGCTTCTTCTCGTGTAGTTT
Bcp1-sp-mCherry GG-R	TTGAAGACAACGAAGGTACCCTTGTACAGCTCGTC
BIrA-GG-F	TTGAAGACAAAATGAAGGATAACACCGTGCC
BirA-GG-R	TTGAAGACAAAAGCTCAGAGATCCTCCTCACTGATG
NTF-GG-F	TTGAAGACAAAATGAATCATTCAGCGAAAACC
NTF-GG-R	TTGAAGACAAAAGCTCAAGATCCACCAGTATCCTCA
PT4p-GG-F	TTGAAGACATCTCAGGAGGACTCGATCCACAACAAAGATT
PT4p-GG-R	TTGAAGACAACTCGCATTGACTCTCTCAAGTTGGTTTTTGG

Appendix B. qPCR Data
See attached Excel file.