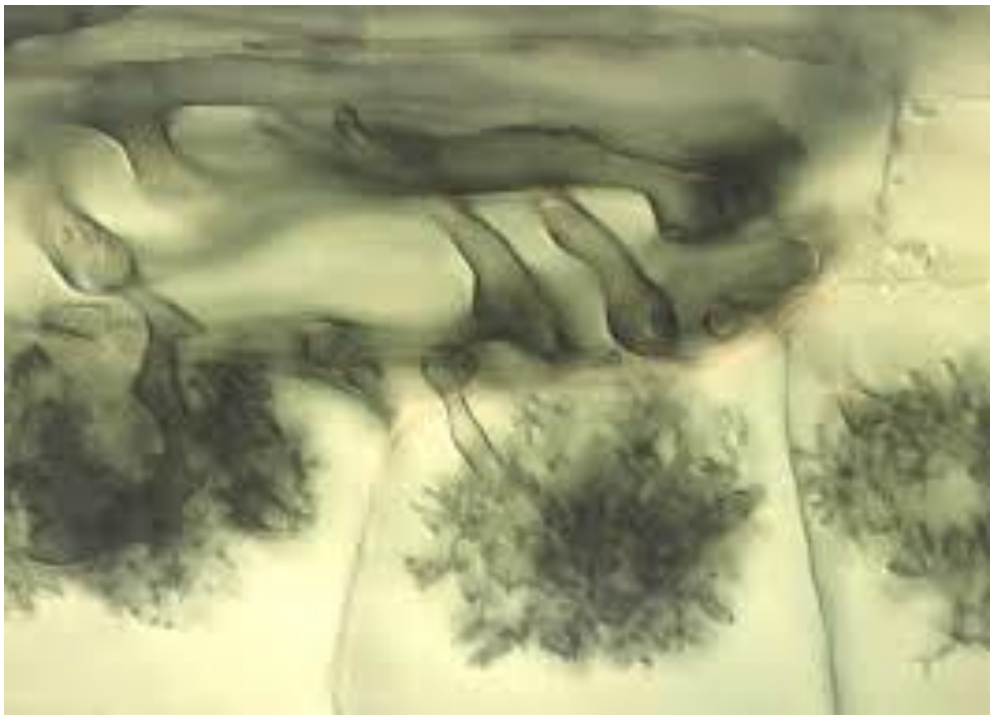
The role of AM effectors in endosymbiosis

Wageningen University

M.Sc. Biotechnology Thesis



Student: Fangyuan Wu
Reg No: 910418975050
Chair group: Molecular biology
Supervisor: Dr.Ir. E. Limpens
May 2014 – September 2014 (24 ECTS)

WAGENINGEN UR
For quality of life

Abstract

Arbuscular mycorrhizal (AM) fungus can colonize root cortical cells to build an endosymbiotic relationship with almost all land plants. This means AM fungus possesses a broad compatibility to adapt to different plant species. At the heart of this symbiosis is the formation of arbuscule. However, the molecular mechanisms underlying the ability of the AM fungus to adapt to a broad host range have not been well elucidated. We hypothesized that AM effectors play a role in the formation and development of arbuscules to facilitate the achievement of highly efficient compatibility. Also we predicted that arbuscules specific AM effectors might be able to translocate into plant cells to suppress host defense responses. To test this, we studied the role of three putative effector genes of AM fungi. These genes are: *RirT167520*, *RirT266090* and *RirT065700*. To study whether these effectors suppress plant defense we used *Nicotiana benthamiana* leaves as a system to monitor defense responses. Firstly we tested whether the selected effectors are well expressed in *N. benthamiana* leave cells. Therefore, constructs of effector fused to GFP were made and these constructs were infiltrated into *N. benthamiana* leaves. RirT167520 localized to the nucleolus and nucleoplasm, RirT266090 to the cytoplasm and possibly nucleus as well, and RirT065700 to the cytoplasm and nucleus. These localizations suggest that these effectors might be translocated into the plant cells and might have their functions in these sites. However, this cannot prove effector translocation since these proteins expressed inside the plant cells without signal peptide. Next we designed defense response assays using flg22 as a trigger for defense responses. The reactive oxygen species (ROS) evaluation as well as qPCR applied on this study failed to detect the occurrence of defense responses. The callose deposition assay provoked defense responses but less than our expectations. This suggests that the length of treatment time as well as the concentration of flg22 used for our assays were not sufficient to detect defense responses. The assays still need to be further optimized before the effect of AM effectors can be tested. To study whether AM fungi use effectors in a plant-species specific manner, it was necessary to know whether effectors are expressed in a plant species specific manner. Since the *Rhizophagus irregularis* DAOM197198wur strain (WUR strain) we used was cultivated for a long time on a chicory root culture, we tested whether there would be a difference in compatibility between our strain and a *Rhizophagus irregularis* BEG21 strain (Utrecht strain). The results show that the fresh weight of *Medicago truncatula* and *N. benthamiana* inoculated with the Utrecht strain was much lower than the ones inoculated with the WUR strain, although the mycorrhization level with the Utrecht strain was higher. This might be due to some contaminants within the sand mixture containing the Utrecht strain. Alternatively, the Utrecht strain fungi can obtain much more photosynthates from plants compared to the WUR strain fungi.

Contents

Introduction

Arbuscular mycorrhizal (AM) fungi which belong to the Glomeromycota order possess the ability to colonize root cortical cells to build an endosymbiotic relationship with 70-90% of land plants [1]. The fungi supply mineral nutrients to the host plants in exchange for photosynthates, and provide protection for hosts against pathogens and environmental stress [2, 3]. After spore germination, some fungal branched hyphae access the root of host, and penetrate the cell walls of cortical cells. Ultimately, fungi colonize the root and in the inner root cortical cells a highly branched structure is formed, called an arbuscule, which serves as an interface for nutritional exchange (Figure 1) [1]. The formation of arbuscule is the heart of the endosymbiosis. However, the molecular mechanisms underlying the ability of the AM fungus to adapt to a broad host range and the formation of these arbuscules have not been elucidated. This is in part due to the fact that there was no available AM fungus genome sequence. Recently, the genome sequences of two *Rhizophagus irregularis* DAOM197198s isolates have been published [4, 5]. This is the first AM fungus genome that has been revealed. The genome sequences can thus provide a way of understanding the molecular mechanisms of how a single AM fungus can intracellularly colonize such a wide variety of plant species.

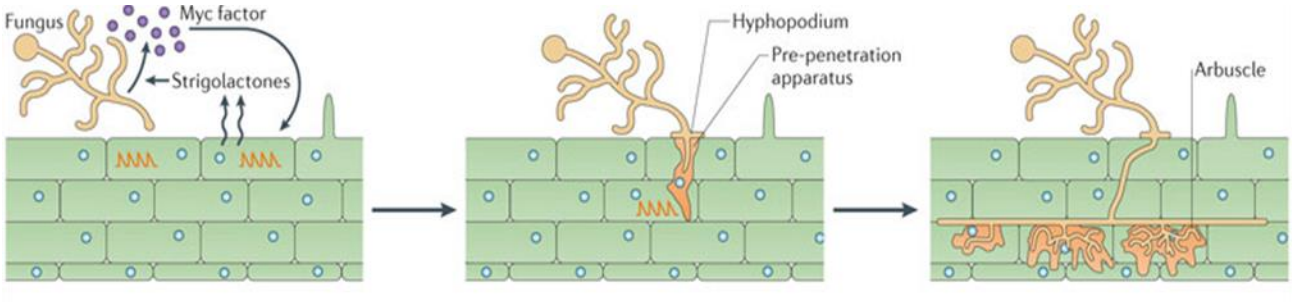


Figure 1. The formation of Arbuscular Mycorrhiza. Strigolactones released by plant roots are detected by AM fungi, resulting in hyphal branching. Afterwards, fungi start secreting Myc factors which in turn are detected by the plant and induce a symbiotic signaling pathway. The hyphae penetrate the roots forming the hyphopodium which allows the fungi start to spread in the inner cortical cells. A pre-penetration apparatus which is important for infection is formed prior to colonization. Eventually, fungi colonize the roots and in the inner root cortical cells an arbuscule with a highly branched structure is formed [1].

Myc factors produced by AM fungi are (lipo)chitooligosacharides (LCOs) that activate a symbiotic signaling pathway. This pathway is required for root colonization as well as arbuscule formation [6-8]. A recent paper has reported that, SP7, as the first identified AM effector, also plays a role in endosymbiosis. This AM effector was shown to able to translocate into the nucleus of the host cells where it interacts with pathogenesis-related transcriptional factor ERF19 to suppress host defense response [9]. This gives us an indication that not only myc factors, but

also AM effectors play a role in AM endosymbiosis. Secreted effectors have been found in pathogenic fungi/oomycetes. These can also have an intracellular lifestyle in host cells. These effectors in pathogenic fungi/oomycetes were found to facilitate plant invasion by modifying the structure and function of host cells or by suppressing host defense mechanism [10-12]. Therefore, it is hypothesized that AM effectors play a role in the formation and development of arbuscules to facilitate the achievement of highly efficient compatibility (Figure 2).

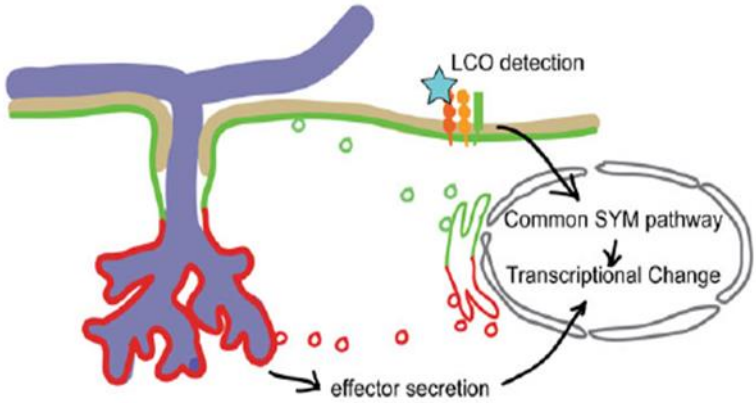


Figure 2: A proposed AM effectors scheme that takes place in the formation and development of arbuscules in host plant cells [13].

With the recent reveal of first genome sequence of an AM fungus, *R.irregularis* DAOM197198wur [5], it allowed us to identify putative secreted effectors. The selection of secreted proteins was based on the existence of a signal peptide and absence of mitochondrial signals and transmembrane domains [5]. Moreover, by comparing RNA abundance in arbuscules with extraradical hyphae/ spores, effectors that show strong up-regulation in arbuscules were considered as putative effectors.

AM fungi penetrate the roots, spread in between the cortical cells and eventually colonize inner cortical cells. It seems that intracellular growth of AM fungi is allowed by host plants, and AM fungi do not trigger host immune responses since elsewise the intracellular growth would be constrained. Nevertheless, the hosts must execute a control against the mycorrhization in roots as arbuscule formation is confined to the inner cortical cells [14]. It has been reported that AM fungi can activate some metabolic pathways involved in immune responses in hosts, however, their influence is not intense [15]. Till now, the reason for this weak activation of immune responses is still unclear. Several mechanisms responsible for the weak defense responses have been proposed in last ten years. During symbiotic interactions, symbiosis-associated plant proteins might be influenced by a fungal elicitor (such as LCOs) to suppress the expression of defense-related genes [15]. Another possibility could be that symbiosis-associated fungal proteins acts as suppressors during defense reactions [16]. In our study, we hypothesize that AM

fungi secrete effectors that are able to translocate into the host plant cells and act as suppressors to suppress host defense responses (Figure 2).

Significance of study

AM fungi facilitate uptake of phosphorous as well as nitrogen from the soil into plants. The result of these supplies is improved growth and productivity in host plants [1].They also protect host plants from pathogens and environmental stress. Additionally, AM fungi are beneficial to ecosystem by improving soil quality and carbon cycle [3]. Therefore, understanding the molecular mechanisms of how AM fungi associate with plants to build a mutualistic relationship may help to optimize mycorrhization; thus, facilitating crops to obtain maximal benefit in a more sustainable manner.

Research hypothesis

AM effectors play a role in the formation and development of arbuscules.

In this research we aim to answer the following questions:

- Do arbuscules-specific effectors suppress plant defense response inside plant cells?
- Do AM fungi use effectors in a plant-species specific manner?

To answer these we:

- Selected three "arbuscules-specific" effectors
- Tested the subcellular localization in *N. benthamiana* leave cells
- Designed defence response assays using *N. benthamiana* leaves
- Studied whether effectors are expressed in a plant species specific way

Approach

Selection of arbuscules-specific effectors

To study the role of AM effectors in arbuscule formation, first of all, effectors need to be selected that are specifically (or enriched) expressed in arbuscules. Therefore, this selection will be based on RNA sequence reads that have been mapped to putative effector genes of *R. irregularis* DAO197198wur strain. By comparing RNA abundance in arbuscules with extraradical hyphae/ spores, effectors that show strong up-regulation in arbuscules.

Tests on the subcellular localization in N. benthamiana leave cells

To test whether the selected effectors are well expressed in the cells, effector genes fused to GFP will be expressed in *N. benthamiana* leaves. Fluorescence signal could be detected using

confocal microscope. If they were well expressed, they can be used in the defense assay as well as studies on potential subcellular localization which may indicate a putative translocation to plant cells.

Design of defence response assays using N. benthamiana leaves

To study whether these effectors could suppress host defense response, an efficient defense response assay should be developed. PAMPs, like flg22 peptide and chitin, will be infiltrated into *N. benthamiana* leaves. Several defense response assays will be used as follows.

The first assay is ROS (reactive oxygen species) evaluation. ROS, an indication of plant immune responses, can be rapidly produced in response to stress [17]. The second one is qPCR analysis to monitor a defense response on a genetic level. Some Pattern-triggered immunity (PTI) marker genes would be selected in this assay. The third one is callose deposition, which is a plant immune response and is thought to play a role in penetration resistance after exposure to pathogens[18, 19]. The callose deposits can be visualized by aniline blue staining.

Studies on whether effectors are expressed in a plant species specific way

To test whether effectors are expressed in a plant species specific way, the WUR strain was used on different plant species. Since this strain was cultivated for a long time on a chicory root culture, we also wanted to test whether there would be a difference in compatibility between this WUR strain and the Utrecht strain.

Materials and Methods

Cloning of effector genes

Putative effector genes mapped to arbuscule specific or strongly upregulated RNA reads (data from [20] & professor Krajinski) were chosen for our study. 40 putative effector genes have been revealed. Effector genes *RirT167520, RirT266090* and *RirT065700* from these 40 candidates were selected in our study. Some RNA/seq information of these 3 effectors was showed in Appendix 3.

Previous experiments showed that if constructs including signal peptides fused to GFP were infiltrated into *N. benthamiana* leaves, these proteins were observed to have a high localization at endoplasmic reticulum (ER). This suggested that the signal peptides from *R. irregularis* effector proteins are not properly processed in *N. benthamiana* leaves. Therefore constructs were made to express effectors without their signal peptide.

Gene-specific primers (Appendix 1) to amplify the effectors *RirT167520, RirT266090* and *RirT065700* without signal peptides were designed using Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). To make sure genes of interest are cloned into 245 pDONR™221 vector (Invitrogen), these primers were bound to *att*B adaptors. Since the length of adaptors are 30bp, *attB*-PCR products were obtained by double amplifications, therefore two pairs of primers were used for each effector genes. The first pair of primers used in the first amplification were gene-specific primers together with 15bp of the latter part *att*B adaptors. The whole *att*B adaptors were used as second pair of primers in the second amplification. cDNA was synthesized from one microgram of RNA which was isolated from *M. truncatula* mycorrhized roots using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., USA). The volume of a PCR reaction was 20µl containing 4µl 5x Phusion HF buffer, 0.5µl dNTPs, 1µl DNA template, 0.5µl Phusion polymerase, 0.5µl 10-time-diluted Forward primer, 0.5µl 10-time-diluted Reverse primer and 13µl sterile MiliQ water. Due to the long length of *RirT065700* (1810bp), the annealing temperature as well as extension time in the cycles were different in PCR reaction in *RirT065700* compared to *RirT167520* (316bp) and *RirT266090* (277bp). The PCR program for *RirT167520* and *RirT266090* was as follows: initial denaturation at 98 $°C$ for 30 s followed by 40 cycles of: denaturation at 98 $°C$ for 10 s, annealing at 60 $°C$ for 30 s and extension at 72 $°C$ for 20 s. Lastly 5 min of an extension step at 72 $°C$ was done. For *RirT065700*, the annealing temperature was 63 $°C$ and cycle extension time was 1 min. The conditions for PCR reactions in two amplifications were the same except for primers. The PCR products were checked by 1% agarose gel electrophoresis. The DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Technologies, USA). Subsequently, these fragments were inserted in 245 pDONR™221 vector according to the manufacturer's instructions. After BP reactions, these constructs were transformed into electrocompetent *E. coli*

(DH5α) cells which subsequently grown on LB agar plates supplemented with kanamycin at 37°C overnight. Colony PCR was conducted on colonies grown on plates using the first pair of primers and Taq polymerase instead of Phusion polymerase. The volume of a PCR reaction was 20µl. PCR program for *RirT167520* and *RirT266090* was as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of: denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1 min. Lastly 7 min of an extension step at 72 °C was done. For *RirT065700*, the annealing temperature was 58 °C and cycle extension time was 2.5 min. These correct plasmids were extracted using the Omega miniprep kit and further confirmed by sequencing. Correct constructs underwent an LR reaction with destination vector 291 or 292 which contains a GFP tag at the C-term or N-term. Likewise, the recombinant constructs were transformed into DH5α cells and then a colony PCR was performed. These correct plasmids were further confirmed by digestion using restriction enzymes (Fermentas®) *Hind*III and *EcoRI* for *RirT167520, SaII* and *KpnI* for *RirT266090* as well as *RirT065700*.

Agroinfiltration

These constructs with GFP fusions were transformed into *Agrobacterium tumefaciens* C58 cells and grown for 2 days in LB medium complemented with gentamicin, spectinomycin and rifampicin. Afterwards, these cells were infiltrated into *N. benthamiana* leaves using a needleless syringe. Leaves were detached after 3 days, and checked for GFP signal using confocal microscope.

Design of a defence response assay

Chitin, a key compound of fungal cell walls, functioning as PAMPs can trigger defense response in plant cells theoretically [21]. However, infiltrating *N. benthamiana* leaves with chitin did not show any obvious up-regulation of PTI marker genes in experiments done by Toolbox using 100 µg/mL $(GlcNAc)_6$ as well as our preliminary experiment using 10 µM $(GlcNAc)_6$. Therefore flg22, a bacterial peptide epitope was instead [22]. Three defense response assays were performed with infiltration of flg22 (Genscript) into *N. benthamiana* leaves using a needleless syringe.

ROS evaluation

DAB staining used for ROS evaluation was followed the protocol represented in Appendix 5. Schematic overview of infiltration is shown in (Figure 3).

qPCR

The infiltration in leaves of 4-week-old *N. benthaminia* plants were showed in (Figure 3). After 6 h, leaf disks from infiltrated areas were detached with a 2 ml microtube for RNA isolation. cDNA was obtained from one microgram of RNA using iScript cDNA Synthesis Kit (Bio-Rad

Laboratories, Inc., USA) and used for subsequent qPCR reaction with My iQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). Three PTI marker genes *NbCYP71D2* [23], *NbACRE132* [24] and *NbPal* [25] were chose and *NbEF-1a* [26] was used as a reference gene. qPCR was conducted in a 10µl volume containing: 0.5 µl of cDNA, 5µl of iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., USA) and 4.5µl of both 1200µM forward and reverse primers. qPCR program was set as follows: 95 °C for 3 min, 39 cycles of two-step of 95°C for 10 s and 60 °C for 30 s, then 95 °C for 10 s and 65 °C for 5 s. Each qPCR reaction was carried out in triplicate.

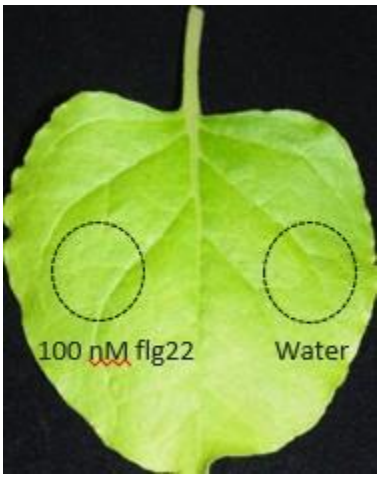


Figure 3. Schematic overview of infiltration with flg22 and water in a piece of *N. benthaminia* leaf performed in ROS and qPCR assay.

Callose deposition

Callose deposition assay for *N. benthamiana* leaves were adapted from Nguyen et al. [27]. The infiltration with 40 µM of flg22 in leaves of 4-week-old *N. benthaminia* plants was showed in (Figure 4). After 24h, leaf disks from infiltrated areas were cut off.

These disks were cleared away chlorophyll by incubation in 96% ethanol at 37°C until the clearing was thorough. The cleared leaf disks were washed with 70% ethanol for two times, followed by three times with sterile MiliQ water. The disks were vacuum-infiltrated with 1% aniline blue in 150 mM K_2HPO_4 (pH 9.5/KOH) and subsequent incubated in the dark overnight. The stained disks were mounted with 60% glycerol on slides. Afterwards, callose deposits were viewed under using a Leica microscope with ultraviolet light. The number of callose deposits were counted in 10 randomly picked visual fields of each disk at low magnification (×10).

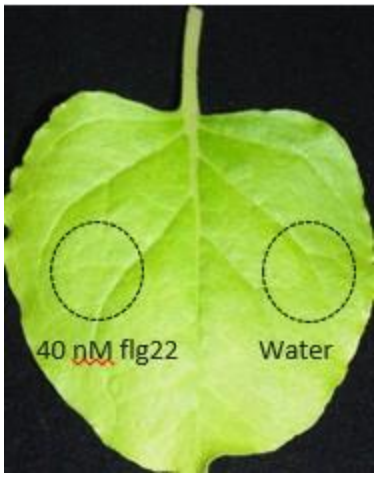


Figure 4. Schematic overview of infiltration with flg22 and water in a piece of *N. benthaminia* leaf performed in callose deposition assay.

Comparison of differences in compatibility between the WUR strain and the Utrecht strain in colonizing different plant species

Plant material, inoculation and growth conditions

Hydrobeads together with sand were autoclaved and mixed in equal proportions. This mixture was divided into 3 groups with different inoculations. The WUR Strain group was inoculated with the *R. irregularis* DAOM197198wur strain (WUR strain) obtained from chicory root culture [5], The Utrecht Strain group consisted of a sand mixture containing *R. irregularis* isolate BEG21 (provided by prof. Pieterse, Utrecht University), and Control group with no strain. 7-day-old seedlings of *N. benthamiana*, 5-day-old seedlings of *M. truncatula*, 7-day-old seedlings of chive and 5-day-old seedlings of tomato previously germinated (Appendix 6 for seed germination) on Fahraeus medium (Appendix 7) were transplanted to pots. Each plant species with each treatment had 2 pots. Except for tomato with 3 plants in one pot, the other plant species had 5 plants. All the plants were watered twice a week. In the first three weeks, these plants were watered only with demi water. Afterwards, demi water was replaced by ½ Hoagland medium (Appendix 8) supplemented with 20µM KH_2PO4. After 4 weeks, plants from one pot for each species with each treatment were harvest.

Biomass and mycorrhization analysis

Plants were uprooted and then sand and hydrobeads were washed away. The fresh weight of the complete plant was measured for each plant. By trypan blue staining (Appendix 9) the half of the roots of the plants, intraradical mycelium (arbuscules, vesicles, intercellular and intracellular

hyphae) was observed using microscopy. The level of mycorrhization and arbuscules were quantified based on the Trouvelot method (Appendix 10) [28].

Results

Intracellular localization of selected effectors in host cells

To test whether *RirT167520, RirT266090* and *RirT065700* are well expressed in the cells, a GFP tag was fused to the effectors at the C-terminus or N-terminus, separately. These recombinant constructs were expressed in *N. benthamiana* leaves under the control of the CaMV 35S promoter. Infiltrated leaves were detached after 3 days, and checked for GFP signal using confocal microscopy. If they were well expressed, they can be used in the defense assay as well as studies on potential subcellular localization which may indicate a putative translocation to plant cells.

RirT167520 localizes to the nucleus

Both nGFP:RirT167520 and RirT167520:cGFP were observed to have a clear localized signal in the nucleus of *N. benthamiana* cells. More specifically, these proteins concentrated in the nucleolus and also in the nucleoplasm with a punctate distribution (Figure 5).

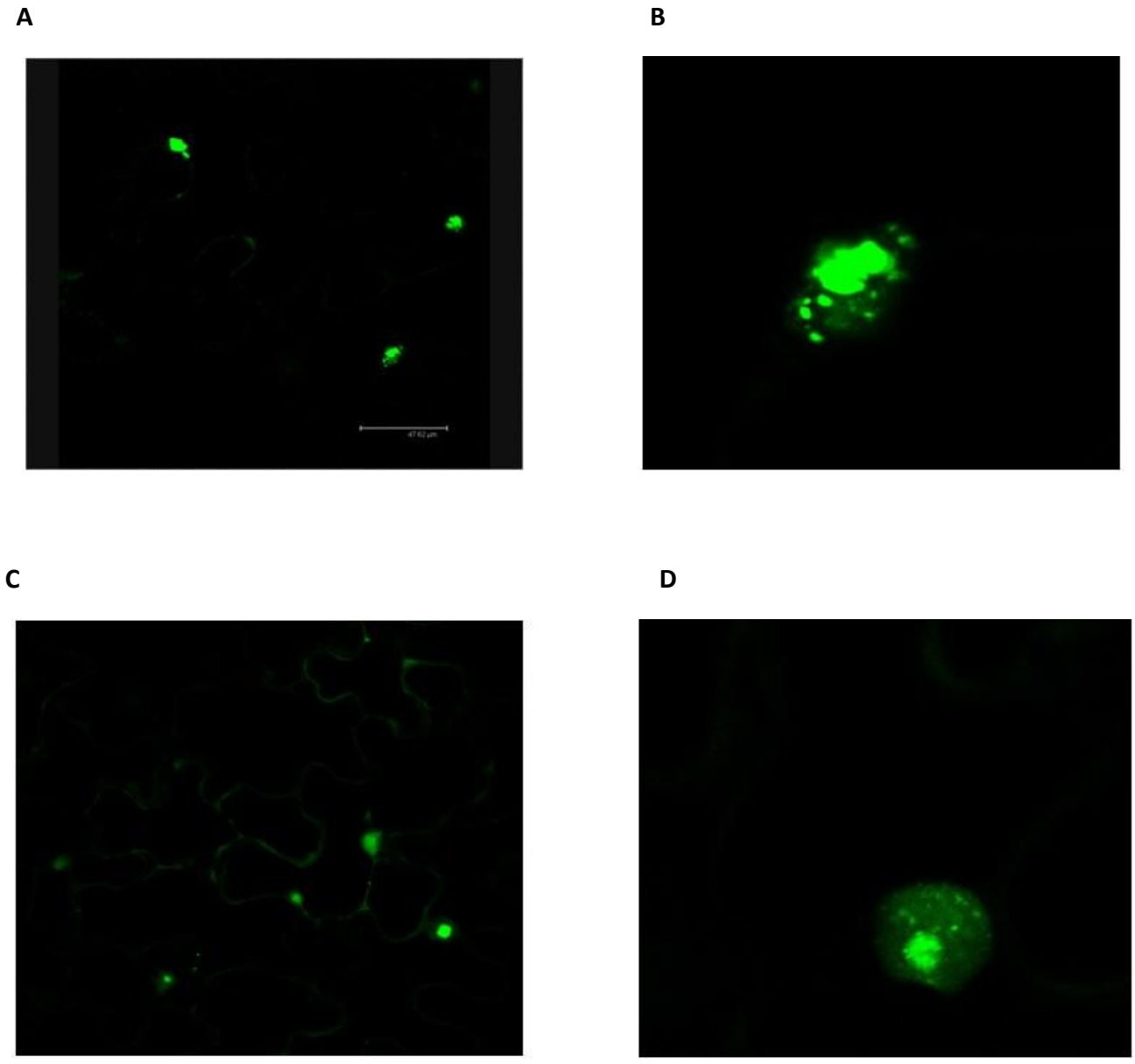


Figure 5. GFP signal localization of nGFP:RirT167520 (A&B) and RirT167520:cGFP (C&D) in *N. benthamiana* leaves.

RirT266090 localizes to the cytoplasm and possibly nucleus

nGFP:RirT266090 proteins were observed to localize to the cytoplasm of *N. benthamiana* cells. RirT266090:cGFP localized not only to the cytoplasm, but also to nucleus (Figure 6).

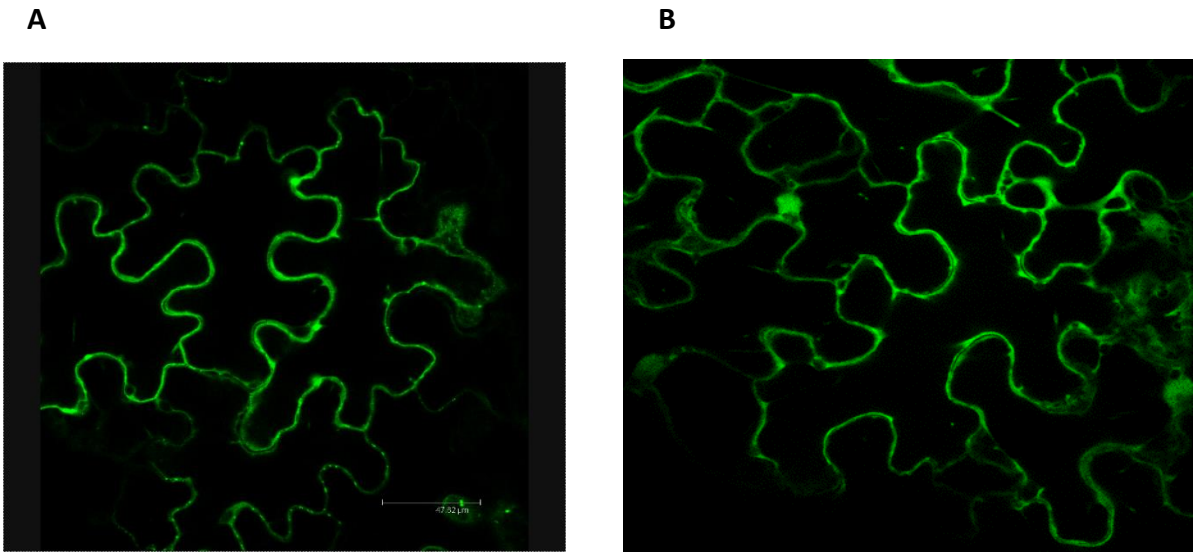


Figure 6. GFP signal localization of nGFP: RirT266090 (A) and RirT266090:cGFP (B) in *N. benthamiana* leaves.

RirT065700 localizes to the cytoplasm and nucleus

Both nGFP:RirT065700 and RirT065700:cGFP were weakly expressed in *N. benthamiana* cells. Both constructs showed GFP signal in the cytoplasm as well as in the nucleus of *N. benthamiana* cells. In more detail, in the nucleus they were excluded from the nucleolus (Figure 7).

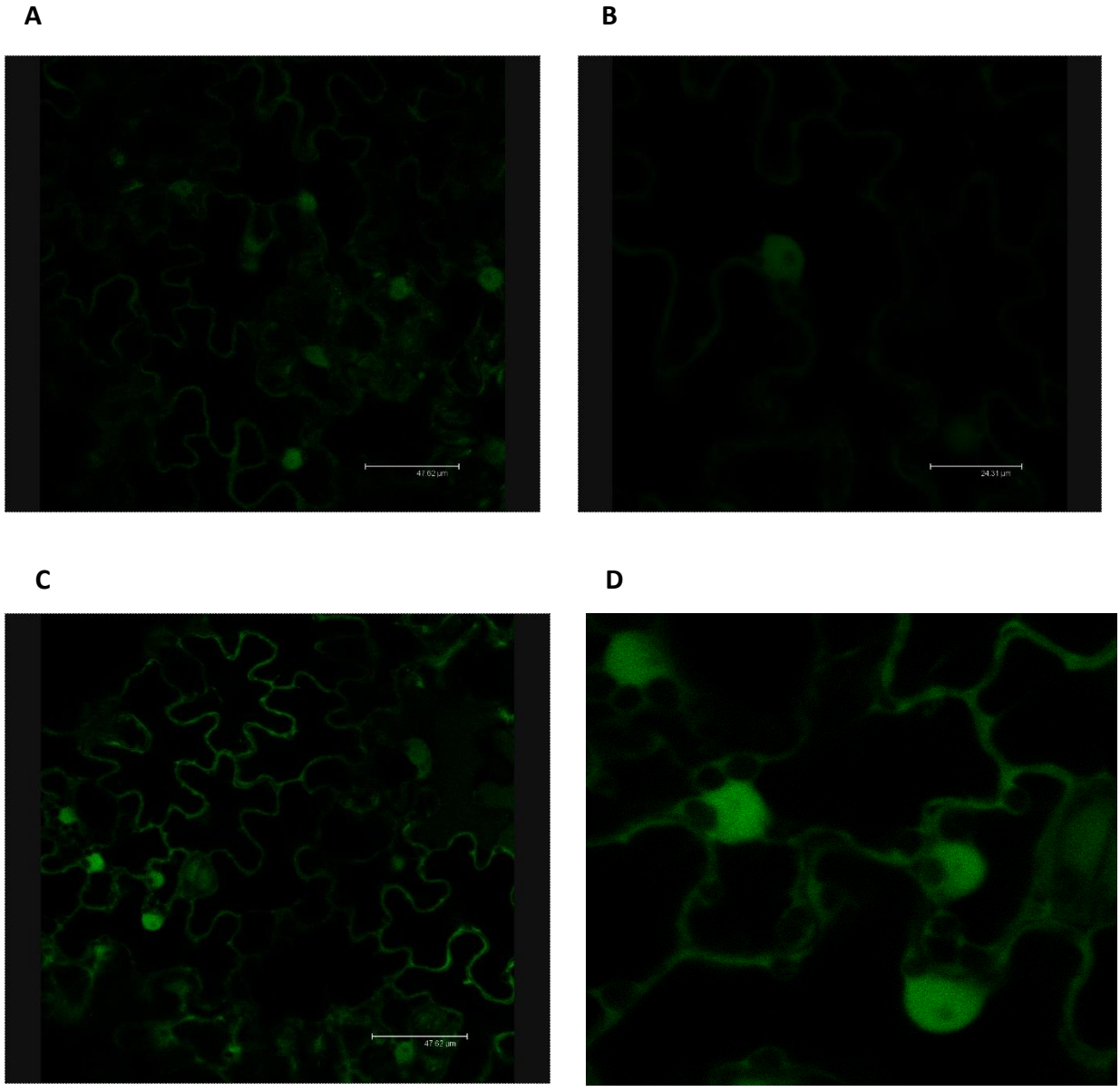


Figure 7. GFP signal localization of nGFP:RirT065700 (A&B) and RirT065700:cGFP (C&D) in *N. benthamiana* leaves.

Design of defense response assays

To study whether RirT167520, RirT266090 and RirT065700 suppress host defense response, an efficient defense response assay should be developed. Flg22, functioning as PAMPs can also trigger a defense response [29]. Our defense response assays were designed for infiltrating flg22 into *N. benthiama* leaves. Three assays were performed: ROS evaluation, qPCR and callose deposition.

ROS evaluation

The production of ROS can be rapidly increased in reaction to stress which indicates an occurrence of immune responses [17]. In our experiment, DAB staining was used for ROS (H_2O_2) evaluation. If a defense response happens, significant dark-brown precipitates could be expected [30]. 100 nM of flg22 was infiltrated into *N. benthiama* leaves. After 25 min or 1 h, hydrogen peroxide was detected by DAB staining. The results of ROS evaluation are shown in (Figure 8). Although some dark-brown precipitates were generated, no significant difference was observed between flg22- treated leaf discs and water-treated discs in both 25 min and 1 h incubation of flg22, suggesting that the production of ROS in leaves in reaction to flg22 at these two infiltration times was too low to be detected.

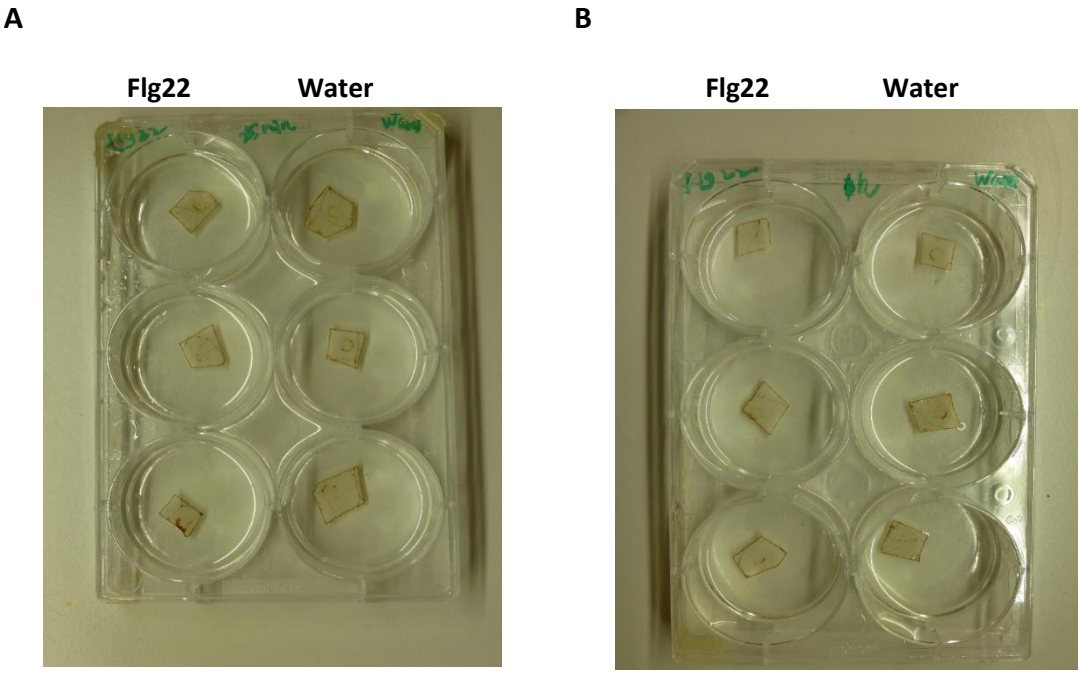


Figure 8. The ROS severity in *N. benthamiana* leaves after 25 min (A) and 1 h (B) treated with flg22 and water.

qPCR

To monitor a genetic response in *N. benthiama* cells, qPCR was performed with 100 nM of flg22 infiltrated into *N. benthamiana* leaves. , in analogy to the performance in [31]. RNA was extracted after 6h and cDNA was subsequently synthesized. qPCR was conducted with 3 PTI marker genes, *NbCYP71D2* [23], *NbACRE132* [24] and *NbPal* [25] were chose and *NbEF-1a* was used as a reference gene [26]. The result is displayed in (Figure 9), showing no obvious up-regulation of these marker genes in flg22-treated samples compared to mock samples. This indicates infiltration with 100 nM of flg22 for 6 hours was not sufficient to detect transcriptional activation of PTI marker genes.

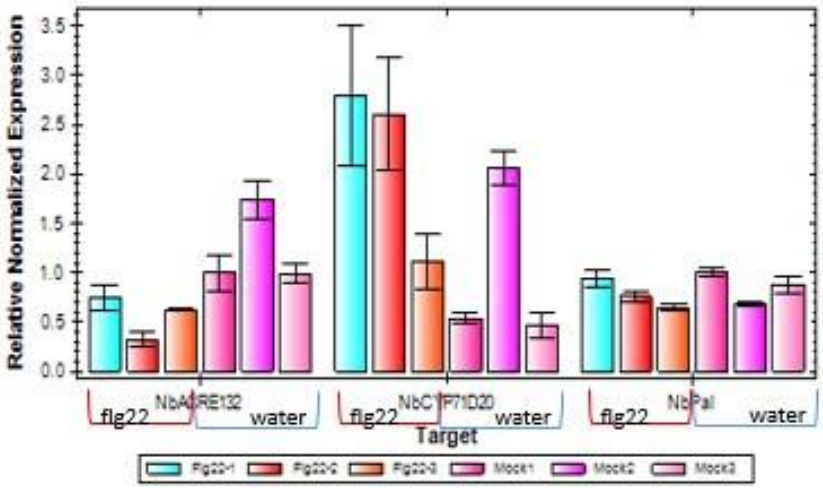


Figure 9. Relative normalized expression of PTI maker genes *NbACRE132, NbCYP71D2* and *NbPal* in reaction to flg22 in *N. benthiama* leaves. *NbEF-1a* was used as a reference gene. Flg22-1, Flg22- 2, Flg22- 3 are flg22 treated samples; Mock1, Mock 2 and Mock 3 are mock-treated with water samples.

Callose deposition

Callose deposition is also a plant immune response induced upon the perception of PAMPs, such as flg22 [19]. After infiltration with flg22 for 24 h, aniline blue staining was performed to visualize callose deposits in *N. benthiama* leaf discs. The results are displayed in (Figure 10A), showing that more callose deposits were generated in flg22-treated discs compared to mock discs. Quantification of these callose deposits was done according to 10 randomly picked fields in one leave disc. The average number of callose deposits in flg22-treated disc was 23.2, which was about 8 folds more than that of water-treated disc (Figure 10B). The result suggests that the callose depositon assay applied in our study provoked defense responses in flg22-treated discs. However, compared to strong callose deposition generated in Arabidopsis cells with the same treatment [19], our results were less than expected.

A

B

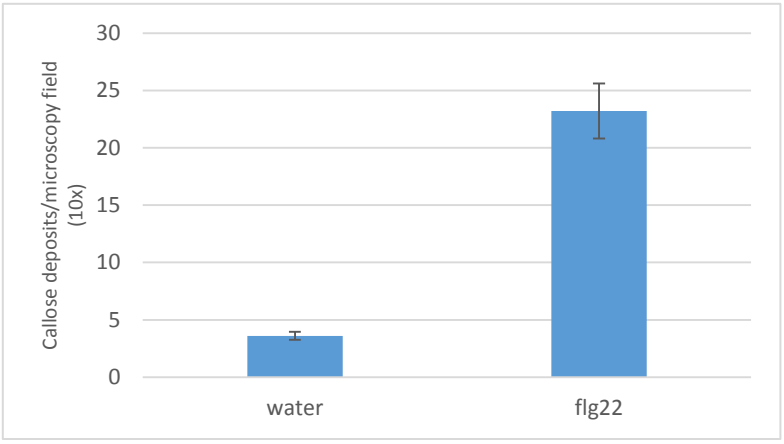


Figure 10. Callose deposition after (40 µM) infiltration. (A) *N. benthiama* leaf discs stained with aniline blue 24 hours after infiltration with flg22 or water. Right image, reference image of callose deposition in Arabidopsis disc with exposure to flg22 [19]. (B) Average number of callose deposits after infiltration. Error bars represent the standard deviation (n = 10 fields).

Mycorrhization and biomass analysis

To compare the differences in compatibility between the WUR strain (*R. irregularis* DAOM197198w) and the Utrecht strain (*R. irregularis* BEG21) in *M. truncatula*, *N. benthamiana*, chive and tomato, frequency of mycorrhization (F%), intensity of mycorrhization (M%) and arbuscule abundance (A%) of root segments were determined. Due to delayed seed germination of tomatoes inoculated with the Utrecht strain, we did not harvest them yet. No

colonization was found in all plants in Control group. Tomatoes inoculated with the WUR strain also did not show any colonization. It might be that we need to wait for a longer time to check for it. These three parameters in *M. truncatula* and *N. benthamiana* inoculated with the WUR strain were lower by more than 50% compared to inoculation with the Utrecht strain (Figure 11). However, the fresh weight of *M. truncatula* and *N. benthamiana* in WUR Strain group was heavier compared to those in Utrecht Strain group and Control group, respectively (Figure 12). In chives, these three parameters in WUR Strain group were comparable to those in Utrecht Strain group (Figure 11). Comparable results were found also for the fresh weight in chives inoculated with the WUR strain or the Utrecht strain or no strain (Figure 12). Although no colonization was found in tomatoes inoculated with the WUR strain, the average fresh weight of tomatoes in WUR Strain group was around 2.45 g, which was about five times larger than the average in Control group (Figure 12).

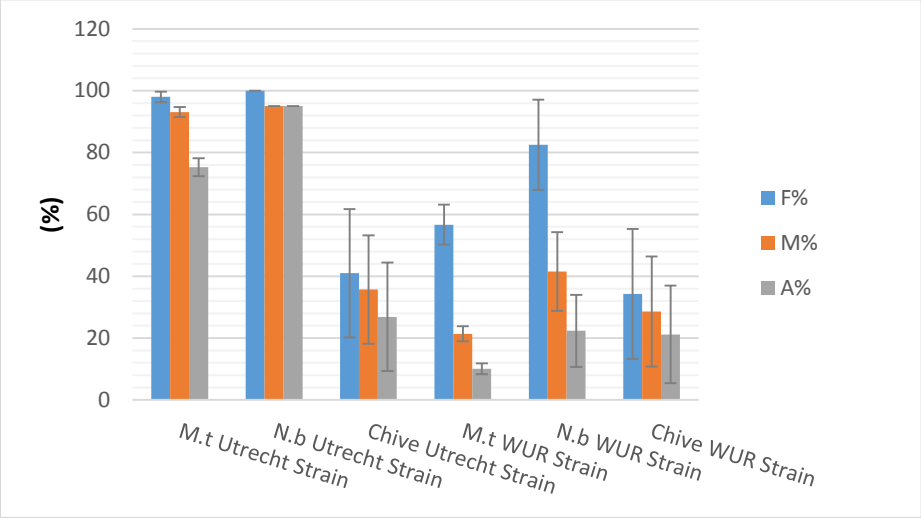


Figure 11. Frequency of mycorrhization (F%), intensity of mycorrhization (M%) and arbuscule abundance (A%) of *M. truncatula*, *N. benthamiana* and chives inoculated with the Utrecht strain or the WUR strain. Half of the roots from all plants were used. Error bars indicate the standard deviation.

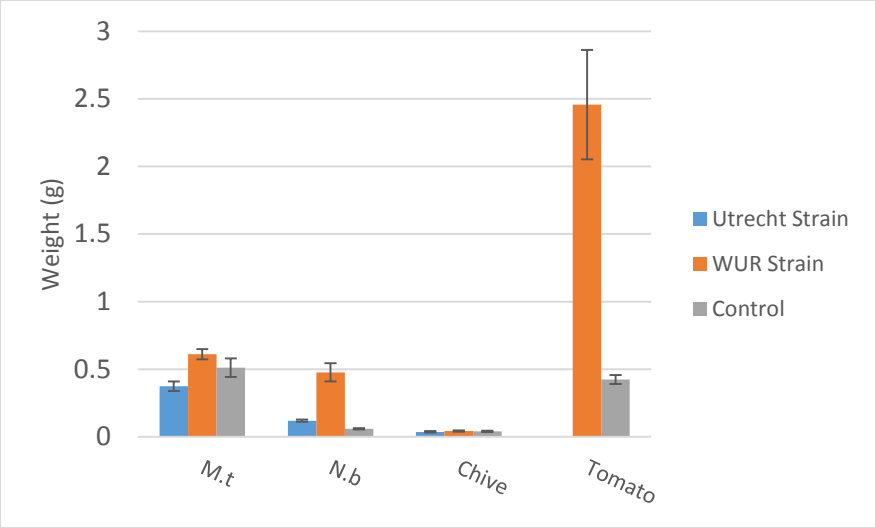


Figure 12. Fresh weight of *M. truncatula*, *N. benthamiana*, chives and tomato colonized with the Utrecht strain and the WUR strain. Error bars indicate the standard deviation.

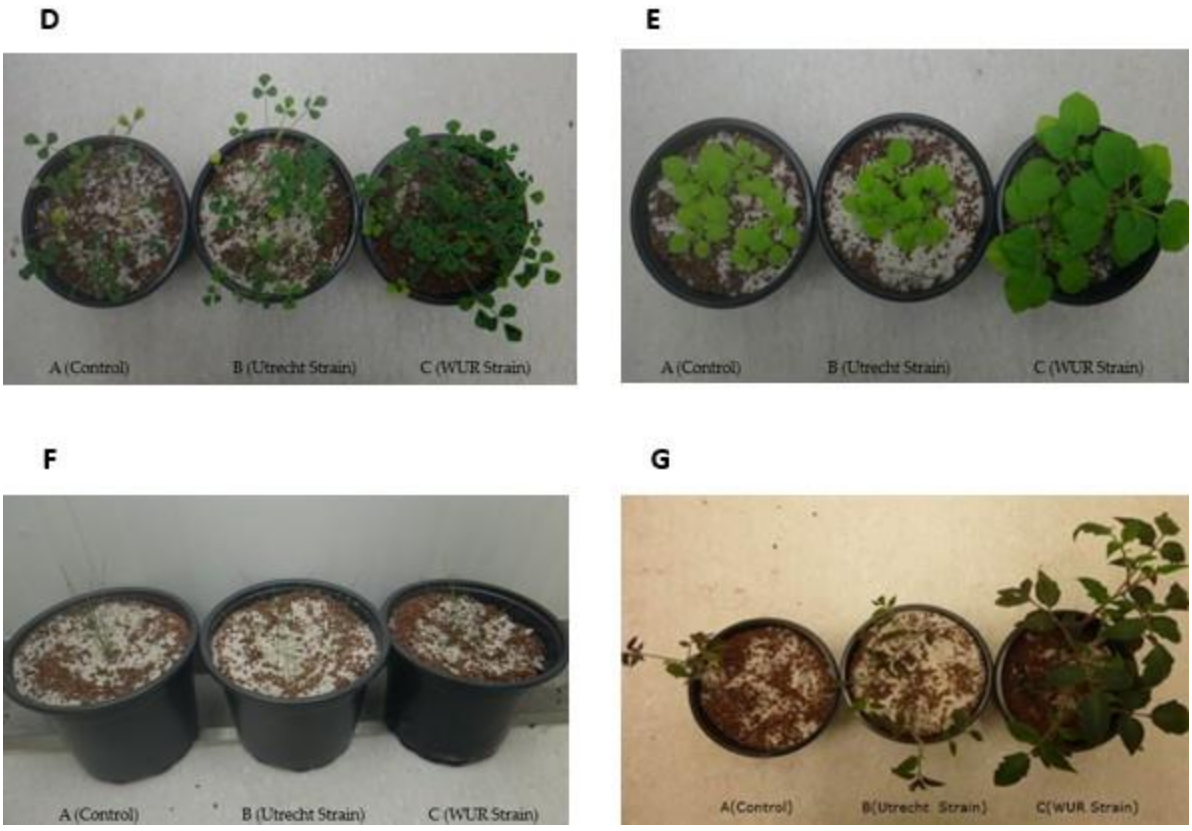


Figure 13. Phenotype of 4-week-old *M. truncatula* (D), *N. benthamiana* (E), chives (F) and tomato (G) with three incubations (A: no strain (control), B: the Utrecht strain, C: the WUR strain)

Discussion

To study whether the candidate effectors (RirT167520, RirT266090 and RirT065700) suppress plant defense response inside plant cells, it was necessary to design defense response assays. *N. benthamiana* leaves were used in our study. Firstly we needed to test the expression of these effectors in leave cells and at the same time we studied their subcellular localization. To study whether AM fungi use effectors in a plant-species specific manner, it was necessary to know whether effectors are expressed in a plant species specific manner. Since the WUR strain we used was cultivated for a long time on a chicory root culture, we also wanted to test whether there would be a difference in compatibility between our strain and the Utrecht strain.

Localization

Both nGFP:RirT167520 and RirT167520:cGFP proteins show a clearly localized signal in the nucleus of *N. benthamiana* cells, especially in the nucleolus but also as dot-like structures in the nucleoplasm. This suggests that RirT167520 might be translocated into the plant cells and might have a function in the nucleolus. Nucleolus is a pronounced nuclear subcompartment. It provides the site for transcription, processing as well as assembly of rRNAs and regulates cell cycle [32]. Moreover, it is also involved in stress response, by functioning as a stress sensor [33]. Such nucleolus localization suggests RirT167520 might interact with host proteins on a transcriptional level. HaRxL44, an effector from *Hyaloperonospora arabidopsidis* (*Hpa*), was also found to localize to the nucleoplasm and nucleous *in planta*. Mediator, a large multiprotein complex, acts as a transcriptional coactivator associating with RNA polymerase II. HaRxL44 proteins were showed to interact with and degrade a Mediator complex subunit 19a (Med19a), which localizes to the nucleoplasm as well as nucleolus and is a positive transcriptional regulator of *A. thaliana* immunity upon *Hpa* invasion [34].

RirT266090 with a GFP tag at the N-terminal localized to the cytoplasm of *N. benthamiana* cells. Proteins with a GFP tag at the C-terminal localized not only to the cytoplasm, but also to nucleus. This nuclear localization might be the result of protein diffusion from cytoplasm since the molecular weight of RirT266090:cGFP is 39 kDa which is less than the nuclear exclusion limit of 45 kDa [35]. These GFP signals suggest that RirT266090 might be translocated into the plant cells and might be a cytoplasmic effector. In the oomycete *Phytophthora infestans*, AVR3a, a cytoplasmic effector, acts as a cell death suppressor that interacts with and stabilizes host E3 ligase CMPG1. This late enzyme is required for INF1-triggered cell death [36, 37]. Hence, these two examples give us an indication that future research can try to identify interacting proteins in host cells which might give a clue on what the effectors might be doing.

Neither nGFP: RirT065700 nor RirT065700:cGFP proteins were highly expressed. This might be because in plant cells these proteins were degraded, or the GFP construct was broken down. There proteins showed a nucleic localization, however, outside the nucleolus.

Western blot would be necessary to check whether the GFP fragment was cleaved off from the GFP constructs. This cleaving off can affect judgment of localization.

However, our selected assays cannot prove effector translocation due to a lack of expression of signal peptide inside the plant cells. A hairy root transformation would be a better method to study localization of these effectors, since they are used in the roots by the fungus.

Alternative approaches to test the translocation of AM effectors into plants cells were proposed. Based on the theory of specific binding of antigen and antibody, immuno-localization was raised. The tested effector can be targeted by a specific antibody which can be further detected by a secondary antibody tagged with fluorescent. Then the localization can be observed by detecting fluorescent signals with confocal microscopy. Other option could be to produce a fluorescent effector fusion protein (for example purified from E. coli) and add it to plant cells to see if it taken up by the plant cells. Or introduce a GFP tagged version with signal peptide into a (hemi)biotrophic fungus that can be transformed to study potential translocation, similar to what was done in the work on SP7.

Defense response assay

The ROS evaluation as well as qPCR applied on this study failed to detect the occurrence of defense response. The callose deposition assay seemed to work but less than our expectations. Several studies have reported that flg22 can induce defense response in *N. benthamiana* cells, however the length of treatment time as well as the concentration of flg22 used for defense response assays varies in different studies [27, 31, 38]. Our results indicate that, despite its wide use in literature, application of flg22 to *N. benthamiana* leaves is not as straight forward as presented in literature. Therefore, it would be necessarily to set different time points and test different concentrations to determine optimal experimental conditions in future experiments. *Pseudomonas syringae*, a plant pathogen, has a type III secretion system which means that it can deliver effectors to host cells to attenuate defense reactions [39]. Leaves inoculated with *P. syringae* hrcC mutants would be another option. Due to a deficiency in type III secretion system, it has been shown that the *P. syringae* hrcC mutant is more efficient in triggering callose depositions in *N. benthamiana* leaves (Klaas Bouwmeester, Phytophathology, Wageningen University, personal communication).

Mycorrhization and biomass analysis

The fresh weight of *M. truncatula* and *N. benthamiana* inoculated with the Utrecht strain was much lower than the ones inoculated with the WUR strain, though the mycorrhization level with the Utrecht strain was higher. This might be due to some contaminants within the sand mixture containing the Utrecht strain. These contaminants might inhibit the growth of *M. truncatula* and *N. benthamiana*. Alternatively, the Utrecht strain fungi can obtain much more photosynthates from plants compared to the WUR strain fungi, and act in a somewhat parasitic way. Strikingly, tomatoes inoculated with the WUR strain performed quite well even though they were not colonized at all. Since the chosen WUR strain was cultivated for a long time on a chicory root culture, some original properties of this strain might have been changed. Tomatoes in Utrecht Strain group should be harvested to check the colonization and fresh weight as well as phenotype. These results should be compared to those of tomatoes in WUR Strain group. In future, it is necessary to equal the amount of inoculum spore in each pot to ensure equivalent conditions for infection and repeat this experiment. To study whether AM fungi use effectors in a plant-species specific manner, it is necessary to know whether effectors are expressed in a plant species specific manner. The other half of the roots of the plants will be used for RNA/seq analysis. This analysis can help us select effectors that are expressed in all these four plant species and show arbuscule specific/enhanced expression. These selected effectors will be used for functional analyses on their role in arbuscule formation and development.

References

1. Parniske, M., *Arbuscular mycorrhiza: the mother of plant root endosymbioses.* Nature Reviews Microbiology, 2008. **6**(10): p. 763-775.
2. Kitahara, R., et al., *A unique mitovirus from Glomeromycota, the phylum of arbuscular mycorrhizal fungi.* Archives of virology, 2014: p. 1-4.
3. Sikes, B.A., *When do arbuscular mycorrhizal fungi protect plant roots from pathogens?* Plant Signal Behav, 2010. **5**(6): p. 763-5.
4. Tisserant, E., et al., *Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis.* Proceedings of the National Academy of Sciences, 2013. **110**(50): p. 20117-20122.
5. Lin, K., et al., *Single nucleus genome sequencing reveals high similarity among nuclei of an endomycorrhizal fungus.* PLoS genetics, 2014. **10**(1): p. e1004078.
6. Harrison, M.J., *Signaling in the arbuscular mycorrhizal symbiosis.* Annu. Rev. Microbiol., 2005. **59**: p. 19-42.
7. Bucher, M., S. Wegmüller, and D. Drissner, *Chasing the structures of small molecules in arbuscular mycorrhizal signaling.* Current opinion in plant biology, 2009. **12**(4): p. 500-507.
8. Maillet, F., et al., *Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza.* Nature, 2011. **469**(7328): p. 58-63.
9. Kloppholz, S., H. Kuhn, and N. Requena, *A Secreted Fungal Effector of< i> Glomus intraradices</i> Promotes Symbiotic Biotrophy.* Current Biology, 2011. **21**(14): p. 1204-1209.
10. Win, J., et al. *Effector biology of plant-associated organisms: concepts and perspectives*. in *Cold Spring Harbor symposia on quantitative biology*. 2012. Cold Spring Harbor Laboratory Press.
11. Ellis, J.G., et al., *Recent progress in discovery and functional analysis of effector proteins of fungal and oomycete plant pathogens.* Current opinion in plant biology, 2009. **12**(4): p. 399-405.
12. Hogenhout, S.A., et al., *Emerging concepts in effector biology of plant-associated organisms.* Molecular plant-microbe interactions, 2009. **22**(2): p. 115-122.
13. Huisman, R., et al., *Endocytic Accommodation of Microbes in Plants*, in *Endocytosis in Plants*. 2012, Springer. p. 271-295.
14. Lambais, M., W. Ríos‐Ruiz, and R. Andrade, *Antioxidant responses in bean (Phaseolus vulgaris) roots colonized by arbuscular mycorrhizal fungi.* New Phytologist, 2003. **160**(2): p. 421-428.
15. GIANINAZZI‐PEARSON, V., et al., *Cellular and molecular defence‐related root responses to invasion by arbuscular mycorrhizal fungi.* New Phytologist, 1996. **133**(1): p. 45-57.
16. Shiraishi, T., et al., *The role of suppressors in determining host-parasite specificities in plant cells.* International review of cytology, 1997. **172**: p. 55-93.
17. Devasagayam, T., et al., *Free radicals and antioxidants in human health: current status and future prospects.* Japi, 2004. **52**: p. 794-804.
18. Chen, X.-Y. and J.-Y. Kim, *Callose synthesis in higher plants.* Plant Signal Behav, 2009. **4**(6): p. 489-492.
19. Ellinger, D. and C.A. Voigt, *Callose biosynthesis in arabidopsis with a focus on pathogen response: what we have learned within the last decade.* Annals of botany, 2014: p. mcu120.
20. Gaude, N., et al., *Arbuscule‐containing and non‐colonized cortical cells of mycorrhizal roots undergo extensive and specific reprogramming during arbuscular mycorrhizal development.* The Plant Journal, 2012. **69**(3): p. 510-528.
21. de Jonge, R., et al., *Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants.* Science, 2010. **329**(5994): p. 953-955.

22. Sun, Y., et al., *Structural basis for flg22-induced activation of the Arabidopsis FLS2-BAK1 immune complex.* Science, 2013. **342**(6158): p. 624-8.
23. Heese, A., et al., *The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants.* Proceedings of the National Academy of Sciences, 2007. **104**(29): p. 12217-12222.
24. Serrano, M., et al., *Chemical interference of pathogen-associated molecular pattern-triggered immune responses in Arabidopsis reveals a potential role for fatty-acid synthase type II complex-derived lipid signals.* Journal of Biological Chemistry, 2007. **282**(9): p. 6803-6811.
25. Zhu, Q., et al., *Transcriptional activation of plant defense genes.* Current Opinion in Genetics & Development, 1996. **6**(5): p. 624-630.
26. Li, X.-Y., et al., *Characterization and expression analysis of the SNF2 family genes in response to phytohormones and abiotic stresses in rice.* Biologia Plantarum, 2011. **55**(4): p. 625-633.
27. Nguyen, H.P., et al., *Methods to study PAMP-triggered immunity using tomato and Nicotiana benthamiana.* Molecular plant-microbe interactions, 2010. **23**(8): p. 991-999.
28. Trouvelot, A., *Mesure du taux de mycorhization VA d'un systeme radiculaire. Recherche de methodes d'estimation ayant une significantion fonctionnelle.* Mycorrhizae: physiology and genetics, 1986: p. 217-221.
29. Kim, M.G., et al., *Two< i> Pseudomonas syringae</i> Type III Effectors Inhibit RIN4-Regulated Basal Defense in< i> Arabidopsis</i>.* Cell, 2005. **121**(5): p. 749-759.
30. Bhat, K.S. and S. Nayak, *Flavin nucleotides in human lens: regional distribution in brunescent cataracts.* Indian journal of ophthalmology, 1998. **46**(4): p. 233.
31. Huang, P.Y., et al., *The Arabidopsis LecRK‐VI. 2 associates with the pattern‐recognition receptor FLS2 and primes Nicotiana benthamiana pattern‐triggered immunity.* The Plant Journal, 2014.
32. Pendle, A.F., et al., *Proteomic analysis of the Arabidopsis nucleolus suggests novel nucleolar functions.* Molecular biology of the cell, 2005. **16**(1): p. 260-269.
33. Fuhrman, L.E., et al., *Nucleolar proteins suppress Caenorhabditis elegans innate immunity by inhibiting p53/CEP-1.* PLoS genetics, 2009. **5**(9): p. e1000657.
34. Caillaud, M.-C., et al., *A Downy Mildew Effector Attenuates Salicylic Acid–Triggered Immunity in Arabidopsis by Interacting with the Host Mediator Complex.* PLoS biology, 2013. **11**(12): p. e1001732.
35. Galy, V., I.W. Mattaj, and P. Askjaer, *Caenorhabditis elegans nucleoporins Nup93 and Nup205 determine the limit of nuclear pore complex size exclusion in vivo.* Molecular biology of the cell, 2003. **14**(12): p. 5104-5115.
36. Bos, J.I., et al., *Phytophthora infestans effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1.* Proceedings of the National Academy of Sciences, 2010. **107**(21): p. 9909-9914.
37. Kamoun, S., *Groovy times: filamentous pathogen effectors revealed.* Current opinion in plant biology, 2007. **10**(4): p. 358-365.
38. Segonzac, C., et al., *Hierarchy and roles of pathogen-associated molecular pattern-induced responses in Nicotiana benthamiana.* Plant physiology, 2011. **156**(2): p. 687-699.
39. Bouwmeester, K., et al., *The lectin receptor kinase LecRK-I. 9 is a novel Phytophthora resistance component and a potential host target for a RXLR effector.* PLoS pathogens, 2011. **7**(3): p. e1001327.
40. Daudi, A. and J.A. O'Brien, *Detection of Hydrogen Peroxide by DAB Staining in Arabidopsis Leaves.*

Appendix

Appendix 1: Primers used in the study

TZ-attB1-F	**GGGGACAAGTTTGTACAAAAAAGCAGGCTTC**
TZ-attB2-R	**GGGGACCACTTTGTACAAGAAAGCTGGGTC**
TZ-RirT167520-F2	**AAAAAAGCAGGCTTCATGTTTTCAAAAGAGGATTTAGTACCTG**
TZ-RirT167520-R2	**CAAGAAAGCTGGGTCGGTTTGGAAACGAATTCTTTT**
TZ-RirT266090-F2	**AAAAAAGCAGGCTTCATGCAAGCATATAAGGCCACAATAAA**
TZ-RirT266090-R2	**CAAGAAAGCTGGGTCACAGTCAAAACTCCAATTACCAA**
TZ-RirT065700-F2	**AAAAAAGCAGGCTTCATGATTCCAGCAAGAATTCATAATGTTG**
TZ-RirT065700-R2	**CAAGAAAGCTGGGTCTCTGATTTTATTAATATCTCTTCTAATTTCA**
TZ-NbACRE132-F	**AGCGAAGTCTCTGAGGGTGA**
TZ-NbACRE132-R	**CAATCCTAGCTCTGGCTCCTG**
TZ-NbCYP71D20-F	**ACCGCACCATGTCCTTAGAG**
TZ-NbCYP71D20-R	**CTTGCCCCTTGAGTACTTGC**
NbPal-F	**TCGAGTTGCAGC CTAAGG**
NbPal-R	**TCTTCCAAATGCCTCAAGTC**
FY-NbEF1α-F	**TGGACACAGGGACTTCATCA**
FY-NbEF1α-R	**CAAGGGTGAAAGCAAGCAAT**

Appendix 2: Candidate effector gene sequences.

> RirT167520 (without signal peptide)
TTTTCAAAAGAGGATTTAGTACCTGTCCAAGAGATTAATCCTAAACCTCTTATAATCAAAAAAGTTGGACACAATAA
ATTGATTGCAGAAGTTACATGGGATGGAACACTTGAAAATGATAATGTACCGGTTAGAACCAAATTTAGATGTTTT
TCTGATGCTGTGACTGTTAAAGGTCCTAAGCATGCTTTATTCGGTGACCGTAAGGTCAATTTTGAAATAAAGGTTC
ACAAAAAGAATGTCAATGTGAAATGTCGATATGGGGTTCAAGATGGTTCCACTTTCATAAAAAGAATTCGTTTCCA
AACCTAA

> RirT266090 (without signal peptide)
CAAGCATATAAGGCCACAATAAAACAGACTTTGGGCGTTTTGTGCAGATTTTGGGTAGAAGATGCAAATCATAATC
GCATAGCTGGTGATGGAAAAAGACACTACCATACTTGTGATGGCGCAGATAAGGTCATTGAATTTGGCAATCAAC

AGTACTATATTGTTGCGAAAGTTGAAGCTAGTTTGCAGCTAGAAAAAGTCCGAGGTCCTTTCGATGGCGATCATTC
GTGTTTTTTTTATGGCACTATTGGTAATTGGAGTTTTGACTGTTAA

> RirT065700 (without signal peptide)

ATTCCAGCAAGAATTCATAATGTTGATAATGATGAACATCTTCATTTGAGAGCCTATACTAATGAAATTCAAAAGCG
TCATGAACCGTTTAAAATTATTCAAACCGATTATTTTAAACCTTTTGTACATTTACCTTTTACTAATAAACCAGTAACT
CGTTATTATCAATTGACATTAAAAAAAGTTAAATTATCTCCTGATGGTTTTGAAAGAACTGTTTGGAGTGTAAATGG
TCAATATCCTGCTCCAATTATTCGTGCAAATAAAGGAGATAGGATGATCATAAATGTTGAAAACAAATTTGGTGAT
CCAGCAGCCGTCCATTGGCATGGTGTGTTCCAACATGGTACAAATTGGTACGATGGAGTTCCAGGACAAACTCAAT
GTCCAATTCCAAATGATGTTTCATTTATTTACAATTTTACTACTGGAGACCAACACGGTACCTTTTGGTATCATTCTC
ATTTTATGGCACAATACGCTGATGGTTTACGAGGAGCATTAATTGTCCACGATCCAGATGATCCATATTTAAAAGA
ATATGATTATGAATATGTCATTACATTATCTGACTGGCATCATAGAACAACTGGTGAAATCTTACCAAATTTCATAT
CTCCAACTTATACTGGTAAACGACCCGTTCCTGATTCACCACTTTTGAGCGGTCGTGGTAGATATAACTGTAATGGG
GCTCCAGATGGATCTAAATGCAAACCAAATGCTCCATTGGCAGTTTATAATGTTAAAAAGAATAAAAAATATAGAT
TTCGTATAATCAATTCTGCAGCAGATGCTTTCTTCATATTCTCTATTGATGAACATAAATTAAAACTTATTGAATCAG
AAGGTATATATATTAAACCAACTATTATTGAAAAATTACCTATTAATGTTGGACAACGTTATTCTGTAATTGTTAAT
GCTGATCAACCGATTGGAAAGTATTGGATTCGTGCAACTATTGATAAAAGATGTGTCCTAATTAATAATGCGACAA
TTAATTTTAATTCTTCTATTGATTGGAATGGTCTTGGTATTCTTAAATATGAAGGATCAAAAAATGATAAACCTAAA
TCAAAAGAATTTCCCGAAAATTTCAAAATTTGTCGTGATCCTGATCCAAAACATTTGAAAACTCTTCAACCCGTTAC
AAAATATGATGGAAATGTTAGTGATTTTTTCAATATTACCGTTAAATTTCAAAGAGAAGGTGATGGAATAGTAAAA
GCTGTAATGAATAATAGTTCATTCATACCACAATTTAATGATCCAACCATAAATAAAATTATAAGACATATCCCACC
AGATGAATTACCAAAAGAACAAAATTCATTAATTTTTGATAATAAAAATGGTATAGTAGAAATTGCTTTATGGAAT
AATAATACTGATGAACATCCATTTCATATGCACGGACACGTTTTTGGCGTAATGTTTGTTGGTGAAAAAAATGAAT
ATCCTGATGAAAAAAAATATGATAAGAAAAATCCTGTAATTCGTGATAATGTGACCGTTCCTGGTTTTGGATATTT
GGTTATACGTTTCATCGCTGATAATCCTGGTATTTGGGCTTTTCATTGTCATATTGAATGGCATGTAGAACTTGGTA
TGGTTCTTCAATTAGTAGAACTACCTAGTATTTTAATGAATGAAACTATACCAAATGATGCTTCATCTTTATGTTTTA
AAAATGATTATCAAAAGAAGAGAAATCCTACGACACCATTTCATAATCGGGAAAGAATGTTTAATCCTGTTATAAT
TAATGAAATTAGAAGAGATATTAATAAAATCAGATAG

Appendix 3: Properties of the selected candidate effectors

Effector ID	Gene Length(bp)	arb1 - ARB_trimmed RNA-Seq-1 - RPKM	erm1 - HS1-4_trimmed RNA-Seq-1 - RPKM
RirT167520	**316**	**903.7735**	**0**
RirT266090	**277**	**427.8176**	**0**
RirT065700	**1810**	**264.574**	**1.796317**

Appendix 4: Agroinfiltration of *N. benthamiana* leaves

1. *Agrobacterium* strains with tested vectors were grown in 10 ml LB medium with antibiotics at 28 °C overnight in 50 ml tube.

2. *Agrobacterium* with vector p19 was grown in 10 ml LB medium with antibiotics (kanamycin 50 µg/ml, tetracyclin 5 µg/ml) at 28 °C overnight in 50 ml tube.
3. The tubes with grown bacteria were centrifuged 10 minutes at 4 000 *x g*.
4. The pellet was resuspended in 10 ml MMAi medium.
5. The OD (600) was measured on spectroscope. The suspensions were diluted to the final OD = 1.0 and they were incubated for 1 hour at RT.
6. 5 ml *Acrobacterium* with p19 vector (**not required;** alternatively 2B) was mixed with 5 ml *Agrobacterium* with tested vector.
7. The mixed *Agrobacterium* was injected to the leaves of *Nicotianatabacum* – 3 leaves of 2 plants with only some areas (for confocal microscopy), 4 whole leaves of 1 plant (for protein extraction). Injected areas and leaves were marked with permanent marker.
8. The infiltrated plants were grown 2 days and then the samples were collected and used for confocal microscopy/protein extraction/frozen at -80 °C.

MMAi medium (1 liter):

20 g sucrose

5 g MS basal salts

2 g MES

MQ to 1 liter

2 ml 1 M NaOH (pH 5.6)

1 ml 200mM acetosyringone

LB medium (1 liter):

10 g Tryptone

5 g Yeast extract

10 g NaCl

Adjust pH to 7.0

Appendix 5: ROS measurement adapted from Daudi et al. [40]

Materials and Reagents

1. 4-week-old *N. benthamiana* plants
2. DAB non-acidified powder (Sigma D8001)
3. Tween 20 viscous liquid molecular biology grade
4. Sodium phosphate (Na_2HPO_4) electrophoresis grade
5. Aluminum foil

Equipments

1. Magnetic stirrer and stirring bar
2. pH meter
3. 1ml needless syringes
4. 6-well microtiter plate
5. Dessicator
6. Shaker
7. Water bath

Procedure

1. Preparation of DAB staining solution

a. In 100 ml flask, add 50 mg DAB and 45 ml sterile H_2O for a final 1 mg/ml DAB solution.
b. Add small magnetic stirrer and reduce pH to 3.0 with 0.2 M HCl (to dissolve DAB).
c. Cover tube with aluminium foil since DAB is light-sensitive.
d. Add 25 µl Tween 20 (0.05% v/v) and 2.5 ml 200 mM Na_2HPO_4 to the stirring DAB solution.
2. Staining leaves with DAB solution
a. 100nM of flg22 and water were injected directly into two sides in one leaf via a 1 ml needleless syringe. Sample 3 leaves in one plant.
b. Leave the plants for 25min or 1h.
c. Sample the leaves after 25min or 1h by manually removing each leaf from the plant and placing in a 6-well microtiter plate.
d. Apply 5 ml of the DAB staining solution to the leaf or leaves in the well. Adjust the volume to ensure that leaves are immersed.
e. Ensure that the DAB solution is taken up by the leaf by gently vacuum infiltrating the leaves. This is achieved by placing the 6-well plates in a dessicator and applying gentle vacuum for 5 min.
f. Cover the 6-well plate with aluminium foil (since DAB is light-sensitive).
g. Place the plate on a standard laboratory shaker for 5 hours at 80 rpm shaking speed.
h. Following the incubation, remove the foil and replace the DAB staining solution with bleaching solution (ethanol:acetic acid:glycerol 3:1:1).
i. Place the 6-well plate carefully in a boiling water bath at 95°C for 15 mins. This will bleach out the chlorophyll but leave the brown precipitate formed by the DAB reacting with the hydrogen peroxide.
j. After 15mins of boiling, replace the bleaching solution with fresh bleaching solution and allow to stand for 30 mins. Samples at this stage can be stored at 4°C for up to 4 days with no detrimental effects observed in our hands.
k. Leaves can be directly visualized for DAB staining. Photographs are recommended on a plain white background under uniform lighting.

Recipes

1. DAB staining solution (Please see procedure 1)
2. 200 mM Na_2HPO_4 (pH > 6.8)
3. Bleaching solution: ethanol : acetic acid : glycerol = 3:1:1

Appendix 6: Seed Sterilization and germination

1. *M. truncatula* seeds are sterilized by incubating with concentrated sulphuric acid (H_2SO_4) for 10 min; chive seeds are sterilized by incubating with bleach for 10 min; Tomato and *N. benthamiana* were sterilized with 25% bleach for 20 min

2. Make five rinses with sterile water.

3. For *M. truncatula* seeds, add bleach to the rinsed seeds and incubate for 10 minutes, then rinse seven times with sterile water.

4. Place the seeds on Fahraeus agar and incubate upside down at 21°C .

Appendix 7: Fahraeus medium preparation

Stock solutions	**Stock concentration**		**Volume (ml)** **(for 1 liter of 1x medium)**	**Final Concentration**
Macronutrients	g/l	M		
$MgSO_4$ 7 H_2O	123.2	0.5	1.0	0.5 mM
KH_2PO_4	95.3	0.7	1.0	0.7mM
Na_2HPO_4, $2H_2O$	71.2	0.4	2.0	0.8mM
Fe-EDTA	20 mM		2.5	50 µM
Micronutrients				
$MnSO_4$, $CuSO_4$, $ZnSO_4$ H_3BO_3, Na_2MoO_4	1mg / ml Each		0.1 each	0.1 µg / l each

- Adjust pH to 6.5.
- Add 0.9% daishin agar. Autoclave
- CaCl2 (1 M stock solution) must be added to the medium after autoclaving and just before use, since it co-precipitates with phosphate ions.
- Sterile macronutrient stock solutions are stable at room temperature. Store at 4 °C once bottles have been opened.
- Prepare separate solutions of 5.6 g/l FeSO4 and 7.4 g/l Na2EDTA by heating at 50 °C and mix.
- Store micronutrient solutions at –20 °C.

Appendix 8: Hoagland medium

	Stock solution g/L	Working solution ml/L
Macro-elements		
KNO_3	101	9
$Ca(NO_3)_2x4H_2O$	236	9
$MgSO_4x7H_2O$	246	3.6
Micro-elements		1.8
H_3BO_4	1.95	
$MnCl_2x4H_2O$	0.36	
$ZnSO_4x7H_2O$	0.57	
$CuSO_4x5H_2O$	0.125	
$(NH_4)_2MoO_4xH_2O$	0.087	
$CoCl_2x6H_2O$	0.087	
Fe—EDTA		1.8
$FeSO_4x7H_2O$	5.56	
Na_2EDTA	7.45	

Appendix 9: Trypan blue staining used for staining arbuscular mycorrhizal fungus.

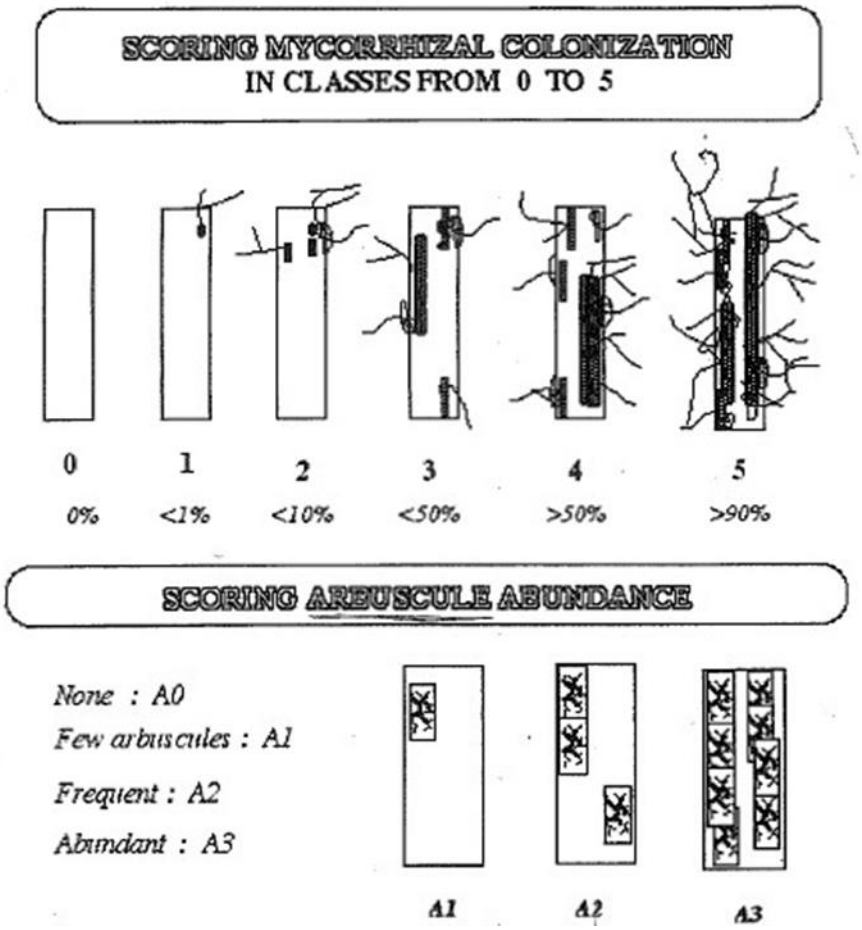
Stock solutions
- 10% (w/v) Potassium hydroxide
- 2% (w/v) Trypan blue
- Lactoglycerol solution (combine 300ml Lactic acid, 300ml Glycerol, 400ml double-distilled

Procedure
a. Submerge the roots in KOH (10%) and heat at 90°C for 20 min.
b. Decant the KOH and rinse the roots twice with deionized water.
c. Prepare Trypan blue staining solution by mixing 25ml of Trypan blue stock and 1000ml Lactoglycerol
d. Cover roots in Trypan blue staining solution and place at 90C for 3-5 mins (DO NOT LEAVE THEM LONGER or they will turn completely blue)
e. Decant the stain into a waste bottle and place the stained roots in glycerol.
f. Mount roots in glycerol on slides for microscopy (NOTE – Do not mount in lactoglycerol, it destroys the microscope!). The fungus will be stained blue and should be clearly visible within the roots. If the fungus has not stained enough, repeat the staining step. If the roots have stained too much, place them in lactoglycerol and they will destain.

g. The roots from the mock-inoculated controls should be stained and examined. These serve as a control and will indicate the quality of the growth conditions. Obviously they should not contain any mycorrhizal fungi.

Appendix 10: Mycorrhization scoring

Roots were cut into 1cm segments and stained using the Trypan blue staining and mounted on slides in glycerol.



Calculations

1. Mycorrhization frequency:

F% = 100 (N-n0)/N

n0 – Number of segments without infection;
N – Total number of segments observed (infected and non infected)

2. Intensity of mycorrhization:

M% = (95*n5+70*n4+30*n3+5*n2+n1)/N

n5, n4 … are the numbers of segments assigned to the class 5, class …

(Mycorrhization scoring continued…)

3. Arbuscule % within mycorrhized parts:

a% = (100*mA= + 50*mA- +10*mA)/100

mA=, mA- and mA were calculated as follows:
mA- = (95*n5A- +70*n4A- +30*n3A- + 5* n2A- +n*1A-)* F%M%*(N-n0)

5A-, n4A- are the number of segments assigned to the class 5A-, 4A- …
mA = calculated in the same way as mA-

4. Presence of arbuscules in all the root apparatus:

A% = a%*M%/100