Reverse genetic study of four *Medicago truncatula* defence genes to elucidate involvement in arbuscular mycorrhizal symbiosis

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ABSTRACT

The arbuscular mycorrhizal (AM) symbiosis is a mutualistic relationship between plants and AM fungi that increases global plant nutrition. Although the benefits of forming the AM symbiosis is significant for around 80% of terrestrial plants, little is known about the molecular pathways involved in facilitating the interaction. In this thesis, we sought to understand the involvement of plant defence-associated genes in regulating the beneficial arbuscular mycorrhizal (AM) symbiosis. Based on GeneChip and RNA sequencing data, we identified four genes in the model legume *Medicago truncatula* with increased expression during AM symbiosis that are predicted to encode proteins associated with host defence pathways. We hypothesized that these proteins are involved in regulating AM symbiosis, either by: a) mediating the suppression of defence pathways to enable fungal colonization of root cells, or alternatively: b) by restricting the growth of the symbiont to prevent over-proliferation in host tissues. Two of the four genes encode putative Toll/inkerleukin-1 nucleotide-binding leucine rich repeat (TIR-NB-LRR) receptors, a group of intracellular receptors that allow plants to identify non-self-microorganisms (Zhai *et al.*, 2011). One gene encodes a leucine-rich repeat (LRR) receptor kinase, a transmembrane receptor that activates signal transduction pathways upon perception of microbe associated antigens (Torii, 2004). The fourth gene encodes an enhanced disease susceptibility-like (EDS1-like) defence protein that mediates signals involved in resistance (Wang *et al.,* 2014). To screen the involvement of these defence genes of interest, we used a reverse genetics approach to compare the mean root length colonized of mutant knockout *Medicago truncatula* plants inoculated with *Rhizophagus irregularis* to wildtype plants. Results showed that the TIR-NB-LRRs had no consistent role in the AM symbiosis, while the the EDS1-like gene and the LRR-kinase displayed promising preliminary results for a potential role in regulating the mutualism.

RESUME

La symbiose mycorhizienne arbusculaire (AM) est une relation mutualiste entre les plantes et les champignons AM qui augmente la nutrition globale des plantes. Bien que les avantages de la formation de la symbiose AM soient importants pour environ 80% des plantes terrestres, on en sait peu sur les voies moléculaires impliquées dans la facilitation de l'interaction. Dans cette thèse, nous avons cherché à comprendre l'implication des gènes associés à la défense des plantes dans la régulation de la symbiose mycorhizienne arbusculaire (AM) bénéfique. Sur la base des données de séquençage de GeneChip et d'ARN, nous avons identifié quatre gènes dans la légumineuse modèle Medicago truncatula avec une expression accrue au cours de la symbiose AM, qui devraient coder des protéines associées aux voies de défense de l'hôte. Nous avons émis l'hypothèse que ces protéines sont impliquées dans la régulation de la symbiose AM, soit en: a) médiatisant la suppression des voies de défense pour permettre la colonisation fongique des cellules racinaires, ou encore: b) en limitant la croissance du symbiote pour empêcher la prolifération excessive chez l'hôte tissus. Deux des quatre gènes codent pour des récepteurs putatifs Toll / inkerleukin-1 nucleotide-binding leucine rich repeat (TIR-NB-LRR), un groupe de récepteurs intracellulaires qui permettent aux plantes d'identifier les non-auto-micro-organismes (Zhai et al., 2011) . Un gène code pour une kinase de récepteur de répétition riche en leucine (LRR), un récepteur transmembranaire qui active les voies de transduction du signal lors de la perception des antigènes associés aux microbes (Torii, 2004). Le quatrième gène code pour une protéine de défense de type susceptibilité améliorée à la maladie (EDS1) qui médie les signaux impliqués dans la résistance (Wang et al., 2014). Pour cribler l'implication de ces gènes de défense d'intérêt, nous avons utilisé une approche de génétique inverse pour comparer la longueur moyenne des racines colonisées de plantes mutantes knockout Medicago truncatula inoculées avec Rhizophagus irrégulis à des plantes de type sauvage. Les résultats ont montré que les TIR-NB-LRR n'avaient aucun rôle cohérent dans la symbiose AM, tandis que le gène de type EDS1 et la LRR-kinase affichaient des résultats préliminaires prometteurs pour un rôle potentiel dans la régulation du mutualisme.

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ABBREVIATIONS

AM - Arbuscular mycorrhizal
Bp – Base pairs
DMI – Does not make infections
DPI - Days post inoculation
EDS1 – Enhanced disease susceptibility 1
ETI - Effector triggered immunity
FST - Flanking sequence tags
MtBCP1 – *Medicago truncatula* Blue Copper Binding Protein 1
CTAB - Cetyl trimethylammonium bromide
GOI - Gene of interest
LB - Lysogeny
GS - Gene-specific
GUS - Beta-glucuronidase
HR - Hypersensitive response
Kb – Kilobases
KOH - Potassium hydroxide
TIR-NB-LRR – Toll/inkerleukin-1 nucleotide-binding leucine rich repeat
LCOs - Lipooligosaccharides
NDR1 - Not-race specific disease resistance protein
PAD 4 - Phytoalexin deficient 4
RT-PCR - Reverse transcriptase polymerase chain reaction
SA – Salicylic acid
Myc factor - Mycorrhizal factor
MTI - Microbe associated molecular pattern-triggered immunity
P – Phosphorus
MAMP – Microbe associated molecular pattern
N - Nitrogen
qPCR - Quantitative polymerase chain reaction
R – Resistance
CC- Coiled coil
SAG101 - Senescence associated gene 101
SiRNAs - Short interfering RNAs
SP7 - Secreted protein 7
SYM - Symbiosis
UROP - Undergraduate research opportunity program
LRR-kinase – Leucine rich repeat receptor kinase

CHAPTER ONE

Introduction

1.1. Overview of the arbuscular mycorrhiza

Throughout a plant's life cycle, it will engage in interactions with countless micro-organisms that span a spectrum from pathogenic to mutualistic. Accordingly, plants have evolved sophisticated mechanisms to distinguish friend from foe: supporting the development of beneficial symbioses, whilst suppressing the growth of potential pathogens. Moreover, even within the context of a beneficial association between plant and microbe, the host must strictly regulate the growth of its partner, as these microsymbionts frequently impose a measurable metabolic burden on their host (Gutjahr & Parniske, 2017). While plant biologists have made significant inroads in improving our understanding of the ways by which plants defend themselves against pathogenic microorganisms, our knowledge of how host plants control and regulate the growth of beneficial symbionts is poor.

Within the context of beneficial interactions, the plant-arbuscular mycorrhiza is amongst the most globally significant (Harrison, 2005). It has been speculated that plant terrestrialization was enabled by AM fungi around 450 million years ago, perhaps explaining why 80% of angiosperms form this symbiosis (Harrison, 2005). In this mutualism, arbuscular mycorrhizal (AM) fungi proliferate within host roots and act as biofertilizers by absorbing soil nutrients and transferring them to host plants in exchange for fixed carbon (Koide & Elliott, 1989). Of notable importance is the transfer of otherwise unavailable soil phosphorus (P) to host plants by AM fungi. Globally, P poor environments are widespread and limiting to plant growth making it particularly crucial that the availability of this nutrient is improved by the AM symbiosis (Gerdemann, 1968; Harrison, 2005). In addition to increased nutrient acquisition, AM fungi alleviate drought stress, contribute to greater soil stability, and heighten defence responses

against pathogens (Koide & Elliott, 1989; Berruti *et al*., 2015) making the ecological consequences of the relationship substantial (Harrison, 2005).

Despite the AM symbiosis being one of the most common and ancient symbioses (Taylor *et al*., 1995) much is still unknown about the symbiotic machinery (Bravo *et al*., 2016). Understanding the underlying genetic mechanisms involved in facilitating the arbuscular mycorrhiza could help improve plant growth while reducing fertilizer use, which come at great economic and environmental costs (Berruti *et al*., 2015). The current project has been undertaken to fill gaps in the knowledge by testing four *Medicago truncatula* defence genes for their involvement in the root colonization success of the arbuscular mycorrhizal fungus *Rhizophagus irregularus*.

1.2. Fertilizer use

Nitrogen and phosphorus are essential nutrients required for the development of plant proteins and nucleic acids (Conley *et al*., 2009). Due to their scarcity in soil, industrial fertilizer applications are widely used to supply food crops with these nutrients. However, aquatic ecosystems are negatively affected by run-off from terrestrial fertilizer applications (Conley *et al*., 2009). N and P in particular cause eutrophication, a phenomenon that reduces water quality and can result in human and animal health concerns (Conley *et al*., 2009). The requirement for fertilizer coupled with the negative downstream ecological effects makes management difficult in the context of agricultural practices. A proposed solution to this issue is the utilization of biofertilizers as an alternative to exogenous fertilizer applications (Borde, 2009; Berruti *et al*., 2015).

Eutrophication of lakes, caused by nutrient run-off, results in cyanobacterial blooms, which create toxins, and decrease oxygen availability in the water (Conley *et al*., 2009). The toxins created by the cyanobacteria can cause illness and death in humans if contaminated water is consumed (Codd *et al*., 2006). Additionally, the loss of economically important fish is common in eutrophicated lakes. For example, the loss of the high-value lake whitefish (*Coregonus clupeaformis*) in Lake Erie in the 1960's was attributed to eutrophication caused by phosphorus. This issue was so consequential that Lake Erie was considered the "Dead Sea of North America" during that time (Ludsin *et al*., 2001).

A study on small lakes in Northwestern Ontario by Schindler *et al*., (1974) found that the addition of nutrients caused significant eutrophication. They concluded that the addition of P in particular was the key driving factor of eutrophication of the experimental lakes. In fact, when nitrate and sucrose were added to the lakes in the absence of phosphorus, eutrophication did not occur. Although studies such as these have resulted in management strategies to control nutrient inputs, fertilizer applications are still widespread and often necessary in agriculture systems (Conley *et al*., 2009). One practical solution used to address this problem is the addition of AM fungi to field plots to exploit the nutrient transfer in the AM symbiosis (Zhang *et al*., 2016; Tawaraya *et al*., 2012).

In paddy fields in northeast China, N run-off caused by anthropogenic activities caused pollution in a major source of freshwater in the area (Zhang *et al*., 2016). To alleviate this contamination, the researchers reduced N fertilizer use by 20% and added the AM fungi *Glomus mosseae* in a field study. They found that N run-off was reduced by 27% compared to the control, with no addition of AM fungi (Zhang *et al*., 2016). Similarly, the addition of the AM fungi *Glomus R-10*, to welsh onion (*Allium fistulosum* L.) in greenhouse trials showed that

inoculated plants required around 3x less P than their uninoculated counterparts (Tawaraya *et al*., 2012).

These studies, and others (Durani *et al*., 2018; Kumar *et al*., 2016; Oliveira *et al*., 2016) , show the potential of AM fungi to act as biofertilizers thereby reducing commercial fertilizer inputs into terrestrial ecosystems. Discovering the role of host plant genes in regulating the AM symbiosis has the potential to unlock the power of this mutualism (Berruti *et al*., 2015). Implications of genetic research on the plant-AM symbiosis includes improved water quality (Codd *et al*., 2006), reduced mortality of commercially important fish (Ludsin *et al*., 2001) and improved plant health (Zhang *et al*,. 2016).

1.3. General information on arbuscular mycorrhizal fungi

AM fungi are part of the phylum glomeromycota, the most globally widespread of all fungal phyla (Harrison, 2005), despite being composed of only 334 known species (Schüßler, 2020). As plant-derived carbon is required for AM fungi to sustain life, they are defined as obligate symbionts (Ropars, 2016). To their benefit, a low plant specificity allows one AM fungal species to form mycorrhizal symbiosis with numerous terrestrial plant species (Sanders, 2003). Conversely, host plants appear to prefer certain AM fungal species based on their quality (Grman, 2012).

AM fungal spores are multi-nucleate units formed in soil by the extraradical mycelium of a mature AM fungus (Seddas *et al*., 2009). Once signalling hormones from the host plants are received, the dormant spores germinate, the newly formed hyphae move towards the host plant, a hyphopodium is formed, and the cell is penetrated (Seddas *et al*., 2009). The mycelium

proliferates intra and intercellularly, and eventually arbuscules are formed in the root cortical cells through hyphal differentiation (**Figure 1.1**) (Seddas *et al*., 2009).

The microscopic size of AM hyphal strands allows for access to soil pores that are unavailable to roots (Allen, 2011). This, coupled with the extended range of the mycelium network increases the surface area by which nutrients are acquired (Allen, 2011). Through unclear mechanisms, soil nutrients are transferred from the extraradical mycelium to the arbuscules where the exchange of commodities occurs (Field *et al*., 2015).

The AM fungus *Rhizophagus irregularis* is multi-nucleate with thousands of nuclei being shared within the hyphae through anastomosis (Ropars, 2016). The predicted nuclear genome size of this species is around 130-148 Mb depending on the isolate (Chen *et al*., 2018) however, the genome organization including the number of chromosomes is currently unknown (Hijri *et al*., 2007). A large roadblock in the study of these organisms is the inability to transform the genome likely due to them being obligate symbionts that are multinucleate (Ropars, 2016). This makes reverse genetic studies on the AM fungal genome impossible at the moment. Consequently, research focusing on the symbiotic machinery involved in the plant-AM symbiosis is biased towards the plant genome. The experiments conducted in this thesis use *R. irregularis* as the fungal symbiont due to the access to commercial spores.

1.4. Molecular plant – AM fungal interactions

Despite the importance of the plant-AM symbiosis, little is known about the plant genes and pathways involved in facilitating this interaction (Bravo *et al*., 2016). In general, it is clear that AM fungi largely affect the gene expression of host plants according to genetic databases hosting RNA-seq, qPCR and RT-PCR expression data, such as the *Medicago truncatula* Gene

Expression Atlas (Benedito *et al*., 2008). The plant genes currently known to play a role in the symbiosis have in large part been discovered via commonalities with the rhizobia-legume symbiosis (Parniske, 2008; Genre *et al*., 2013). The latter association is evolutionarily more recent, and significantly more information is available on the molecular pathways involved (Kistner & Parniske, 2002). Reverse genetics is another common technique employed to discover symbiosis-related genes whereby mutant plants with null genes are observed for a phenotype (Bravo *et al*., 2016).

A few key studies have advanced the field by identifying critical genes and pathways in the AM symbiosis using these methods. The described pathways have revealed that host plants and AM fungi have pre-contact communication, that AM fungi are recognized by host plants, and that plant cells undergo many physical alterations to accommodate the interaction (Berruti *et al*., 2015; Seddas *et al*., 2009; García-Garrido & Ocampo, 2002; Buee *et al*., 2000). In this section, a review of pathways and plant genes involved in the AM symbiosis will be discussed, and gaps in the knowledge will be identified.

1.4.1. Rhizosphere

The rhizosphere is a limited area of topsoil that is affected by root secretions and is habitat to the greatest diversity of species in the world (Berendsen, 2012). Per gram of soil, there is estimated to be an astounding 10^{11} microbes (Berendsen, 2012). The influence of the rhizosphere on plants is so significant that the microbial DNA contained in it is referred to as a plants second genome (Berendsen, 2012). The microbial diversity and composition of the rhizosphere greatly influences plant health positively, negatively or neutrally. To successfully navigate the barrage of microbes in the soil, plants have cultivated sophisticated methods to

interact with this rich environment (Laffont *et al*., 2015). With the incredible diversity of plants and microbes in the rhizosphere, it comes as no surprise that upon perception of a soil fungus, the interactions within the plant cell and between the plant and microbe are complex and intertwined (Laffont *et al*., 2015).

1.4.2. Pre-contact communication

Through the use of signalling molecules, host plants and AM fungi communicate in the rhizosphere before they ever come into physical contact. This is the first step in the formation of the symbiosis (Rich *et al*., 2017). Root exudates from *M. truncatula* cause significantly greater hyphal branching of AM fungi compared to non-host root exudates (Buee *et al*., 2000). Conversely, AM fungal signalling molecules are involved in root branching and transcription factor activation in host plants (Maillet *et al*., 2011; Rich *et al*., 2017).

Strigolactones are plant signalling molecules that induce germination and hyphal branching of AM fungal spores in the rhizosphere (López-Ráez *et al*., 2011). Although it is unclear by which mechanism(s) AM fungi recognize strigolactones, the end result of extensive hyphal branching is often necessary to increase the probability of connecting with the host roots (López-Ráez *et al*., 2011). It follows that host plants with diminished strigolactone synthesis have significantly less colonization by AM fungi (Gómez-Roldán *et al*. 2008). Interestingly, strigolactones are exuded in greater quantities by hosts when they are P deficient, essentially recruiting the aid of these microsymbionts to increase nutrition (López-Ráez *et al*., 2011). This not only allows plants to enlist support when necessary, but also signals to the AM fungal spores that there is an impoverished host plant in the vicinity, allowing them to save germination and hyphal branching for the appropriate time (López-Ráez *et al*., 2011).

As a complement to strigolactones, there are mycorrhizal factors (Myc factors), signalling molecules released by AM fungi that trigger symbiosis-related molecular pathways in host plants (Akamatsu *et al*., 2016). Key Myc factors are lipooligosaccharides and chitin oligomers which similar to the effect of strigolactones on spores, increase branching and growth in host roots (Maillet *et al*., 2011; Genre *et al*., 2013). In *M. truncatula*, these molecules also initiate the common symbiosis signalling pathway (SYM pathway) (MacLean *et al*., 2017) subsequently activating calcium spiking (Genre *et al*., 2013) and a family of GRAS transcription factors (Hohnjec *et al*., 2015), that signal hundreds of genes potentially involved in early symbiosis development (Rich *et al*., 2017).

The does not make infections (DMI)1 (nuclear channel protein), DMI2 (LRR receptor-like kinase) and DMI3 (nuclear protein kinase) genes have been identified as a key proteins in perceiving symbiosis signals (Genre *et al*., 2013) and inducing a nuclear calcium spike that is required for activation of downstream responses involved in AM symbiosis (Oldroyd and Downie, 2006). As described by Seddas *et al*. (2009), *M. truncatula dmi1*, *dmi2*, and *dmi3* mutants prevent fungal penetration at the root epidermis due to irregular growth or shape of the appressorium. This evidence suggests that pre-contact communication dictates the compatibility of AM fungi and host roots. Despite the progress made in uncovering the pre-contact communication pathways, the majority of the genes that are activated by Myc-factors have not been characterized, leaving large gaps in this area (Rich *et al*., 2017).

The success of the AM symbiosis relies on the molecular dialogue and crosstalk occurring prior to physical interactions (Seddas *et al*., 2009; Harrison, 2005). Evidently, the interactions between plants, AM fungi, and the environment is highly complex with many different signalling pathways and molecular components of which we have only scratched the

surface. It is clear that there is a large effort and investment by both symbionts to facilitate this beneficial interaction. Using a reverse genetics approach, additional host plant genes involved in AM symbiosis can be discovered to fill the large gaps in the knowledge.

1.4.3. Recognition of AM fungi

A key component of a successful plant-AM interaction is the recognition of AM fungi by the host plants (Bravo *et al*., 2016). The mechanism used to identify and distinguish these beneficial fungi from pathogenic organisms in the rhizosphere is largely unknown. Although pre-contact communication plays a role in expressing symbiosis genes in host plants, upon physical contact between the organisms, there is still much to learn in fully understanding the components required for a successful molecular connection (MacLean *et al*., 2017). Identifying essential recognition proteins at this stage in the molecular plant-AM fungal interaction is the focus of this thesis.

In response to AM fungal contact, host plants suppress defence gene expression suggesting that they are recognized as beneficial microbes (García-Garrido & Ocampo, 2002). For example, the initial contact between *M. truncatula* and *R. irregularis*, the two species used in this thesis, produces only a transient increase in the expression of defence compound pre-cursors (García-Garrido & Ocampo, 2002). Additionally, when AM fungi first come into contact with plants, there is induction and subsequent suppression of salicylic acid (SA), a phytohormone involved in defence responses (Kapulniki *et al*., 1996). These defence mechanisms are identical to those used against pathogens, however, they are too weak to elicit a response that will prevent the AM fungi from colonizing the cell (Kapulniki *et al*., 1996).

The low defence response intensity during establishment of AM symbiosis implies that consenting host plants are able to recognize AM fungi as separate entities from pathogens and may be down-regulating their defence strategies to align with the beneficial nature of these fungi. Alternatively, AM fungi may have mechanisms similar to those used by pathogens to overcome the host plant defence system. In this case, it is possible that the fungi are silencing the innate defence response of the plants in order to colonize the cells (Kloppholz *et al*., 2011). Although this hypothesis has not been confirmed, effector proteins have been postulated as a mechanism through which this could occur (Kloppholz *et al*., 2011). This thesis aims to address this gap by screening defence-related genes similar to those involved in pathogen recognition for involvement in the AM symbiosis.

1.4.4. Preparation for colonization

After pre-contact communication and recognition of AM fungi, plants undergo significant cellular changes to accommodate the occupancy of cortical cells. Notably, an intracellular symbiosis interface with a large surface area is formed, allowing for the seamless exchange of carbon and nutrients (Balestrini & Bonfante, 2014). To prepare for this interface, there are several morphological changes that occur (Balestrini & Bonfante, 2014). These include the movement of the host plant nucleus towards the site of contact, the central vacuole being fragmented (Genre and Bonfante, 2005) and a pre-penetration apparatus that guides the hyphae inwards (Siciliano *et al*., 2007). The pre-penetration apparatus in particular suggests that the host plant has a high degree of control over the fungal partner since this is the only point of entry into the cell for the hyphae (Siciliano *et al*., 2007). Morphologically, this is an incredible transformation that allows the microsymbiont to live inside the host and inhabit a large volume

of their cells (Balestrini & Bonfante, 2014). Such drastic changes may further signify that the plants not only correctly recognize the species, but that they welcome their inhabitance.

1.4.5. Maintaining the mutualism

Once infection is established, there are techniques employed by the plant to control the level of growth of AM fungi in the roots (Gutjahr & Parniske, 2017). For example, host plants determine the quality of AM fungal partners based on the level of nutrients being transferred and accordingly allocate carbon to better performing individuals (Ji & Bever, 2016; Grman, 2012; Kiers *et al*., 2011). This was shown by Kiers *et al*., (2011) using staple isotope probing, which tracked the carbon being transferred to AM fungal species of differing qualities. Ji & Bever (2016) have also shown that the amount of phosphorus supplied by AM fungi determines the amount of carbon that is provided by host plants in low phosphorus conditions. When an AM fungal species provided more phosphorus to plant roots, that root segment received more carbon than the root segment receiving less phosphorus (Ji & Bever, 2016). Although the mechanism used to identify the quality of the AM fungal partners in this context is unknown, the fitness of the higher-quality individuals increases, while the fitness of "cheaters" decreases, thereby stabilizing the mutualism (Grman, 2012).

Furthermore, Bravo *et al*. (2016) used a reverse genetics approach to identify the acyl carrier protein mutant *fatm* (fat required for AM symbiosis) which resulted in significantly reduced root colonization and incapacitated arbuscule development in *M. truncatula* mutants (Bravo *et al*., 2016). The reduced transfer of fat to AM fungi appears to affect the morphology of the fungus in the cortical cells (Brands *et al*., 2018). These are a few of the many requirements

for the proper maintenance of the symbiosis. More work should be done to identify other key players in the long-term functioning of the interaction.

1.5. Resistance genes

Unlike plant – AM interactions, the mechanisms plants use to protect against economically important pathogenic fungi such as *Fusarium graminearum*, the causal agent of Fusarium Head Blight, are very well studied (Kazan & Gardiner, 2018). Although plants may appear to be passive entities on the surface, they are quite accomplished in defending themselves from disease. Due to the devastation a pathogen can have on plant health, a significant portion of energy is invested into preventing tissue colonization.

Oftentimes, beneficial, neutral and pathogenic fungi are described as separate entities by biologists. However, microbes in the rhizosphere span a continuous spectrum from beneficial to pathogenic (Harrison, 2005) depending on the environmental conditions, species of microbe, and plant host. It is worth noting that although AM fungi are beneficial to most plants, they can be pathogenic if colonization is not strictly controlled by the plant's immune system (Gutjahr & Parniske, 2017).

To elicit a defence response, plants must first recognize that there is a microbe which is accomplished in two ways. Firstly through recognizing the presence of extra-cellular microbe associated molecular patterns (MAMPs) that are conserved in microbes but not present in plants. This is accomplished using leucine rich repeat receptor (LRR) kinases (Torii, 2004). However, successful pathogens have evolved to overcome this roadblock by secreting effector proteins into host plants which, if successful, prevent a defence response (Staskawicz *et al.*,1984). To combat these effectors, plants developed a second layer of defence where intracellular receptors,

nucleotide-binding leucine rich repeat (NB-LRRs) kinases recognize effectors and elicit an appropriate defence response (Duxbury *et al*., 2016).

Based on evidence from the studies outlined above, it is possible that the defense mechanisms used by plants to protect against pathogens are also used to interact with AM fungi (**Figure 1.2**) (Zhai *et al*., 2011). However, the knowledge of the specific host plant proteins involved is severely lacking. In this thesis, we are broadly looking to close some of these gaps through the study of defence-related proteins and their involvement in the AM symbiosis.

1.5.1. Leucine rich repeat receptor kinase genes

Leucine rich repeat receptor (LRR) kinases are transmembrane proteins that in addition to various biological processes, recognize foreign microbes via microbe associated molecular patterns (MAMPs) (Torii, 2004). LRR-kinases are ubiquitous in the plant world for their indispensable ability to recognize non-self-organisms and are very successful in protecting most plants from most microbes (Torii, 2004). For example, recognition of chitooligosaccharides, a component of chitin, elicits a defence response that degrades fungal cell walls and protects plants from pathogens (Wan *et al*., 2008).

Recognition of MAMPs by LRR-kinases produces a signal that confers the appropriate rapid, intermediate, or long-term defence response (**Figure 1.3.**) (Zipfel *et al*., 2010). Examples of such responses include a calcium spike, the release of antimicrobial reactive oxygen species, and the induction of defence related genes (Boller & Felix, 2009). This is the first line of defence against microbes and the successful evasion of colonization using this process is aptly named MAMP-triggered immunity (MTI) (Zipfel *et al*., 2010). Despite the success of this pathway in protecting plant tissues, microbes are not idle bystanders to the defence artillery of plants, in fact,

quite the opposite. As a response to MTI, pathogenic microorganisms secrete effector proteins whose sole purpose is by-passing these basal defence mechanisms (Thulasi Devendrakumar *et al*., 2018).

Based on expression data from the *Medicago truncatula* Gene Expression Atlas, we identified a gene encoding an LRR-kinase (**Table 1.1**) that was expressed in *M. truncatula* roots infected with *R. irregularis* at 28 days post inoculation (dpi) and not in the uninoculated control in the same conditions. Considering the role of these defence genes in identifying non-self-organisms, we tested the involvement of this gene in the mean root length colonized.

1.5.2. Nucleotide-binding leucine rich repeat genes

The second method of recognizing pathogens is accomplished by identifying and inhibiting effectors (Duxbury *et al*., 2016). This is achieved through a key resistance protein family named nucleotide-binding leucine rich repeat (NB-LRR) receptors (Zhai *et al*., 2011). Making up one of the largest plant protein families, there are at least 333 NB-LRR genes that are hypothesized to be non-redundant in *M. truncatula* (Ameline-Torregrosa *et al*., 2008).

If a NB-LRR perceives an effector protein either directly (receptor ligand hypothesis) (**Figure 1.4**) or indirectly (guard hypothesis and decoy hypothesis) the plant recognizes that a pathogenic microbe is trying to overcome MTI (Duxbury *et al*., 2016). As a result, a stronger and more rapid defence response called effector triggered immunity (ETI) is enacted which often results in the hypersensitive response (HR): programmed cell death at the site of infection (Lolle *et al*., 2020). This is a drastic measure taken by plants to prevent disease. If an effector is recognized, the pathogen is not able to colonize the plant tissues. Oppositely, if the effector is not recognized, then the pathogen is able to successfully colonize the plant tissues potentially

resulting in disease symptoms (Adlung & Bonas, 2017). These plant-microbe interactions are labelled incompatible and compatible, respectively (Staskawicz *et al*.,1984).

NB-LRRs are categorized based on their N-terminal domains which can either be the coiled-coil (CC) domain or a Toll/interleukin-1 (TIR) domain (Qi and Innes, 2013). The NB-LRR structure determines which independent signalling pathway is activated. While TIR-NB-LRRs signal upstream of the EDS1 (enhanced disease susceptibility 1) protein, CC-NB-LRRs signal upstream of the NDR1 (non-race specific disease resistance protein 1) protein (Aarts *et al*., 1998). These two downstream proteins activate independent signalling pathways thus the N-terminal domain of the NB-LRRs is important for determining which defence pathway is activated during ETI (Aarts *et al*., 1998).

Identifying NB-LRRs in the AM symbiosis could be a ground breaking development in the plant-microbe field. This is an important piece of information for understanding how the two partners interact to make their relationship compatible. Specifically, whether host plants use NB-LRRs to facilitate the interaction between the plant cells and the AM fungi has been neither confirmed nor refuted (Antolín-Llovera *et al*., 2014). In AM fungi, there have been two effectors identified to date: SP7 (secreted protein 7) (Kloppholz *et al*., 2011) and RiCRN1 (Voß *et al*., 2018). SP7 has been shown to interact with a transcription factor in host roots, ultimately leading to increased root colonization rates (Kloppholz *et al*., 2011), while RiCRN1 is suggested to localize in the nucleus, and downregulation results in impaired arbuscule development (Voß *et al*., 2018).

In this thesis, we consider whether plant cells use TIR-NB-LRR's to not only recognize pathogens and initiate a defence response, but also to recognize beneficial AM fungi. As discussed above, AM fungi, although generally beneficial, are still foreign microbes that need to

be strictly regulated. Therefore, it is possible that the plant cells utilize similar methods in regulating the AM symbiosis as they do in regulating plant pathogens. The increased expression of two genes encoding TIR-NB-LRRs exclusively during AM symbiosis (**Table 1.1**) suggests that there may be a role of these proteins in this mutualism.

1.5.3. Enhanced disease susceptibility 1 protein

Plant defence responses are energetically costly and are therefore strictly controlled using signal cascades (Wang *et al*., 2014). To invest energy appropriately, the response to a virulent pathogen must be different than the response to an avirulent one. Accordingly, both basal resistance and effector-triggered immunity are critically important for the health of a plant (García *et al*., 2010). The enhanced disease susceptibility 1 (EDS1) lipase-like protein plays an important role in organizing the enormous number of intracellular signals and producing the appropriate immune response (García *et al*., 2010). This defence protein is shuttled between the nucleus and the cytoplasm in a coordinated fashion depending on the signal input (García *et al*., 2010). It functions both independently and in conjunction with the PAD4 (phytoalexin deficient 4) and SAG101 (senescence associated gene 101) co-regulators (García *et al*., 2010). Null mutants of this protein have an enhanced susceptibility to infection which suggests that the protein is required for a successful defence response (Wang *et al*., 2014).

In the nucleus, EDS1 positively regulates effector-triggered immunity downstream of TIR-NB-LRRs (Falk *et al*., 1999) and is required to activate the disease response pathway that is derived from TIR-NB-LRR signalling (García *et al*., 2010). In *Arabidopsis thaliana*, EDS1 was found to be positioned downstream of TIR-NB-LRR proteins and upstream of defence gene transcription factors (Feys *et al*., 2005), and the HR (Wang *et al*., 2014; García *et al*., 2010).

Additionally, EDS1 has been postulated as an important regulator of basal immunity since the accumulation of nuclear EDS1 coincides with the upregulation of SA biosynthesis genes which are broadly required for defence against virulent biotrophs and hemi-biotrophs (García *et al*., 2010). Although the mechanism by which EDS1 influences transcription levels is unclear, yeast-two-hybrid analysis have shown an interaction with transcription factors (García *et al*., 2010).

The function of EDS1 in the cytoplasm is not as well characterized, but the careful balance of this protein between the nucleus and cytoplasm points to a unique role (García *et al*., 2010). García *et al* (2010) suggest that signals from the EDS1 complexes in the cytoplasm may be required for a thorough immune response.

In this thesis, we identified a gene encoding an EDS1-like protein that is upregulated during AM symbiosis (**Table 1.1**). The funnelling of multiple signals through EDS1 (García *et al*., 2010) makes this gene a suitable candidate for determining if a successful AM symbiosis relies on suppression of TIR-NB-LRR and/or basal immunity defence signalling. The response that EDS1 confers against pathogens would not be beneficial to the plant-AM symbiosis, particularly when considering the HR and SA accumulation. Accordingly, upon host plant perception of AM fungi, HR is lacking and SA accumulation is repressed (Kapulniki *et al*., 1996). As EDS1 appears to have great control over these responses (Feys *et al*., 2005), it is worth investigating if there is a link between AM fungi perception, TIR-NB-LRRs, EDS1, SA suppression, and the lack of an HR. Particularly, if there a distinct signalling pathway downstream of an EDS1-like protein that negatively regulates defence signals upon interaction with AM fungi. This thesis broadly aims to address this question by screening an EDS1-like mutant in *M. truncatula* for its involvement of mean root length colonization by *R. irregularis*.

1.6. *Medicago truncatula*

Medicago truncatula is a legume species closely related to alfalfa (*Medicago sativa*), one of the most important global forage crops (Cheng *et al*., 2011). Due to the relatively small diploid genome of around 500 Mbp, *M. truncatula* is a model legume plant that is commonly used for mycorrhizal genomics studies (Cheng *et al*., 2011; Krishnakumar *et al*., 2015). The rapid life cycle, self-fertilization and access to online resources add to the favourability of *M. truncatula* for genomic studies (Krishnakumar *et al*., 2015). Additionally, *M. truncatula* also forms a symbiosis with nitrogen fixing bacteria (Benedito *et al*., 2008). Since the pathways involved in the rhizobia-legume symbiosis overlap with those in the AM fungi-legume symbiosis, gene discoveries have been made using orthologues (Kistner & Parniske, 2002).

A comprehensive *M. truncatula* Gene Expression Atlas developed using GeneChip analysis is available online. This gene atlas includes expression of most genes and represents all major organs of the A17 cultivar (Benedito *et al*., 2008). Using this resource, researchers can identify genes that are expressed in organs of interest (Benedito *et al*., 2008). Furthermore, expression of genes under differing conditions can be determined. For example, according to the *Medicago truncatula* Gene Expression Atlas, 6.5% of all genes are upregulated during the AM symbiosis (Bravo *et al*., 2016). This tool assists in narrowing down genes that may be involved in mycorrhizal root colonization (Benedito *et al*., 2008). Using this resource, the four defence-related genes screened in this thesis were identified. Based on their expression pattern, we hypothesized that these genes were involved in regulating the AM symbiosis in some way.

1.7. Transformation of *Medicago truncatula* roots

Plant transformations are a common method in biotechnology used to integrate foreign DNA into the genome of *M. truncatula* due to their accuracy and efficiency (Floss *et al*., 2013; Aggarwal *et al*., 2018). Transient plant transformations are often used in genetic studies to determine the function of a gene of interest (Aggarwal *et al*., 2018; Floss *et al*., 2013).

Expression of the *Escherichia coli* beta-glucuronidase (GUS) reporter gene allows for stable visualization of promoter activity (Jefferson *et al*., 1987). In this thesis, transient *M. truncatula* root transformations using *Agrobacterium rhizogenes* are used to determine the spatial and temporal expression patterns of our genes of interest. To this end, a GUS gene is expressed under the activity of the defence gene promoter of interest, and we are able to visualize where the protein is being expressed.

This is an important aspect of this research as the type of cells that genes are expressed in provide information regarding the role that gene plays. For example, if a defence gene of interest was expressed exclusively in colonized root cortical cells, that may provide evidence for a role in the AM symbiosis.

1.8. *Tnt1* mutant lines

Insertional mutagenesis is frequently used in reverse genetics studies to truncate genes and subsequently identify a mutant phenotype. *Tnt1* is a tobacco retrotransposon that The Noble Foundation has used to create around 21,700 *M. truncatula* insertion lines in the R108 wildtype genome for the purpose of reverse genetics studies. Retrotransposons are class I transposable elements that randomly disperse through the genome and allow for gene tagging (Cheng *et al*., 2011). There are a total of 300,000 individual *Tnt1* inserts in the Noble Foundation Database with an average of 25 in every mutant line, each around 5.3 kb in size (**Figure 1.5**) (Cheng *et al*.,

2011). Flanking sequence tags (FST) are used to identify the presence and location of the *Tnt1* insertions (Cheng *et al*., 2011).

Tnt1 insertions are stable through the life cycle of the plant, and are segregating for the mutation (Cheng *et al*., 2011). The insertions may integrate into one allele, two alleles, or neither allele. This makes the genotype at the gene of interest either heterozygous, *Tnt1* homozygous or WT homozygous (**Figure 1.6**). For the mutant lines used in this thesis, *Tnt1* homozygous plants are required as WT alleles in heterozygous plants may be fully or partially functional and thus prevent the exhibition of a phenotype. As a control, segregating WT plants are used as they are more genetically similar to the *Tnt1* homozygous mutant plants than the R108 WT plants, which lack any *Tnt1* insertions. This is because they should share all or most *Tnt1* insertions, except the one truncating the gene of interest (Cheng *et al*., 2011).

According to Cheng *et al*., (2011), to contribute a phenotype to the *Tnt1* insertion in the gene of interest: 1) the same phenotype should be displayed for all plants that are homozygous for the *Tnt1* insertion in the gene of interest, 2) all plants that display a specific phenotype should also be homozygous for the *Tnt1* insertion in the gene of interest. Ensuring these two criteria are met will assist in ruling out any other *Tnt1* insertions as causing any phenotype.

1.9. Research Justification and Goals

Based on evidence of *M. truncatula* defence gene expression patterns, the suppression of defence related compounds, and the strict regulation of AM fungal growth within host tissues, it is possible that the genes involved in plant immunity may also be involved in the regulation of the AM symbiosis. Accordingly, four *M. truncatula* genes that encode putative defence proteins have been identified and are hypothesized to be involved in the mean root length colonized by *R.*

irregularis. We predict that these proteins are involved in regulating AM symbiosis, either by: a) mediating the suppression of defence pathways to enable fungal colonization of root cells, or alternatively: b) by restricting the growth of the symbiont to prevent over-proliferation in host tissues. To test this hypothesis, a reverse genetic study using *Tnt1* mutant plants from the Noble Foundation has been undertaken.

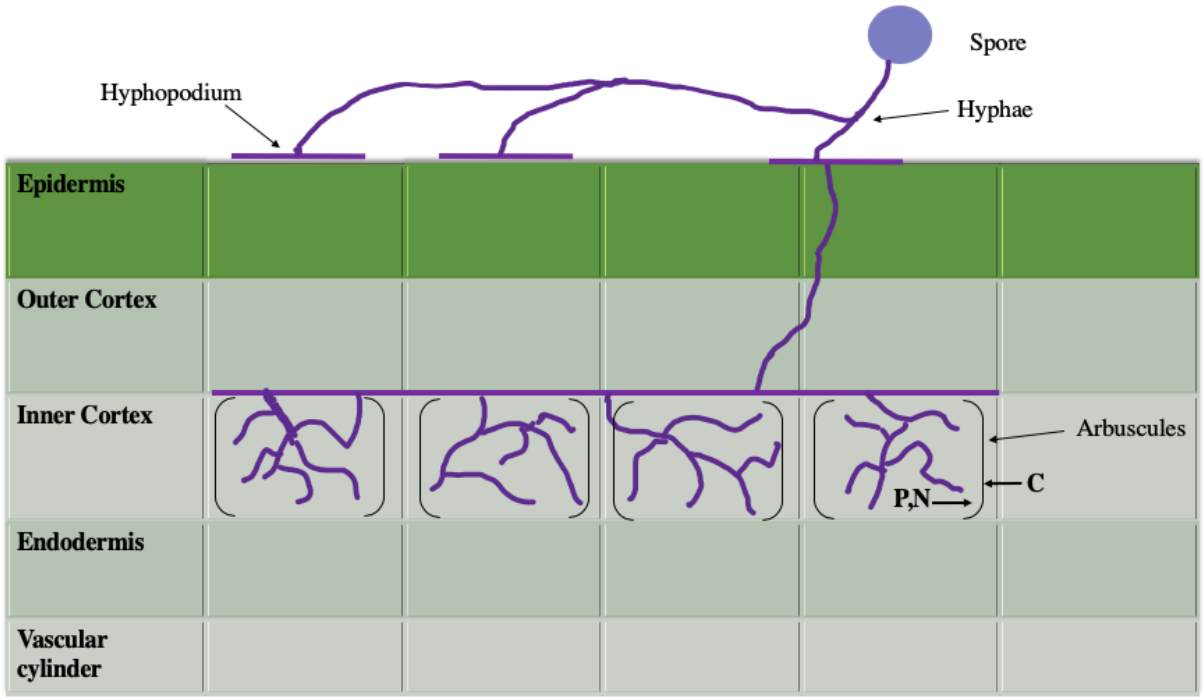

Figure 1.1. The growth pattern of arbuscular mycorrhizal fungi inside host plant roots. Spores in the rhizosphere germinate, move towards the plant root, enter the plant root via differentiated hyphae called hyphopodium, proliferate intra and intercellularly to reach the inner root cortical cells, where the arbuscules are formed. The arbuscules are the site where the AM fungi transfer nutrients in exchange for fixed carbon.

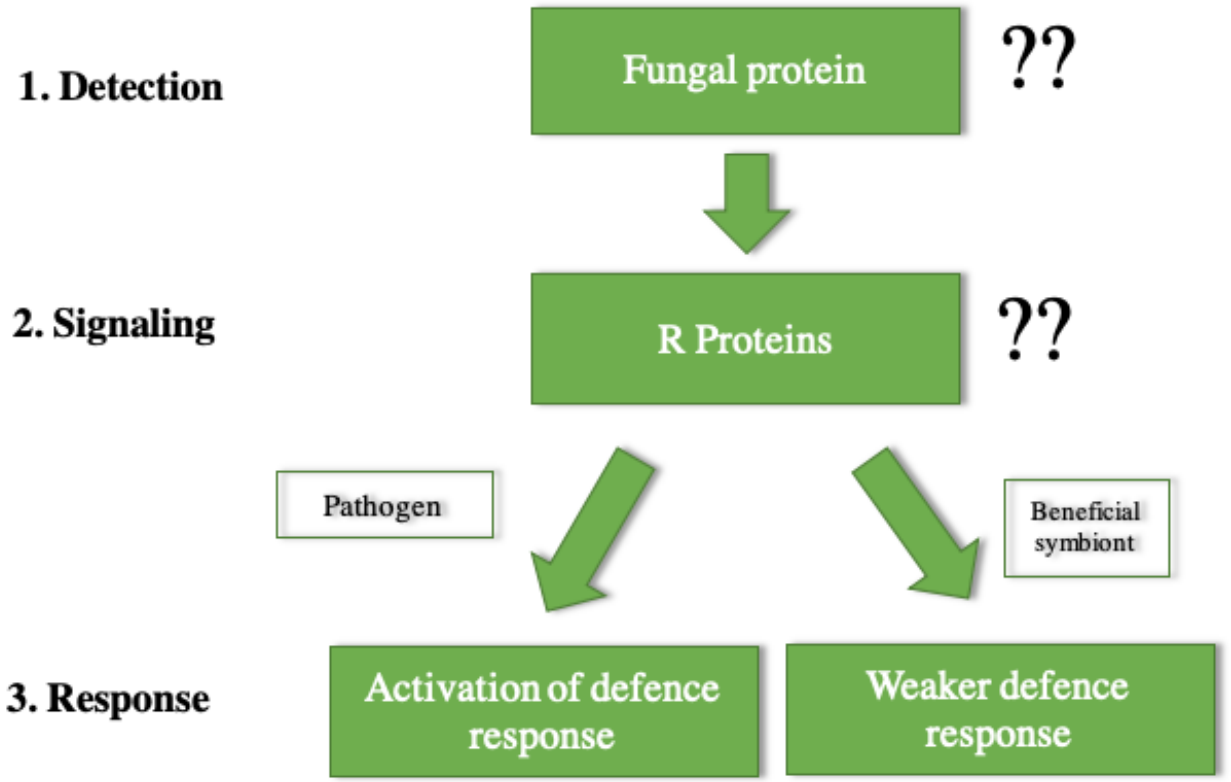

Figure 1.2. The role of fungal proteins in eliciting a response in host plants. The three broad steps in plant responses to microbes are detection, signalling, and a response. The mechanisms of defence responses against pathogens are well studied. Knowledge of the mechanisms by which plants interact with arbuscular mycorrhizal fungi is not clear.

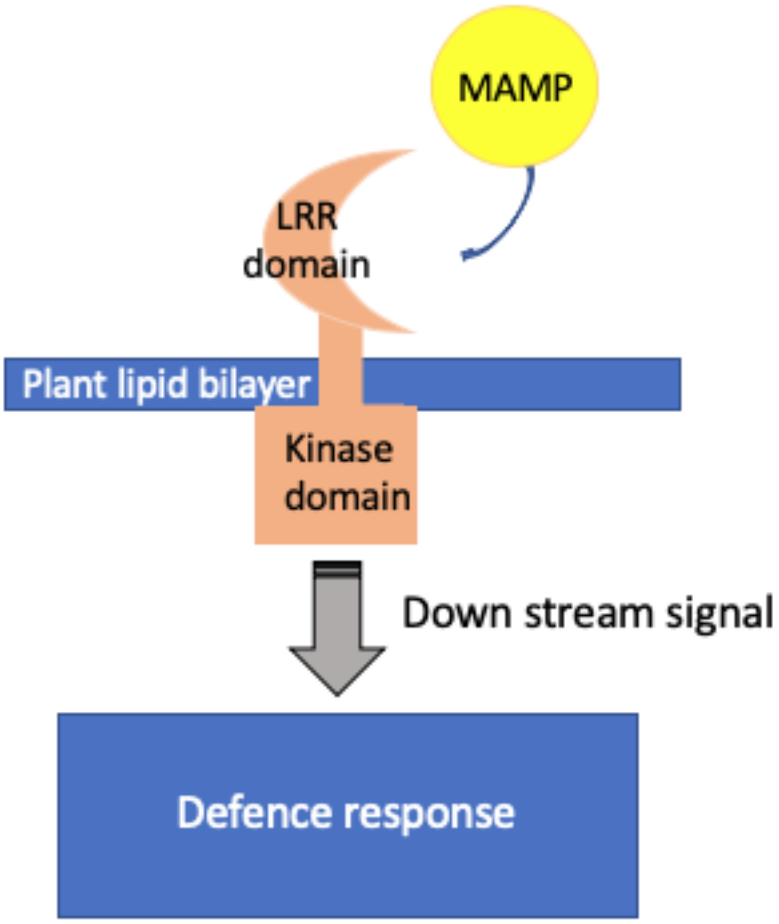

Figure 1.3. Leucine-rich repeat receptor kinase schematic. Microbe-associated molecular patterns (MAMPs) bind to the leucine rich-repeat domain in the extracellular space. The kinase domain recognizes the MAMP and sends a downstream signal which activates a defence response, such as salicylic acid accumulation. This is a defence response that provides most plants with immunity against the majority of pathogens.

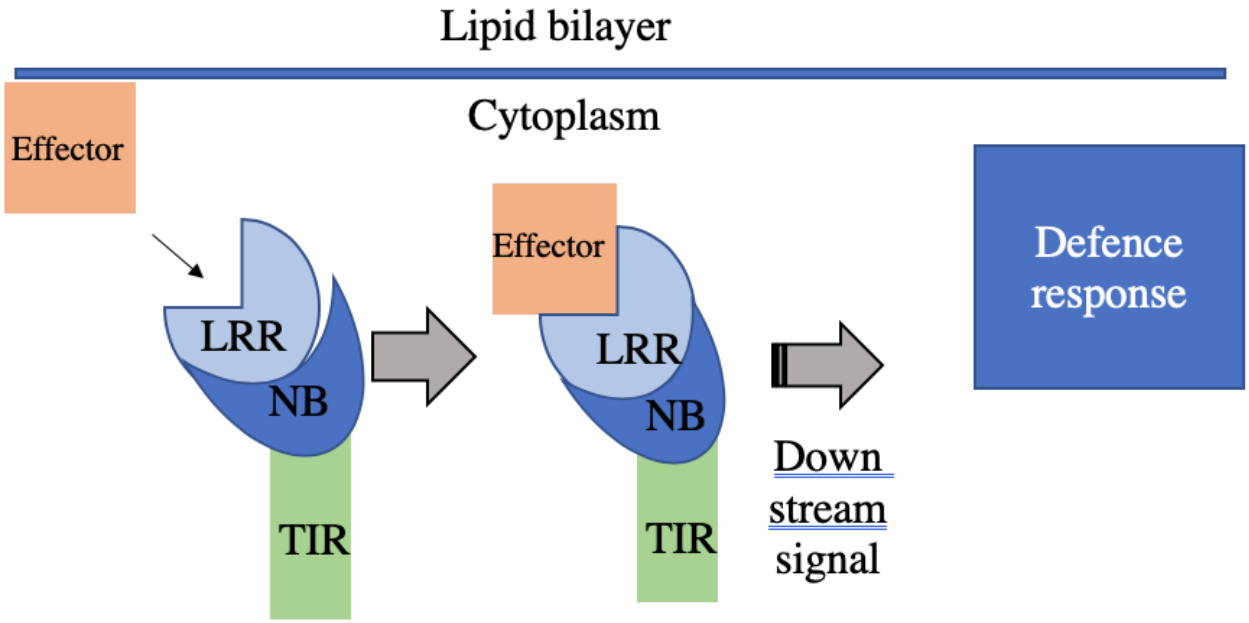

Figure 1.4. Nucleotide-binding leucine rich repeat receptor-ligand hypothesis. A visual representation of the NB-LRR receptor-ligand hypothesis depicting the binding on a fungal effector protein to the LRR domain of the NB-LRR defence protein complex. This interaction results in the recognition of a pathogenic microbe and activation of effector-triggered immunity, commonly in the form of a hypersensitive response.

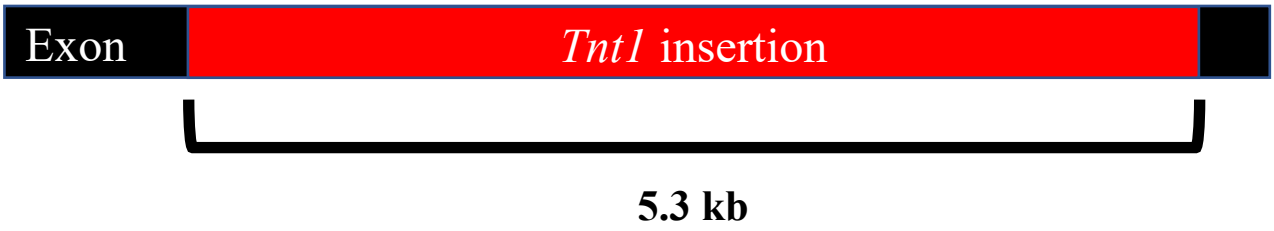

Figure 1.5. The insertion of a *Tnt1* retrotransposon into an exon. In the mutant lines acquired for these experiments, a *Tnt1* retrotransposon was randomly inserted into the genome of *Medicago truncatula* R108 wildtype plants. Due to the large size of the transposon (5.3 kb), insertion into an exon causes a null mutation.

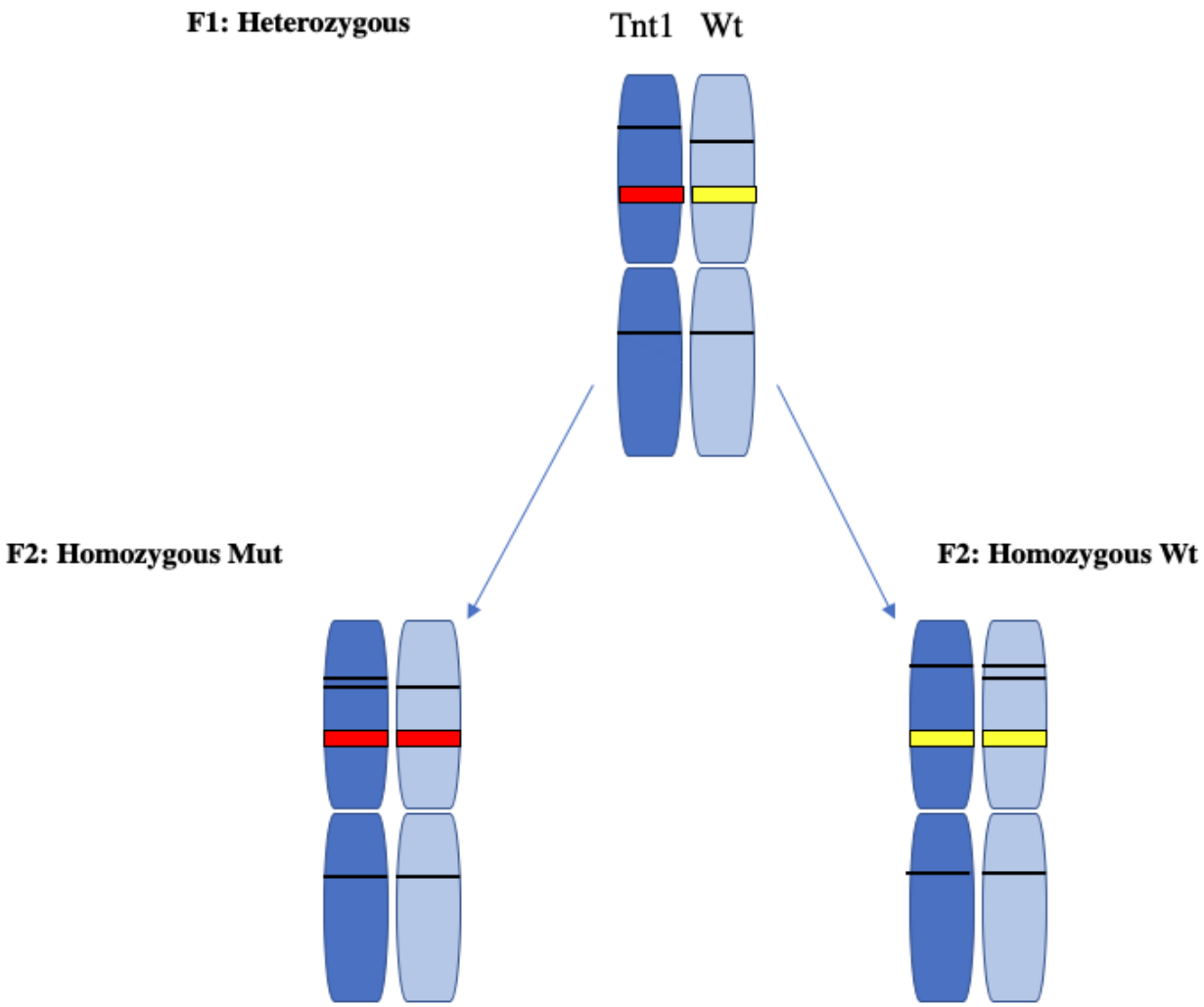

Figure 1.6. The segregation of a *Tnt1* transposon from heterozygous parent plants to homozygous offspring in self-fertilizing *Medicago truncatula* obtained from the Noble Foundation. Red lines depict the gene of interest being truncated by a *Tnt1* retrotransposon. Yellow lines depict a wild-type copy of the gene of interest. Black lines depict the location of background insertions in non-relevant genes.

Gene I.D.	Gene product	Function	Mutant line(s)	*Tnt1* location	Gene size (bp)	Total insertions in line
Medtr5g031270	Toll/interleukin 1 nucleotide-binding-leucine-rich repeat (TIR-NBS-LRR)	Disease resistance protein involved in effector triggered immunity	NF16380	Intron	4,776	43
			NF4789	Exon 2		3
Medtr4g023060	Toll/interleukin 1 nucleotide-binding-leucine-rich repeat (TIR-NBS-LRR)	Disease resistance protein involved in effector triggered immunity	NF15831	Exon 2	6,466	52
Medtr3g078633	Enhanced disease susceptibility 1 (EDS1)	Positive regulator of effector triggered immunity	NF12940	Exon 2	3,405	9
Medtr8g087420	LRR-receptor-like kinase (LRR-kinase)	Positive regulator of MAMP-triggered immunity	NF20332	Exon 2	2,156	50

Table 1.1. Four *M. truncatula* genes with increased expression during AM symbiosis. Each gene has at least one *Tnt1* insertion in one exon. All plant lines were acquired from the Noble Foundation.

CHAPTER TWO

Materials and Methods

2.1. Cloning promoter: GUS constructs

To determine the spatial and temporal expression patterns of three genes of interest (Medtr5g031270, Medtr4g023060, and Medtr8g087420), *M. truncatula* roots were transformed with the GUS reporter gene under the control of the gene of interest (GOI) promoter region (promoter /Gus/NosT:pKm43GWRR). The destination vector (pKm43GWRR) has a DsRED1 fluorescent protein sequence allowing for screening of successfully transformed plant roots (Stepanenko *et al*., 2004). Gateway™ cloning technology was used in this experiment due to the ease of combining multiple donor vectors into a destination vector (Liang *et al*., 2013).

The positive control in this experiment was the promoter region of an *Arabidopsis thaliana* polyubiquitin protein (AtUBQ10) which causes constitutive uniform expression of the GUS reporter gene (AtUBQ:GUSNosT/pKm43GWRR) in *M. truncatula* root cells (Ivanov and Harrison, 2014). A second control was the promoter region of the *M. truncatula* Blue Copper Binding Protein 1 (MtBCP1sp) gene (BCPp:NLSGFPGUS/pkm43GWRR) which causes GUS expression in AM colonized cells (Ivanov and Harrison, 2014). This promoter construct serves as both a positive and negative control as there should be clear GUS expression in mycorrhizal roots, and no expression in non-mycorrhizal roots.

2.1.1 Isolating attB flanked putative promoter regions

High-quality genomic DNA was extracted from *M. truncatula* A17 leaves using a CTAB (cetyl trimethylammonium bromide) plant DNA extraction protocol (OPS Diagnostics, 2016). In order to capture the unknown promoter sequence of the genes of interest, primers were designed to isolate an area of around 1 kb directly upstream of the start codon of the GOI (**Supplemental Table 3.1**) with attB4 and attB1 sequences added to the 5’ ends of each forward and reverse

primer, respectively. These attB sequences were complimentary to the position in the destination vector in which the donor vectors were recombined (Floss *et al*., 2013). PCR was performed with Phusion High-Fidelity Polymerase using the protocol supplied by ThermoScientific™, (2020). The 50 µL PCR product was run on a 0.8% agarose gel at 100 V for one hour. The band containing each putative promoter region was cut out and purified using the GenElute™ Gel Extraction Kit and accompanied protocol (Sigma-Aldrich).

2.1.2. Preparing pDONR 4,1 donor vectors

Using Gateway™ cloning technologies the GOI putative promoter sequences and the AtUBQ control were each cloned into a pDONR™4,1 donor vector (kanamycin selection) which corresponds to the final position in the destination vector (promoter: GUSNosT/ pKm43GWRR). The gel purified product of interest (30-50 ng), the pDONR 4,1 plasmid (75 ng), the BP Clonase II enzyme (1 µL), and TE (Tris-EDTA) buffer were combined and incubated at 25°C overnight. The reaction mix was transformed into chemically competent *E. coli* DH5α using heat-shock (Addgene, 2017), plated on lysogeny (LB) media (Sigma-Aldrich) (50 µg/mL kanamycin) and incubated at 37°C overnight. To confirm the success of the heat-shock reaction, colony PCR was performed using M13F and M13R primers (**Supplemental Table 3.1**) which flank the sequence of interest with the empty pDONR4,1 vector as a negative control. One colony was grown in LB broth (25 µg/ mL kanamycin) overnight at 37°C (25 µg/mL kanamycin). The plasmids were isolated from the overnight cultures using the GenElute™ Plasmid Miniprep kit (Sigma Life Science, 2014).

2.1.3. Creating destination vector

The purified pDONR4,1 (promoter regions), pDONR221 (GUS gene), and pDONR2,3 (NosT sequence) donor vectors (150 ng) were re-combined into the pKm43GWRR destination vector backbone (130 ng) using the Gateway™ LR clonase™ enzyme mix (1 µL) during an overnight incubation at 25°C. The recombination reaction placed the promoter sequence upstream of the GUS gene and NosT terminator sequences (respectively). The purpose of the NosT sequence is to ensure that transcription is terminated after the GUS gene.

To confirm the successful recombination in the destination vector, a PCR was completed using two primer sets: M13F and M13R and GUSF and M13F (**Supplemental Table 3.1**) with the empty destination vector as a control. A single colony was selected and grown overnight in LB broth at 37°C (200 µg/mL spectinomycin and 100 µg/mL streptomycin) and the plasmid was isolated using the GenElute™ Plasmid Miniprep kit (Sigma Life Science, 2014).

To transfect the *M. truncatula* root cells, the infectious bacteria *Agrobacterium rhizogenes* was used. The purified destination vector plasmids were transformed into *A. rhizogenes* ARqual cells via electroporation. The transformed cells were plated on LB media (200 µg/mL spectinomycin and 100 µg/mL streptomycin) and grown for two days at 28°C. The resulting colonies were used to continue into the root transformation protocol.

2.1.4. Sanger sequencing of vectors

To confirm the cloning of the promoter regions was successful, PCR was performed on the four purified donor plasmids (promoter: pDONR4,1) using the M13F and M13R primers and around 1 µg of PCR product was sent to GenomeQuebec for Sanger sequencing. The donor plasmids containing the GUS reporter gene (pDONR 221) and the NosT sequence (pDONR 2,3)

were acquired from the lab of Dr. Maria Harrison at the Boyce Thompson Institute and had been used in previous studies, so were not sequenced.

To confirm the correct recombination of the GOI promoter sequence and GUS gene in the destination vectors (GOI promoter: GUSNosT/pKm443GWRR), PCR was used to amplify the area within the M13F and M13R markers and 1 μg of product was sent to GenomeQuebec for Sanger sequencing.

2.2. *Medicago truncatula* root transformation

The protocol for root transformation was acquired from Floss *et al*. (2013) and was completed as follows.

2.2.1. *Agrobacterium rhizogenes* preparation

A single colony of *A. rhizogenes* ARqua1 containing the destination vector of interest was chosen from LB selection media (spectinomycin 200 μg/mL and streptomycin 100 μg/mL) and suspended into 50 μL of sterile water. The bacteria slurry was plated and spread onto tryptone yeast agar (Sigma-Aldrich) (spectinomycin 200 μg/mL and streptomycin 100 μg/mL) and incubated at 28°C for two days.

2.2.2. *Medicago truncatula* preparation

M. truncatula A17 seeds (30 seeds/construct) were removed from pods and placed in concentrated sulfuric acid for 9 minutes to remove the seed coat before rinsing 5x with distilled water. To sterilize the seeds they were placed in 10% bleach and 0.1% Tween®20, agitated for 10

minutes, and rinsed with sterile dH_20 in the flow hood. They were then shaken for 3 hours at room temperature before being placed at 4°C for 26 hours to imbibe water.

The seeds were then placed in sterile, glass petri plates with a thin layer of water to allow the seeds to stick to the glass via water tension. This allowed the petri plates to be flipped so the radicle was able to grow straight down. The plates were incubated at 28°C for 16 hours in the dark to germinate.

2.2.3. *Medicago truncatula* root inoculation with *Agrobacterium rhizogenes*

The *M. truncatula* seed coats were removed, and the bottom mm of each radicle was cut at a diagonal. The root tips were individually dipped into the prepared *A. rhizogenes* containing the destination vector of interest. The radicles were placed into the F-media (25 μg/mL kanamycin) (Floss *et al*., 2013) and incubated at 18°C with 16 $\mu M/m^2/s$ light (16h light/8h dark) for 5 days. The F-plates were then incubated at 25°C (22°C overnight) with 45 $\mu M/m^2/s$ light. The destination vector (pKm43GWRR) contained a kanamycin resistance gene under the control of a plant promoter, therefore, only successfully transformed roots had kanamycin resistance.

2.2.4. Transformed root screening

After approximately 16 days, the plant roots that were successfully transformed had grown in the F-media and were screened for successful transformation using a Zeiss Axiozoom stereoscope. As mentioned, the pKm43GWRR plasmid had a DsRED gene, allowing the roots to be screened under the 540-590 nm wavelength. Transformed roots were visibly red, and were marked with a sharpie to indicate their transformation.

2.2.5. Transformed root assays

The transformed plants for each construct were planted in Cone-tainers as described in Section 2.4.2. The number of successfully transformed roots were divided in two treatment groups. The first treatment group was mycorrhizal, and the roots were inoculated with 200 spores of the AM fungi *R. irregularis*. The second treatment group was non-mycorrhizal, and was not inoculated. Due to the COVID-19 pandemic, we were unable to harvest the roots and determine where our genes of interest were expressed. However, the transformation process has been re-started beginning from Section 2.4 and is currently in progress.

2.3. *Tnt1* mutants

Five *M. truncatula Tnt1* mutants lines were acquired from the Noble Foundation to screen the four genes of interest for involvement in AM fungal colonization using symbiosis assays. For three of the four genes, one mutant line was acquired, and for the fourth gene, two mutant lines were acquired (**Table 1.1**). Screening of the Medtr8g087420 gene was completed by UROP (undergraduate research opportunity program) student Monique Power under my direction and supervision. All *Tnt1* seeds (approximately 10 for each line) received from the Noble Foundation were sterilized and grown to maturity in the greenhouse to obtain sufficient seed for the symbiosis experiments.

2.3.1. DNA extraction

DNA was extracted using a quick and simple protocol similar to the one described by Chabi Sika *et al* (2015). Briefly, 1-3 leaves were collected from *M. truncatula* in 1.5 mL tubes and crushed into a fine powder with liquid nitrogen using a plastic pestle. Next, 400 uL of DNA

extraction buffer (0.2M TRIS (pH 8), 0.25M NaCl, 0.025M EDTA, 20% SDS) was added to the crushed leaves, vortexed to mix and centrifuged at max speed (12,000 xg) for 7 minutes. The supernatant was removed, an equal volume of isopropanol was added, and the solution was centrifuged at max speed (12,000 xg) for 10 minutes. The isopropanol was decanted, with care not to disturb the pellet, and 500 uL of 70% ethanol was added before an additional 10 minute centrifuge at max speed (12,000 xg). The ethanol was removed and once all had evaporated, the pellet was dissolved in 50 uL of dH20. Isolated DNA was ready to be used right away. Samples were placed at 4°C for short-term storage, -20°C for medium-term storage, and -80°C for long-term storage.

2.3.2. Genotyping

Due to the large library of mutants identified by FSTs at the Noble Foundation, mutant plants were genotyped to confirm the presence of the *Tnt1* insertion of interest, and to determine if the plants were *Tnt1* homozygous, heterozygous, or WT homozygous at the insertion site (Cheng *et al*., 2011).

2.3.2.1. Primer design

The Noble Foundation Genetic Genome Browser (version 2.56) provides a short sequence (around 300 bp) where a *Tnt1* transposon is expected to have integrated. *M. truncatula* gene-specific (GS) primers that flank the integration site were designed using the A17 genome accessed through NCBI for a total amplicon size between 500 - 700 bp (Supplemental Table 3.1). *Tnt1* forward (*Tnt1*_F2) and reverse primer (*Tnt1*_R2) (Supplemental Table 3.1) sequences were obtained from Cheng *et al*. (2011).

2.3.2.2 Polymerase chain reaction (PCR)

PCR was performed using GoTaq™ DNA polymerase and it's accompanied protocol (Promega Corporation, 2002). PCR products were run on a 0.8% agarose gel at 100 volts for 30 minutes. The gels were visualized using ethidium bromide staining and UV light. Overall, two PCRs using two different primer combinations were conducted on each plant of interest to determine their genotype (**Supplemental Table 3.1**).

2.3.2.3. PCR interpretation

The orientation of the *Tnt1* insertion was not specified so the first PCR performed on each mutant line was completed using four different primer combinations: 1) GS_F + *Tnt1*_F2, 2) GS_F + *Tnt1*_R2, 3) GS_R + *Tnt1*_R2, 4) GS_R + *Tnt1*_F2 (Cheng *et al*., 2011) (**Figure 2.1**). This initial PCR provided information on the orientation of the insertion, as only two of the four primer combinations would yield results (**Figure 2.1**). One of the primer combinations was chosen for the ongoing genotyping based on the quality of the band produced on the gel. This primer combination helped determine whether or not the insertion was present, but gave no information on the zygosity of the insertion. If a band at the correct size was present, either a) the plant was homozygous for the *Tnt1* insertion (both copies of the gene were truncated) or b) the plant was heterozygous for the *Tnt1* insertion. If no band was present, the plant is likely WT homozygous at the insertion site. To determine if the plants were homozygous for the *Tnt1* insertion, the appropriate gene-specific (GS) forward and reverse primers were used to perform PCR on each plant.

Once a *Tnt1* homozygote was identified, it was used as a positive control when using a *Tnt1* primer combination and a negative control when using a GS primer combination.

Oppositely, the *M. truncatula* WT plants (A17 or R108 cultivar) were used as a positive control when using GS primers, and a negative control when using *Tnt1* primers.

Plants that were homozygous for the insertion in the gene of interest are henceforth called "*Tnt1* homozygous". Plants that were homozygous WT at the gene of interest but had segregating background insertions are henceforth called "segregating WT". Seeds were collected and grown from the *Tnt1* homozygous and segregating WT plants. If all plants were heterozygous, another generation of plants was grown until homozygosity was achieved (**Table 3.2.1**).

2.3.3. *Tnt1* insertion sequencing

The Noble Foundation, through their *Tnt1* mutant database, provide a short sequence (~300 bp) in which the *Tnt1* transposon is expected to have integrated in each of the mutant lines. However, since the specific integration site is not known, it is possible that this region is not exactly where the transposon is located. The *Tnt1* genotyping provides general information about the location of the fragment (based on amplicon size), however, it is imperative to confirm that the transposon integrated into an exon to ensure that it is not spliced out with the introns, potentially resulting in a functioning protein.

To confirm the exact location of the *Tnt1* insertion in seeds acquired from the Noble Foundation, PCR product amplified using the appropriate *Tnt1* and gene specific primers (**Supplemental Table 3.1**) were sent to GenomeQuebec for Sanger sequencing. Sequencing results were acquired and analysed using SnapGene™software. To confirm the presence of the *Tnt1* transposon, the PCR product sequences were aligned to the insertion sequence. Then, the sequence directly upstream of the insertion was aligned to the respective annotated gene

sequence obtained from NCBI to determine if the insertion was in a coding region (**Figure 3.1**). The insertions in background genes are outlined in **Supplemental Tables 1.1-1.5** for each of the plant lines in this thesis.

2.4. NF mutant line symbiosis assays

To determine if the *M. truncatula Tnt1* mutants showed a phenotype of reduced *R. irregularis* colonization compared to WT plants, symbiosis assays were set-up in Cone-tainers (2 cm radius and 21 cm length). The Cone-tainer method saves space, and allows for more efficient set-up procedures and care of the plants compared to pots. Two plants of the same genotype were planted in each conetainer. There were 4-8 conetainers/replicates for each mutant and control treatment group at 2-3 different time points. The number of replicates depended on germination rates, seed availability and seedling survival rate. If one seedling did not survive, that replicate was not included in the analysis.

2.4.1 Seed sterilization

M. truncatula seeds were sterilized prior to planting to reduce the chances of microbial contamination interfering with the AM symbiosis. The seed sterilization protocol was similar to Cheng *et al*., (2005) and is as follows: the required number of *M. truncatula* seeds were carefully removed from their seed pods and placed in concentrated sulfuric acid for nine minutes to remove the seed coat and allow the seeds to imbibe water. Then, the seeds were rinsed in dH2O five times. A 10% bleach and 0.1% Tween 20 solution was added to the seeds and agitated on a shaker for 10 minutes to sterilize. In a flow hood, the seeds were rinsed with sterile dH20 5x. Then, using sterile technique, they were spread apart on damp filter paper in a petri dish as close

proximity can inhibit germination. The petri plate was placed at 4°C for at least 3 days to break dormancy and allow synchronized germination. After 3 days, the petri plates were placed in a dark drawer overnight to germinate at room temperature. The next day, the plates were placed by a bright window for two days to allow the cotelydons to develop. At this point, the seedlings were ready to be planted.

2.4.2. Cone-tainer preparation

A one-inch marble was placed at the bottom of each Cone-tainer, and covered with five inches of autoclaved sand and turface (1:1 mix). Subsequently, 200 spores (0.250 g) of Myc-Pro brand *R. irregularis* were spread evenly on top of the sand/turface mixture. This inoculum was used as it is guaranteed to have 800 spores/gram. Around 2 inches of autoclaved sand/turface (1:1 mix) was packed on top of the spores and two sterilized and germinated seedlings were planted. Cone-tainers were placed in a growth chamber with the environmental conditions based on optimal *M. truncatula* growth recommendations by the Noble Foundation (Barker *et al*., 2006) (**Table 2.1**). Seedlings were fertilized with half strength Hoagland's fertilizer with 20 µM potassium phosphate (Hoagland and Arnon, 1950) twice weekly. Fertilization began after the first week of growth.

2.4.3. Symbiosis assay harvesting

Around 3, 4, and 6 weeks post planting (wpp), the roots of the AM colonized *M. truncatula* plants were stained with fluorescent dye to visualize the colonization rates under a fluorescent microscope (Bravo *et al*., 2016). The roots were cut from the stem, washed in water, and placed in 50 mL falcon tubes. A 1:1 ethanol/water mixture was added and they were soaked

for at least 15 minutes at room temperature. The ethanol was removed and roots were placed in 20% potassium hydroxide (KOH) overnight at 65°C. KOH clears the roots of proteins and lignins making them transparent so AM fungal visualization is possible (Vierheilig *et al*., 2005). The KOH was decanted, and the roots were covered with phosphate buffered saline and shaken for 10 mins. This was repeated 3x. To visualize the AM fungi via microscopy, a wheat germ agglutinin (WGA), Alexa Fluor™ 488 conjugate was added to each sample (0.2 ng/mL) (Vierheilig *et al*., 2005). WGA is a lectin that attaches to chitin allowing the visualization of fungal structures (Vierheilig *et al*., 2005) when bound to an Alexa Fluor™(ThermoFisher Scientific, 2009). The treated and stained roots were kept at 4°C long-term, with care to avoid lengthened exposure to light as the WGA conjugate is light-sensitive (ThermoFisher Scientific, 2009).

2.4.4. Mean root length colonized quantification

The grid line intersect method was used to quantify mean root length colonized for quantitative analysis and comparison. This method is considered an accurate and consistent way of quantifying AMF colonization in roots compared to other quantification methods (Giovannetti & Mosse, 1980). Additionally, this method reduces bias as it is a systemic counting of colonized roots to determine a total percentage (Giovannetti & Mosse, 1980).

The cleared and stained roots from the *Tnt1* mutant and segregating WT *M. truncatula* samples were spread onto an 8 x 8 cm square petri dish with vertical and horizontal grid lines 1.4 cm apart for a total of 36 squares. The gridlines were systemically scanned using a Zeiss Axiozoom stereoscope, and each time a root crossed a gridline it was recorded as either

colonized or uncolonized. The percentage of colonization was determined for each replicate as follows:

Percent root length colonized = Total number of colonized root segments/(Total number of colonized root segments + total number of uncolonized root segments)*100

The mean root length colonized was averaged for each treatment group (*Tnt1* homozygous or segregating WT) at each time point. In addition to root colonization rates, stained roots were also observed for differential arbuscule development phenotypes (size, shape, amount).

2.4.5 Statistical analysis

Statistical analysis was used to compare the mean root length colonized from each mutant group (*nf15831, nf20332, nf4789, nf16831, nf12940*) to their respective segregating WT control group. Data from each treatment group was subject to a two-tailed t-Test to determine if there were any statistically significant differences ($p < 0.05$) in mean root length colonized. For the NF12940 symbiosis assay a third treatment, the *M. truncatula* R108 WT was included (**Figure 3.3.8**). As such, this assay was subjected to a one-way ANOVA to compare the groups.

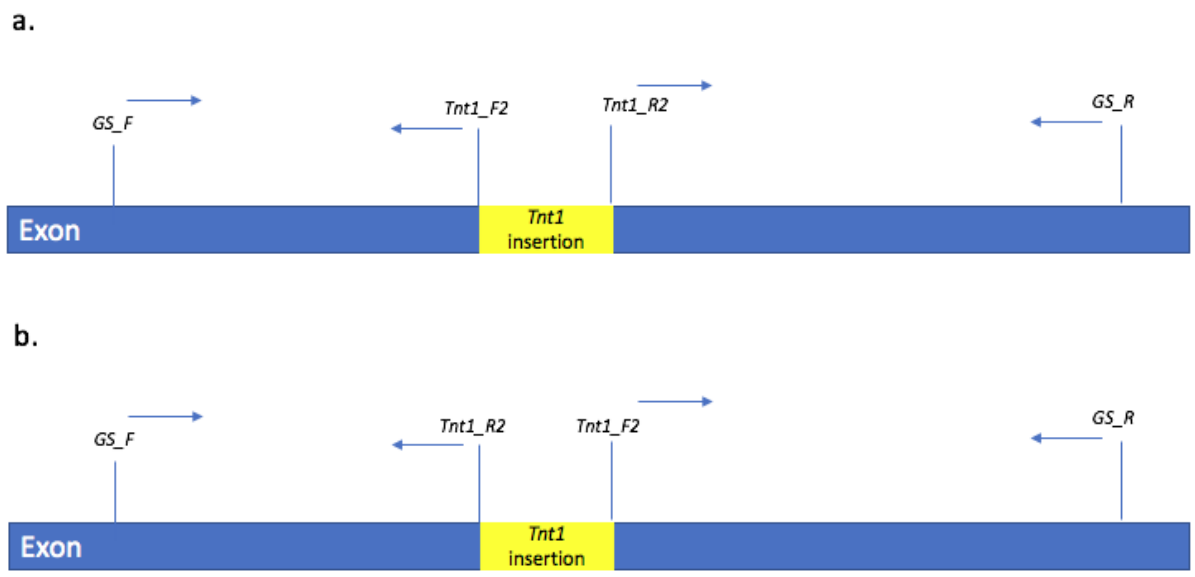

Figure 2.1. Transposon insertion in gene with primer set locations and direction. a) Two of four primer sets used for mutant *M. truncatula* genotyping, GS_F + *Tnt1*_F2, GS_R + Tnt_R2 b) Remaining two primer sets used for genotyping, GS_F + *Tnt1*_R2, GS_R + *Tnt1*_F2. To determine if the plants are homozygous for the *Tnt1* insertion, the GS_F and GS_R primers were used (not shown).

Table 2.1. Growth conditions of symbiosis assays. Optimal conditions obtained from the *Medicago truncatula* Handbook (Barker *et al*., 2006).

Day Temperature	25°C
Night Temperature	22°C
Photoperiod	16h light, 8 hours dark
Photosynthetically active radiation	160 - 200 µmol.m-2 .s -1
Humidity	60%

CHAPTER THREE

Results

3.1. Gateway™ plasmid sequencing

All Gateway™ plasmids involved in transforming *M. truncatula* roots with promoter: GUS constructs were sent to GenomeQuebec for Sanger sequencing to confirm the contents of the plasmids. All pDONR4,1 plasmids had the correct insertion of the promoter regions of interest. The pKm43GWRR plasmids were confirmed to have the promoter region of interest upstream of the GUS gene, as expected. No nucleotide insertions had occurred during PCR amplification.

3.2. Preparation for the symbiosis assays

3.2.1 *Medicago truncatula* seed bulking

The genotypes of the original *Tnt1* mutant seed obtained from the Noble Foundation are outlined in **Table 3.2.1**. Line NF20332 did not immediately have *Tnt1* homozygous and WT homozygous plants and was grown for an additional generation to obtain sufficient seed to begin the symbiosis assay experiment. The four remaining mutant lines had the required genotypes so the next generation (F2) of plants was used for seed bulking for the symbiosis assays, which required 72-126 seeds each, depending on the sample size and controls used. In total, around 192 stock plants were genotyped for the purpose of seed bulking (**Table 3.2.1**). Representative genotyping photos for each plant line are displayed in **Figures 3.2.1-3.2.5**.

Table 3.2.1. Number of *M. truncatula* plants per *Tnt1* mutant line over three generations.

F1				
Plant Line	Homozygous Mut	Homozygous Wt	Heterozygous	Total
NF4789	4	3	3	10
NF16380	2	4	14	20
NF15831	1	4	6	11
NF20331	0	2	5	7

F2				
Plant Line	Homozygous mutant	Homozygous Wt	Heterozygous	Total
NF4789	5	4	3	12
NF16380	19	2	12	33
NF15831	11	11	10	32
NF20331	4	7	14	25

F3				
Plant Line	Homozygous mutant	Homozygous Wt	Heterozygous	Total
NF4789	3	1	0	4
NF16380	0	0	0	0
NF15831	9	18	1	28
NF20332	7	3	0	10

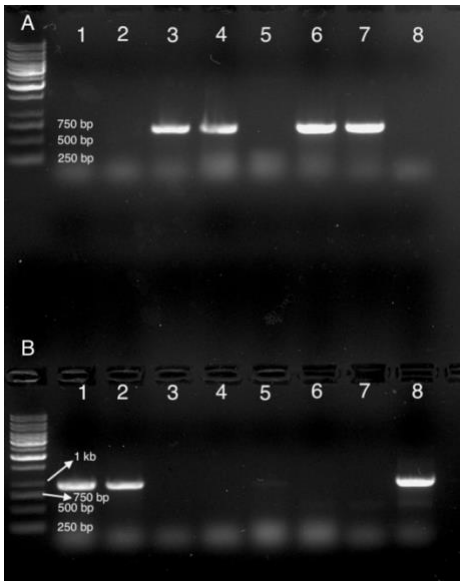

Figure 3.2.1. **Representative photo of genotyping of plant line NF4789** (Medtr5g031270) via PCR. Panel A primers: NF4789_R + *Tnt1*_R combination (556 bp). Panel B primers: NF4789_F + NF4789_R combination (995 bp). Lanes 1, 2, and 5 depict PCR completed with DNA from segregating WT plants at the gene of interest. Lanes 3, 4 and 6 depict PCR completed on *Tnt1* homozygous plants, as expected. Lane 7 is the NF4789 *Tnt1* homozygous positive control. Lane 8 is the R108 WT control.

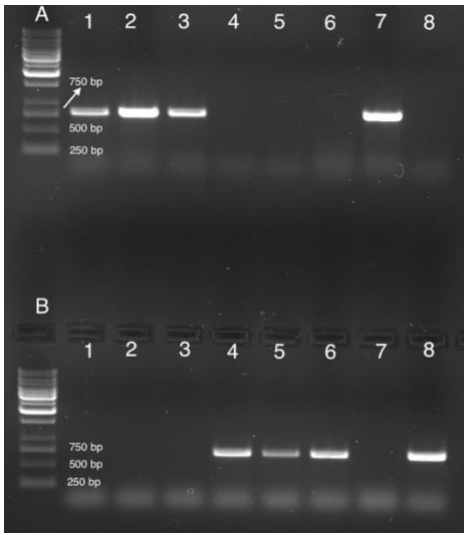

Figure 3.2.2. **Representative photo of genotyping of plant line NF16380** (Medtr5g031270) via PCR. Panel A primers: NF16380_R + *Tnt1*_R (696 bp). Panel B primers: NF16380_F + NF16380_R combination (778 bp). Lanes 1, 2, and 5 depict PCR completed with DNA from segregating WT plants at the gene of interest, as expected. Lanes 1, 2 and 3 depict PCR completed on *Tnt1* homozygous plants, as expected. Lane 7 is the NF16380 *Tnt1* homozygous positive control. Lane 8 is the R108 WT control.

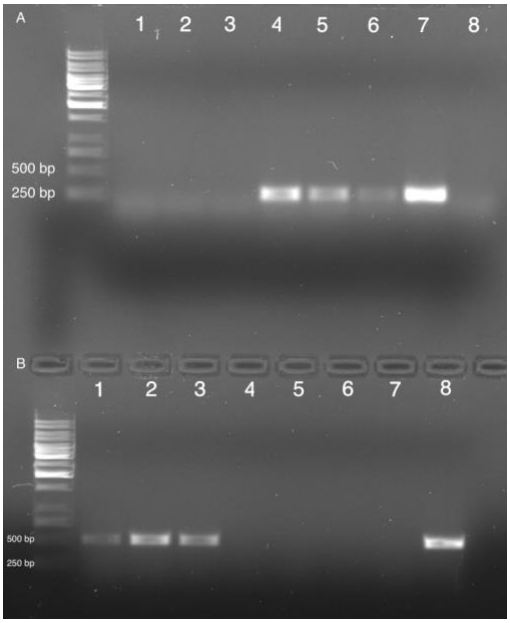

Figure 3.2.3. Representative photo of genotyping of plant line NF15831 (Medtr4g023060) via PCR. Panel A primers: NF15831_F and Tnt1_R (expected size: 358 bp). Panel B primers: NF15831_F and NF15831_R (expected size: 552 bp). Lanes 1-3 depict PCR completed with DNA from segregating WT plants. Lanes 4-6 depict PCR completed on Tnt1 homozygous plants. Lane 7 is a NF15831 homozygous WT positive control. Lane 8 is the R108 WT control. 1 kb DNA ladder (GeneRuler™) used.

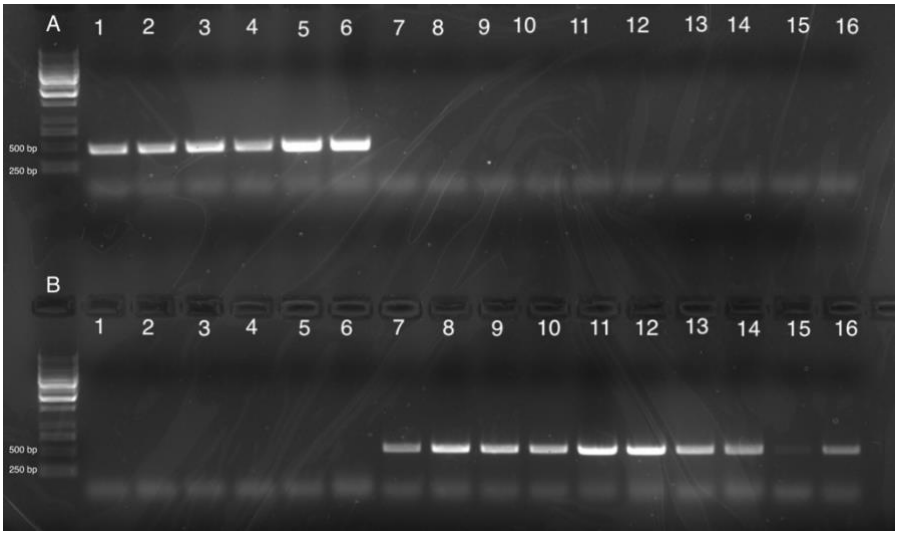

Figure 3.2.4. Representative photo of genotyping of plant line NF12940 (Medtr3g078633) via PCR. Panel A primers: NF12940_F + *Tnt1*_R combination (~475 bp). Panel B primers: NF12940_F + NF12940_R combination (601 bp). Lanes 1-6 depict PCR completed on *Tnt1* homozygous plants. Lanes 7-14 depict PCR completed with DNA from segregating WT plants at the gene of interest, as expected. Lane 15 is a NF12940 homozygous WT positive control. Lane 16 is the R108 WT control.

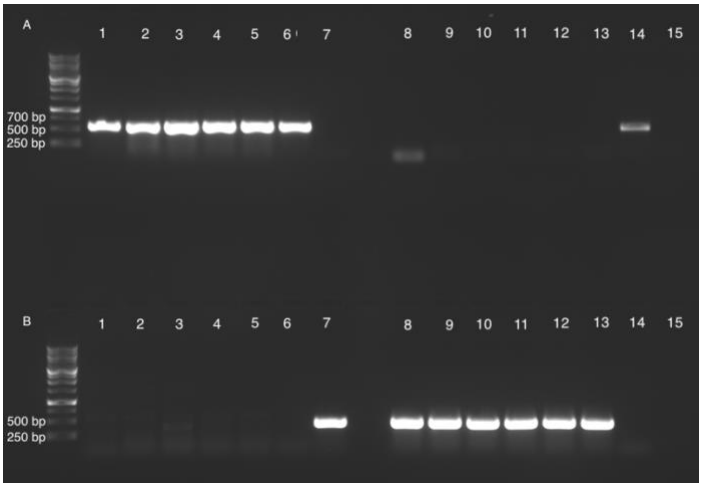

Figure 3.2.5. **Representative photo of genotyping of plant line NF20332** (Medtr8g087420) via PCR. Panel A primers: NF20332_F + NF20332_R (expected size: 569 bp). Panel B primers: NF20332_F + *Tnt1*_R combination (expected size: 311 bp). Lanes 1-6 depict PCR completed with DNA from segregating WT plants at the gene of interest. Lanes 8-12 depict PCR completed on *Tnt1* homozygous plants. Lanes 6 and 13 are the R108 WT controls. Lanes 7 and 13 are the NF20332 *Tnt1* homozygous positive controls. DNA extraction and PCR performed by Monique Power.

3.2.2 NF mutant line sequencing results

Sequencing results of the five *Tnt1* lines showed that four of the five mutant lines (NF4789, NF15831, NF12940, NF20332) had a *Tnt1* insertion in an exon (**Figure 3.2.6**). We can be confident that the proteins resulting from these genes are non-functional due to the insertion location and size (5.3 kb). The insertion in the remaining mutant line, NF16380, integrated into an intron (**Figure 3.2.6**). As a result, it is possible that the coding regions of this gene are still intact, resulting in a mutant line where the protein of interest is either partially or fully functioning.

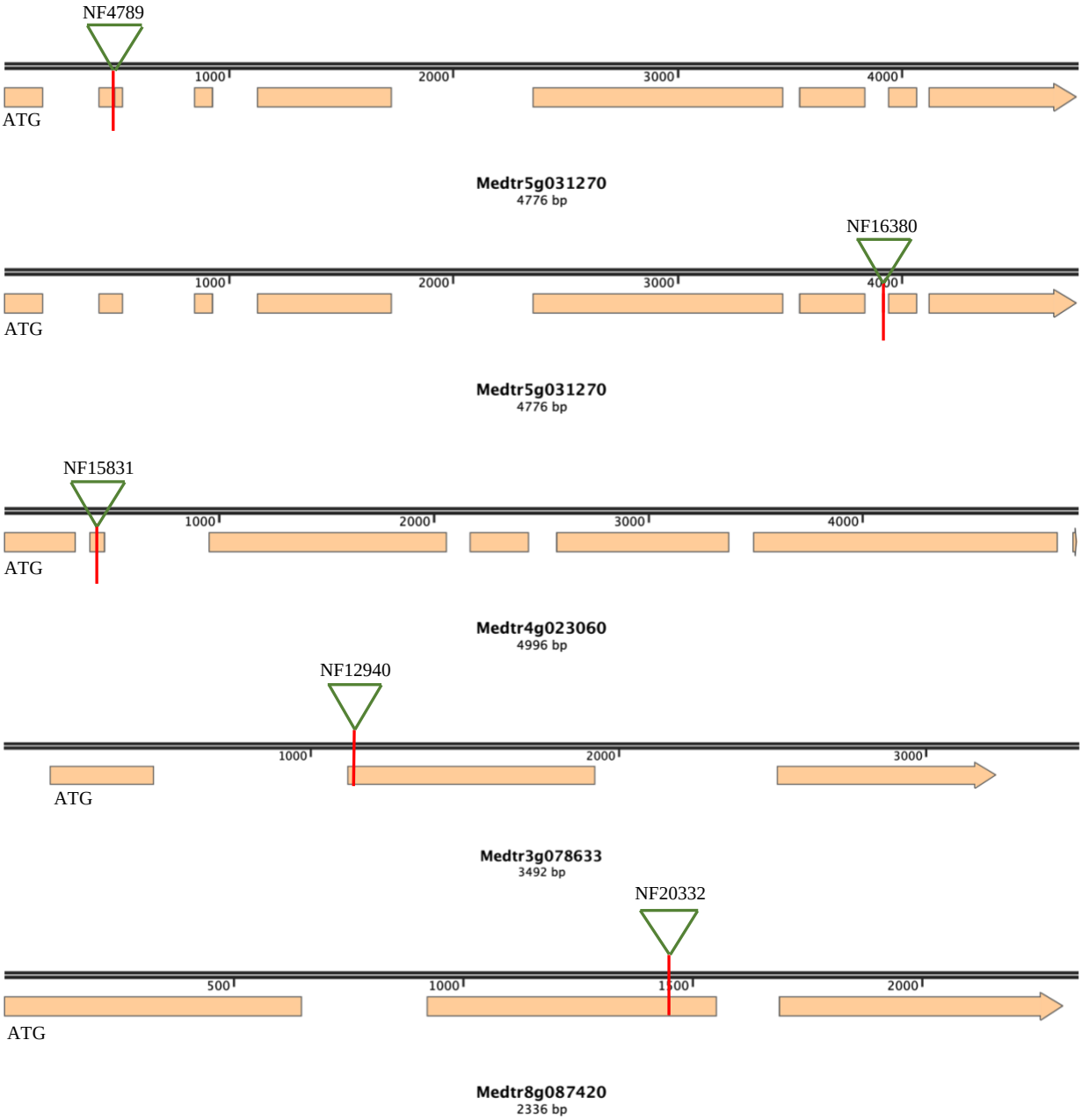

Figure 3.2.6. Insertion sites of five *Tnt1* mutants into four genes of interest. Orange boxes indicate exons, ATG indicates the start codon, red line indicates the location of the *Tnt1* insertion.

3.3. NF mutant line symbiosis assays

Comparisons of root colonization rates between five *Tnt1* mutant lines and their respective segregating WT plants at three different time points are shown in **Figures 3.3.1 – 3.3.8** and **Figure 3.3.10**. In total, nine symbiosis assays were completed to screen genes encoding two TIR-NB-LRR's, one LRR-kinase, and an EDS1-like protein for involvement in the AM symbiosis. Statistically significant colonization differences were observed in assays represented by **Figures 3.3.1, 3.3.5**, **3.3.7, and 3.3.10.**

Of the 618 plants used in the nine symbiosis assays, around 50% (300) were genotyped. Of these, 56 DNA extractions and 23 PCR reactions were completed by undergraduate student Dominique Daniels. The DNA extractions and PCR performed on the NF20332 assay (12 samples) were completed by undergraduate student Monique Power. Representative genotyping photos are shown in **Figures 3.2.1-3.2.5**.

3.3.1. Medtr5g031270 screening

The Medtr5g031270 gene encodes a TIR-NB-LRR hypothesized to be involved in the AM symbiosis due to its upregulation in mycorrhizal plants. The *Tnt1* mutant lines NF4789 and NF16380 (**Figures 3.3.1– 3.3.4**) have transposon insertions truncating this gene. The segregating WT line did not have the Medtr5g031270 gene knocked out, but shared background transposon insertions. There was no consistent statistically significant difference in mean root length colonized between the *Tnt1* mutants and segregating WT. There were no visual differences in arbuscule size, shape or amount in any of the assays conducted. The data relating to the specific plant lines used to screen this gene is further outlined below.

3.3.1.1. NF4789 symbiosis assay

The first symbiosis assay screening the NF4789 mutant line (**Figure 3.3.1**) showed no statistical difference in colonization at 3 wpp between the mutant knockout and the segregating WT (6.10% and 3.20%, respectively). However, there was a statistically significant increase ($p < 0.05$) in mean root length colonized in the mutant knockout compared to the segregating WT at 4 wpp (21.80% and 8.30%) and 6 wpp (46.90% and 32.20%). All of the plants used in the first NF4789 symbiosis assay were genotyped. Based on these results, a repetition of this experiment was conducted to determine if the trend was reproducible.

The second assay (**Figure 3.3.2**) did not show a statistically significant difference in mean root length colonized between the mutant knockouts and segregating WT at 3 wpp (2.35% and 3.50%), 4 wpp (23.60% and 25.80%) or 6 wpp (57.50% and 53.10%). All of the plants used in the second NF4789 symbiosis assay were genotyped.

Accordingly, a third assay was completed to determine with which result it aligned (**Figure 3.3.3**). Similar to the second assay, there was no statistically significant difference in mean root length colonized between the two treatment groups at 4 wpp (11.50% and 11.30%) or 6 wpp (24.90% and 28.10%). Around 23% of all the plants in the third NF4789 assay were genotyped.

3.3.1.2. NF16380 symbiosis assay

A second *Tnt1* mutant line for the Medtr5g031270 gene, NF16380, was also used to screen for a phenotype (**Figure 3.3.4**). The results from this assay comparing the mutant knockout and the segregating WT showed no difference in mean root length 4 wpp (10.70% and 10.10%) or 5 wpp (28% and 27.50%). Sequencing results revealed the location of the *Tnt1*

transposon as being in an intron, not an exon as required (**Figure 3.2.6**). There was no visual differences in arbuscule size, shape or amount in this assay. All the plants in the NF16380 symbiosis assay were genotyped.

3.3.2. Medtr4g023060 screening

A second gene encoding a TIR-NB-LRR, Medtr4g023060, also had increased expression during mycorrhization, according to the Noble Foundation's *Medicago truncatula* Gene Expression Atlas. The mutant line NF15831 was acquired to test the involvement of Medtr4g023060 in the AM symbiosis. Results from two independent experiments did not show a consistent symbiosis phenotype (**Figures 3.3.5 and 3.3.6**). There were no visual differences in arbuscule size, shape or amount in any of the assays conducted. The data relating to the specific plant line used to screen this gene is further outlined below. Around 40% of all plants in the two NF15831 assays were genotyped.

3.3.2.1. NF15831 symbiosis assay

The first assay (**Figure 3.3.5**) screening for Medtr4g023060 involvement in the AM symbiosis showed no difference in mean root length colonized between the mutant and segregating WT at 3 wpp (7.50% and 10%) and 4.5 wpp (43.0% and 32.70%). At 6 wpp there was a statistically significant increase ($p = 0.033$) in the mean root length colonized in the mutant knockout compared to the segregating WT (49.3% and 42.2%). However, the second symbiosis assay (**Figure 3.3.6**) displayed no difference in mean root length colonized at 3 wpp (5.40% and 7.12%), 4 wpp (17.90% and 22.20%) or 6 wpp (47.0% and 49.90%).

3.3.3. Medtr3g078633 screening

The Medtr3g078633 gene encodes an EDS1-like protein that is upregulated in *M. truncatula* during mycorrhization. The NF12940 *Tnt1* mutant line was used to screen this gene for involvement in the AM symbiosis. Results from two independent experiments did not show a consistent symbiosis phenotype (**Figures 3.3.7 and 3.3.8**) although the second assay (**Figure 3.3.8**) was found to have root nodules. There were no visual differences in arbuscule size, shape or amount in any of the assays conducted. Around 25% of all plants used in the two NF12940 symbiosis assays were genotyped. The data relating to the specific plant line used to screen this gene is further outlined below.

3.3.3.1. NF12940 symbiosis assays

Results from the first NF12940 symbiosis assay (**Figure 3.3.7**) displays comparable mean root length colonization (p=0.69) between the mutants (5.05%) and their segregating WT counterparts (4.07%) at 3 wpp. At 4 wpp there was a statistically significant decrease (p=0.027) in mean root length colonized in the mutant group (9.06%) vs. the segregating WT group (16.14%). At 6 wpp, there was a non-significant decrease in the mean root length colonized in the mutant (29.40%) compared to the segregating WT (36.60%) (p=0.15).

Based on these results, another assay was set up to determine reproducibility (**Figure 3.3.8**). The second NF12940 screening assay included a third group, the *M. truncatula* R108 WT. This assay did not show any statistically significant difference in mean root length colonized between the *Tnt1* mutants, the segregating WT or the R108 WT at 3 wpp (5.76%, 4.42% and 8.61%, respectively), 4 wpp (6.35%, 10.57% and 12.77%), or 6 wpp (18.03%, 19.20% and

24.60%). This assay was found to have root nodules which indicates rhizobia contamination of the roots.

When these two independent NF12940 assays were combined to compare the groups with (nod +) and without (nod -) root nodules at each time point, there was a statistically significant difference in mean root length colonized. Within the *Tnt1* treatment group, there was an 11% reduction in the mean root length colonized in the rhizobia assay at 6 wpp ($p = 0.046$). Within the segregating WT group there was a reduction in the mean root length colonized of around 17% in the rhizobia assays at 6 wpp ($p=0.00136$) (**Figure 3.3.9**). This is an interesting, yet unsurprising trend due to the common symbiosis pathway shared between AM fungi and rhizobia (see Section 1.4.2).

3.3.4. Medtr8g087420 screening

Medtr8g087420 is a gene encoding an LRR-kinase that is up-regulated during the AM symbiosis in *M. truncatula.* To screen for the involvement of this gene in the AM symbiosis, one symbiosis assay was conducted by UROP student Monique Power using the NF20332 mutant line. The seed sterilization, cone-tainer set-up, genotyping, root harvesting and colonization quantification were completed by Monique under my direction and supervision. There were no visual differences in arbuscule size, shape or amount in the assay conducted. Exactly 50% of the plants in the NF20332 assay were genotyped.

3.3.4.1. NF20332 symbiosis assay

Results from the NF20332 assay showed a statistically significant decrease ($p=0.0123$) in mean root length colonized in the *Tnt1* mutant group (5.9%) compared to the segregating WT

group (13.6%) at 3 wpp (**Figure 3.3.10**). There was no statistically significant difference in mean root length colonized at 4 wpp between the *Tnt1* mutant group (31.2%) and the segregating WT group (31.3%).

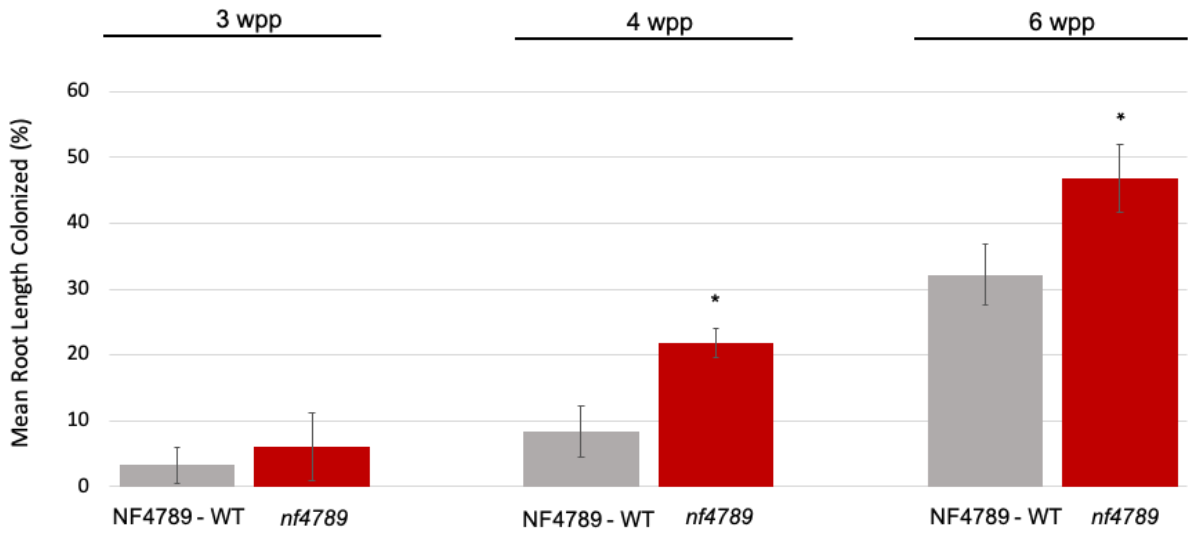

Figure 3.3.1. Screening the involvement of the *Medicago truncatula* Medtr5g031270 gene *(*encodes TIR-NB-LRR) in the AM symbiosis. Comparison of mean root length colonized between the *nf4789* mutant and the NF4789 segregating WT inoculated with *Rhizophagus irregularis* at three time points (3wpp, 4 wpp, and 6 wpp). Data shown is averaged and error bars indicate standard error, n = 5, 6, 5, 6, 3, and 6, respectively. Asterisks indicates p-value < 0.05 in a two-tailed *t*-test comparing mutants and control plants. Three separate graphs represent the independent experiments carried out using the NF4789 mutant line. All of the plants in this assay were genotyped.

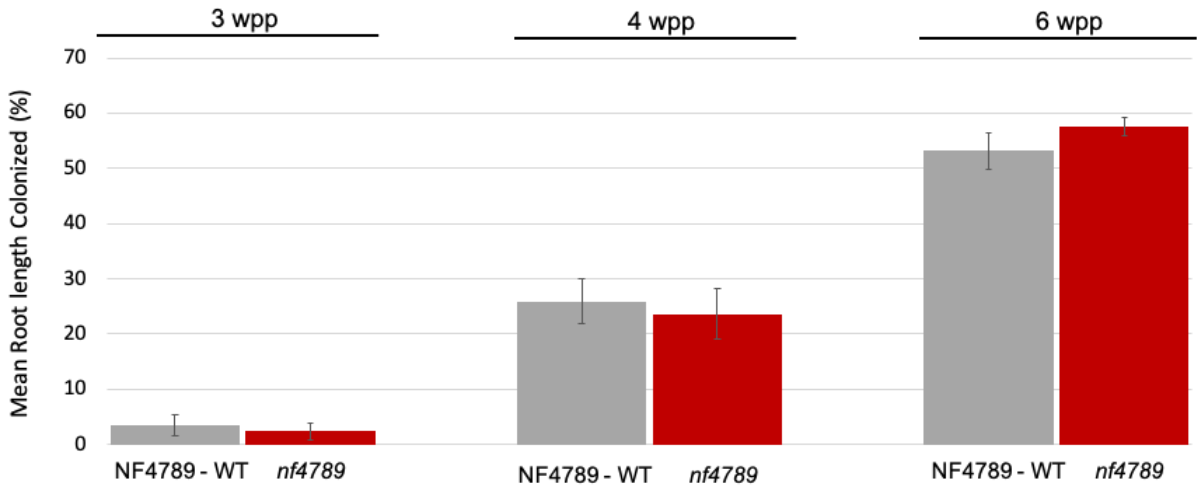

Figure 3.3.2. Screening the involvement of the *Medicago truncatula* Medtr5g031270 gene (encodes TIR-NB-LRR) in the AM symbiosis. Comparison of mean root length colonized between the *nf4789* mutant and the NF4789 segregating WT inoculated with *Rhizophagus irregularis* at three time points (3wpp, 4 wpp, and 6 wpp). Error bars indicate standard error, n = 4, 4, 7, 9, 5, and 8, respectively. Three separate graphs represent the independent experiments carried out using the NF4789 mutant line. All of the plants in this assay were genotyped. Statistical analysis was completed using a two-tailed t-test.

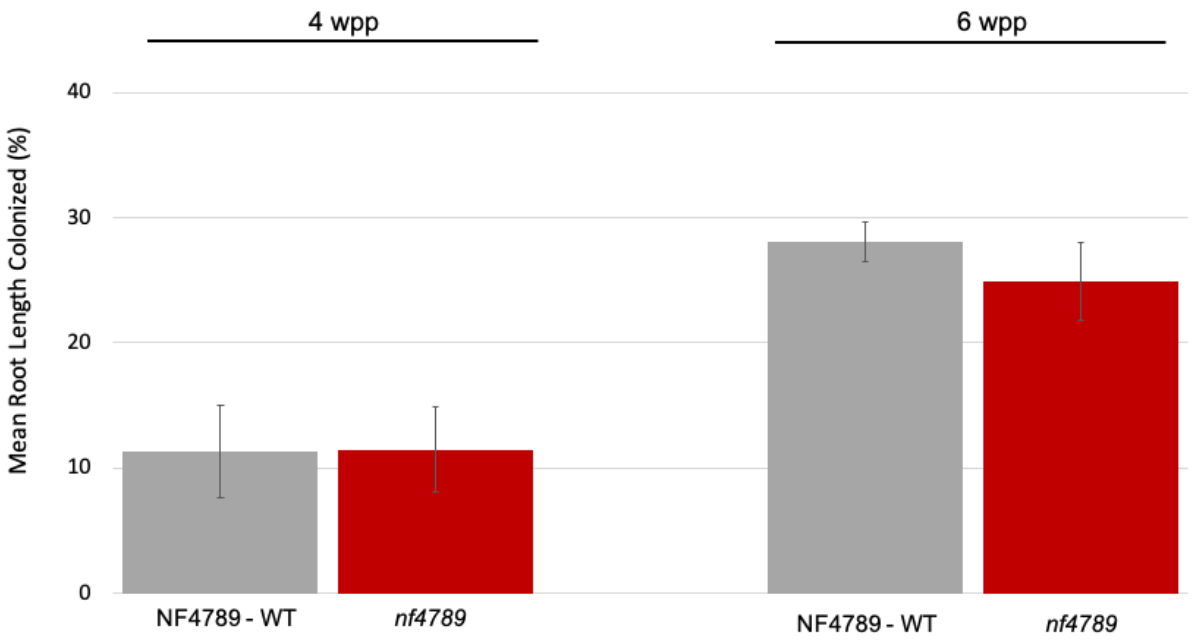

Figure 3.3.3. Screening the involvement of the *Medicago truncatula* Medtr5g031270 gene (encodes TIR-NB-LRR) in the AM symbiosis. Comparison of mean root length colonized between the *nf4789* mutant and the NF4789 segregating WT inoculated with *Rhizophagus irregularis* at two time points (4 wpp, and 6 wpp). Error bars indicate standard error, n = 8, 8, 5, and 6, respectively. Data were compared using a two-tailed t-test ($p>0.05$). Three separate graphs represent the independent experiments carried out using the NF4789 mutant line. Around 23% of all plants used in this assay were genotyped.

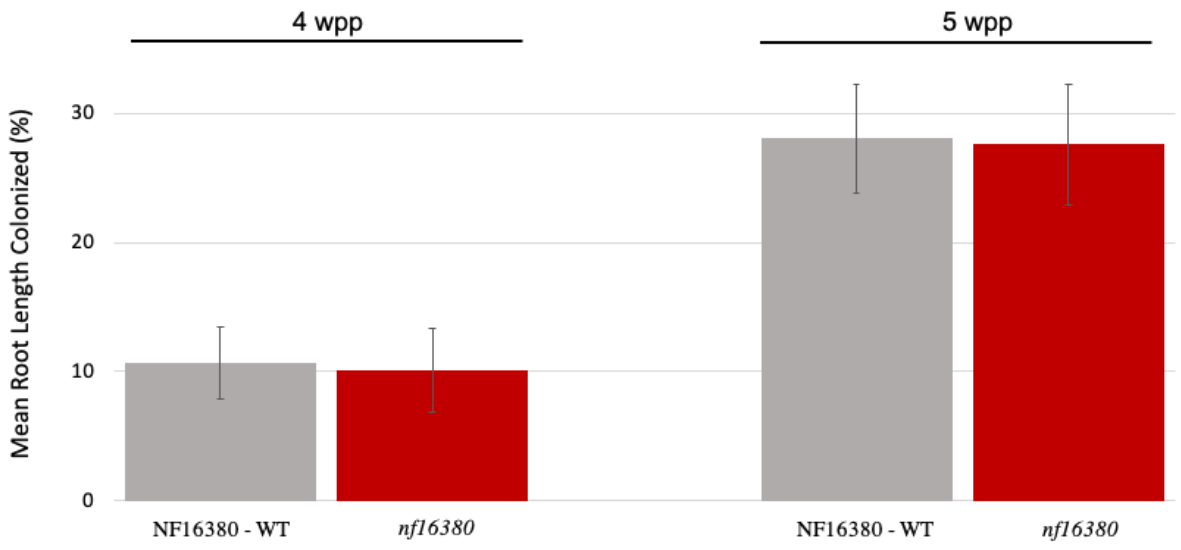

Figure 3.3.4. Screening the involvement of the *Medicago truncatula* Medtr5g031270 gene (encodes TIR-NB-LRR) in the AM symbiosis. Comparison of mean root length colonized between the *nf16380* mutant and the NF16380 segregating WT inoculated with *Rhizophagus irregularis* at two time points (4 wpp and 5 wpp). Data shown is averaged and error bars indicate standard error, n= 6. All of the plants used in this assay were genotyped. Statistical analysis was completed using a two-tailed t-test.

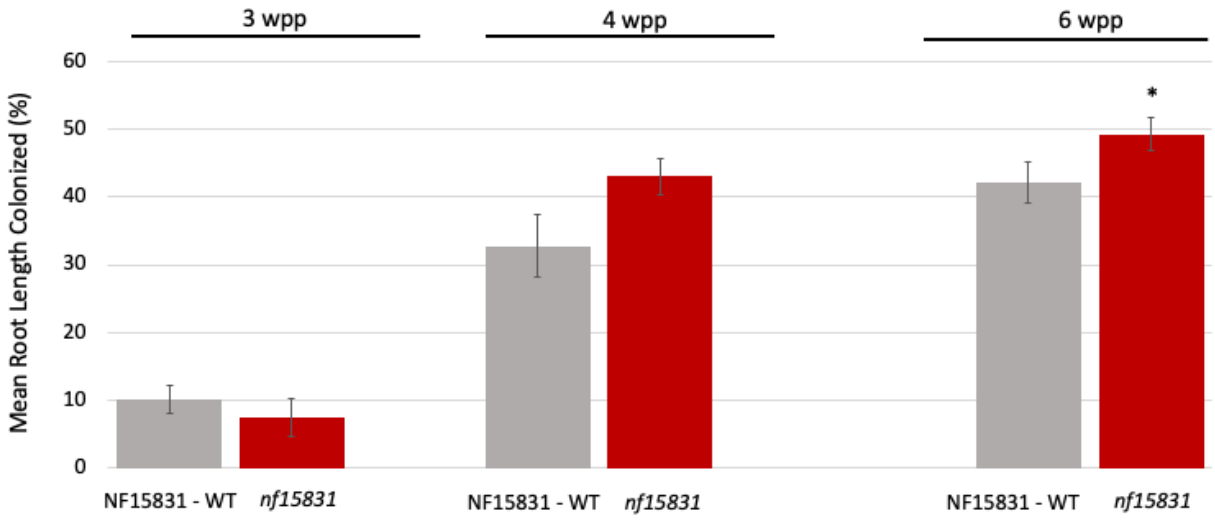

Figure 3.3.5. Screening the involvement of the *Medicago truncatula* Medtr4g023060 gene *(*encodes TIR-NB-LRR) in the AM symbiosis. Comparison of mean root length colonized between the *nf15831* mutant and the NF15831 segregating WT inoculated with *Rhizophagus irregularis* at three time points (3 wpp, 4 wpp, and 6 wpp). Data shown is averaged and error bars indicate standard error, n = 6, 6, 6, 6, 7, and 8, respectively. Asterisks indicates p-value < 0.05 in a two-tailed *t*-test comparing mutants and control plants. Two separate graphs represent the independent experiments carried out using the NF15831 mutant line. Around 40% of all plants used in this assay were genotyped.

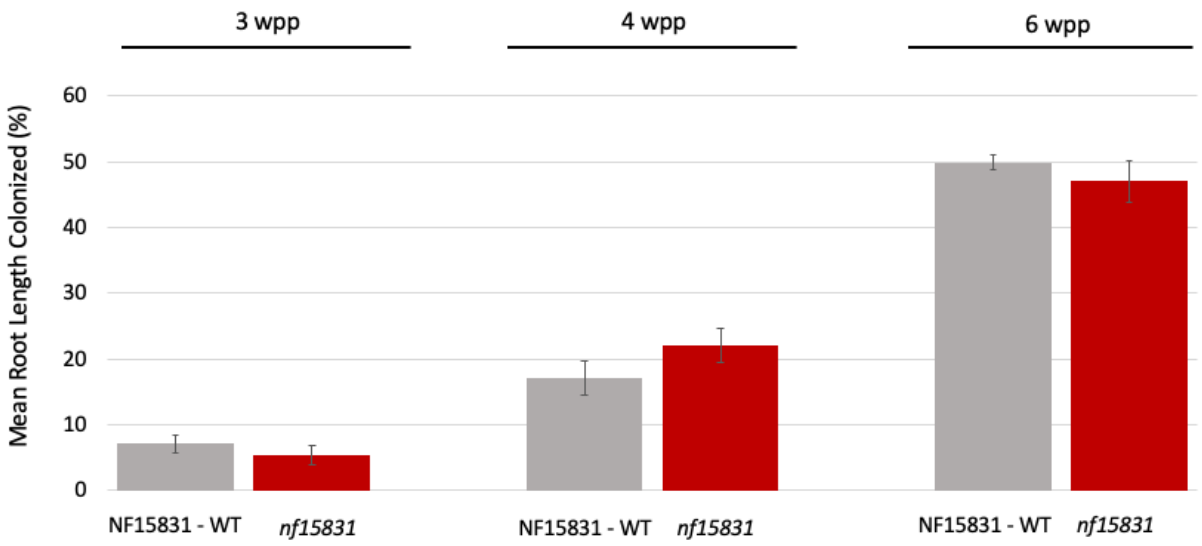

Figure 3.3.6. Screening the involvement of the *Medicago truncatula* Medtr4g023060 gene (encodes TIR-NB-LRR) in the AM symbiosis. Comparison of mean root length colonized between the *nf15831* mutant and the NF15831 segregating WT inoculated with *Rhizophagus irregularis* at three time points (3 wpp, 4 wpp, and 6 wpp). Data shown is averaged and error bars indicate standard error, n = 6, 6, 7, 7, 6, and 7, respectively. Two separate graphs represent the independent experiments carried out using the NF15831 mutant line. Around 40% of all plants used in this assay were genotyped. Statistical analysis was completed using a two-tailed t-test.

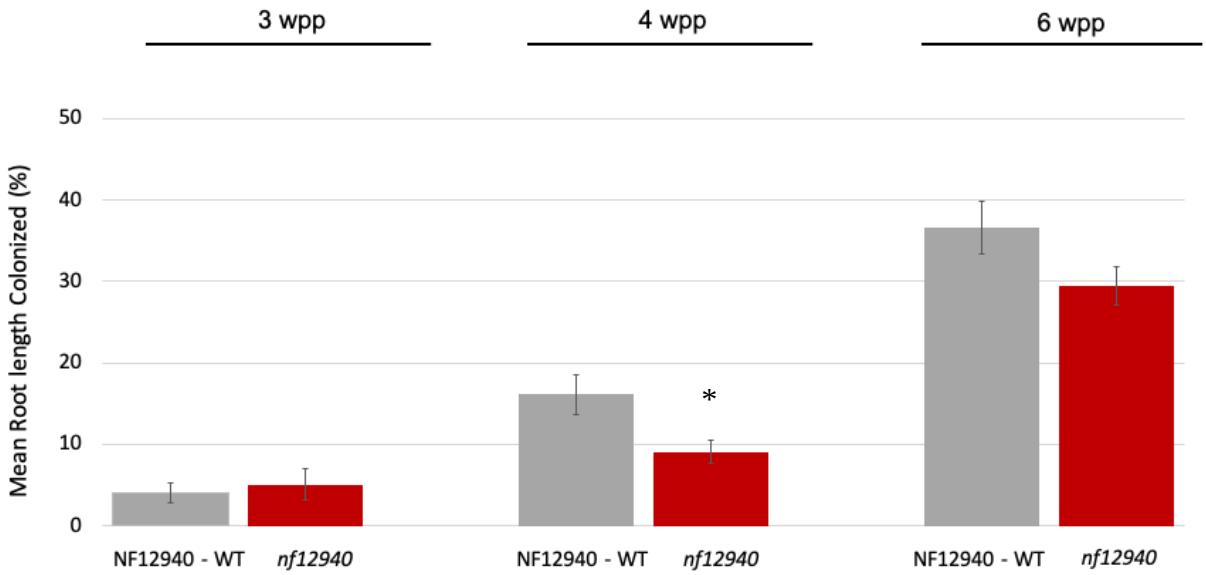

Figure 3.3.7. Screening the involvement of the *Medicago truncatula* Medtr3g078633 gene (encodes EDS1-like protein) in the AM symbiosis. Comparison of mean root length colonized between the *nf12940* mutant and the NF12940 segregating WT inoculated with *Rhizophagus irregularis* at three time points (3 wpp, 4 wpp, and 6 wpp). Data shown is averaged and error bars indicate standard error, n = 6, 6, 8, 7, 6, and 6, respectively. Asterisks indicates p-value < 0.05 in a two-tailed *t*-test comparing mutants and control plants. Two separate graphs represent the independent experiments carried out using the NF12940 mutant line. Around 18% of all plants used in this assay were genotyped.

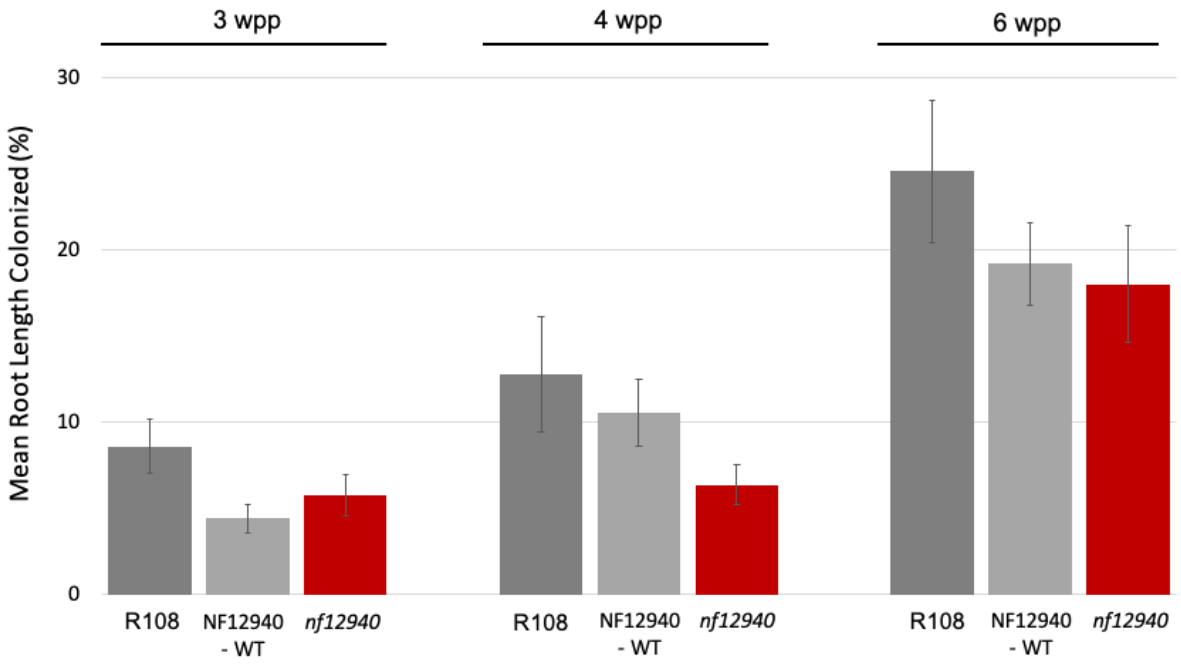

Figure 3.3.8. Screening the involvement of the *Medicago truncatula* Medtr3g078633 gene (encodes EDS1-like protein) in the AM symbiosis. Comparison of mean root length colonized between the *nf12940* mutant, the NF12940 segregating WT and the R108 WT inoculated with *Rhizophagus irregularis* at three time points (3 wpp, 4 wpp, and 6 wpp). Data shown is averaged and error bars indicate standard error, n = 7, 8, 6, 8, 8, 6, 7, 8, 6 respectively. Two separate graphs represent the independent experiments carried out using the NF12940 mutant line. Around 18% of all plants used in this assay were genotyped. At 4 wpp and 6 wpp, the *M. truncatula* roots had root nodules indicating that they were contaminated with rhizobia. Statistical analysis was completed using a one-way ANOVA.

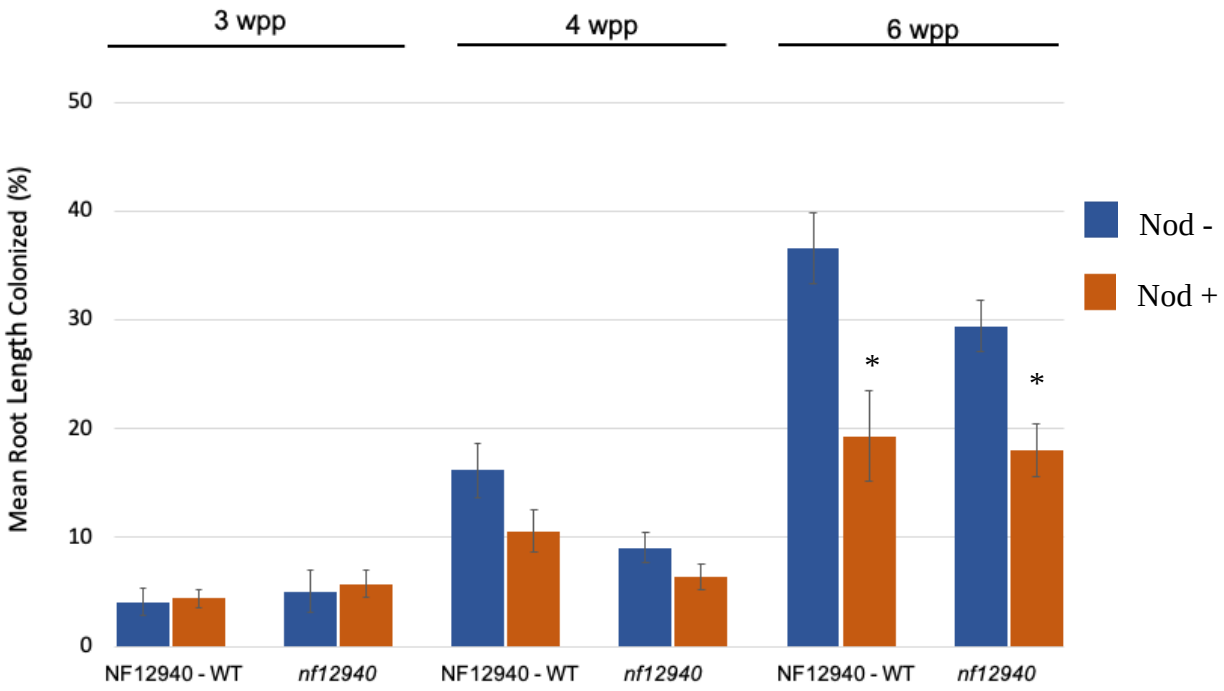

Figure 3.3.9. Mean root length colonized of *Medicago truncatula medtr3g078633* (EDS1) mutant and segregating WT inoculated by *Rhizophagus irregularis* with (nod +) and without (nod -) root nodules at three time points (3 wpp, 4 wpp and 6 wpp). Data shown is averaged and error bars indicate standard error, n = 6, 8, 6, 7, 7, 8, 8, 8, 6, 7, 6, and 8 respectively. Asterisks indicates p-value < 0.05 in a two-tailed *t*-test comparing nod + and nod – groups.

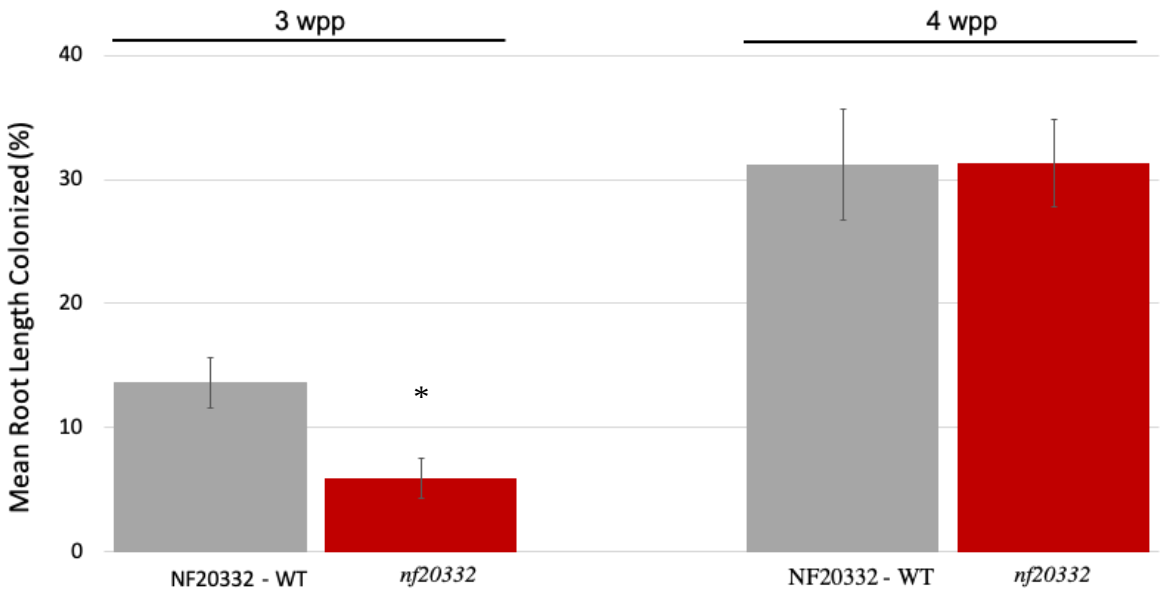

Figure 3.3.10. Screening the involvement of the *Medicago truncatula* Medtr8g087420 gene (encodes LRR-kinase) in the AM symbiosis. Comparison of mean root length colonized of the *nf20332* mutant and the NF20332 segregating WT inoculated with *Rhizophagus irregularis* at two time points (3 wpp and 4 wpp). Error bars indicate standard error, n = 6, 6, 7, and 8 respectively. Asterisks indicates p-value < 0.05 in a two-tailed *t*-test comparing mutants and control plants. Exactly 25% of all plants used in this assay were genotyped.

CHAPTER FOUR

Discussion and Conclusion

4.1. TIR-NB-LRR's in the arbuscular mycorrhizal symbiosis

Seven symbiosis assays were completed to determine if two genes encoding TIR-NB-LRR defence proteins were involved in the mean root length colonized of *Medicago truncatula* inoculated with *Rhizophagus irregularis*. The Medtr5g031270 knockout displayed a statistically significant increase in mean root length colonized in one (**Figure 3.3.1**) out of three NF4789 assays screened (**Figures 3.3.1-3.3.3)**. A second mutant line, NF16380, was also used to screen Medtr5g031270, however, this line did not have the *Tnt1* insertion in a coding region, and therefore we were not confident that this gene was a true mutant. There was no statistically significant difference between the *Tnt1* mutant and the segregating WT in the NF16380 line (**Figure 3.3.4)**. The second TIR-NB-LRR gene screened, Medtr4g023060, displayed a statistically significant increase in the mean root length colonized at 6 wpp in one (**Figure 3.3.5)** of the two NF15831 mutant line assays (**Figure 3.3.5 – 3.3.6**).

There are many possible reasons for these inconsistencies such as natural variability (Section 4.4.1), fully/partially redundant involvement (Section 4.4.2), or the AM fungal species used (Section 4.4.3). The presence of fungal effectors could also be affecting the functioning of the proteins. Two AM fungal effectors have been characterized in recent years (Kloppholz *et al.,* 2011; Voß *et al.,* 2018) and although neither of them have been shown to interact with TIR-NB-LRRs, pathogenic fungi have been shown to evade NB-LRR's to avoid increased defence signalling.

The data does not indicate that these two TIR-NB-LRRs play a clear role in the AM symbiosis. Although there was increased expression of these genes exclusively during the AM symbiosis, post-transcriptional or post-translational regulation could prevent the involvement of

the encoded proteins in the interaction. SiRNAs (short interfering RNAs) were found to target more than 60% of NB-LRR's in *M. truncatula* in order to regulate them post-transcriptionally and it has been postulated that these regulators could be involved in increasing mycorrhizal colonization (Zhai *et al.,* 2011). Ubiquitination confers post-translational regulation of defence genes and has been shown to regulate TIR-NB-LRRs (Cheng & Li, 2012) and LRR-kinases (Trujillo *et al.,* 2008). Therefore, although there is increased expression of the genes encoding the two TIR-NB-LRRs, there are other methods by which plants regulate protein levels.

It is also conceivable that these TIR-NB-LRRs are not required for WT colonization levels. Cereals do not have TIR-NB-LRRs in their genome (McHale *et al.,* 2006), although they do form the AM symbiosis (Sawers *et al.,* 2008). Therefore, TIR-NB-LRRs are not required for a successful AM symbiosis in many plant species such as wheat, rice, corn, and barley. It would be interesting to screen CC-NB-LRRs to determine if they play any clear role in the symbiosis.

Overall, there may be a small role that these TIR-NB-LRRs play in the AM symbiosis based on the statistically significant increase in mean root length colonized in two of the six assays used to screen two TIR-NB-LRRs. This would align with our second prediction in which an increase in mean root length colonized in *Tnt1* mutants likely means that the defence genes are increasing defence signaling against AM fungi. However, the data does not support this as a clear conclusion of these results and it is difficult to generalize due to the inconsistencies between symbiosis assays. More work should be done to determine if these genes are likely to play a role in the AM symbiosis. A key piece of information would be determining where these two TIR-NB-LRR's are expressed based on their promoter activity. Although these genes are upregulated in colonized roots, it is unclear in which cells specifically they are expressed. If they are present exclusively in cells containing arbuscules, for example, this may provide more

information regarding whether they play any role in the AM symbiosis. The plant transformations required for this information are currently ongoing. Current work outside the scope of this thesis is also in progress to screen the two TIR-NB-LRR's (Medtr5g031270, Medtr4g023060) for an overexpression phenotype which may uncover a trend that was not consistently present in the knockout assays.

4.2. EDS1-like protein in the arbuscular mycorrhizal symbiosis

The EDS1 gene receives and organizes signals derived from the TIR-NB-LRR defence pathway. In this thesis, we screened one gene encoding an EDS1-like protein (Medtr3g078633) that was upregulated during the AM symbiosis. Our results showed a phenotype of reduced colonization in one (**Figure 3.3.7**) of the two assays at 4 wpp (**Figure 3.3.7-3.3.8**). The second assay, despite the standard error bars not overlapping, did not show a statistically significant phenotype ($p = 0.07$) at 4 wpp. This assay was contaminated with rhizobia, which was evident based on the nodules that had formed on the *M. truncatula* roots at 4 wpp and 6 wpp. We cannot conclusively determine if rhizobia impacted the root length colonized between the treatment groups in the second assay. For this reason, another experiment was set up with great care taken to ensure there was no rhizobia contamination. The sample size was also increased to 13 to account for any natural variability in the samples. This assay is currently ongoing.

When the first and second NF12940 assays were compared to each other, there was a statistically significant difference in mean root length colonized at 6 wpp (**Figure 3.3.9**). When AM fungi and rhizobia occupy the same roots, the competition for resources increases which can reduce AM fungal colonization (Konvalinková & Jansa, 2016), as seen in these results. The data

was included in the thesis as it serves as a comparison between the mean root length colonized of *Tnt1* mutant plants with and without root nodules.

If a phenotype of reduced colonization is present in the ongoing EDS1 assay, it would be interesting to delve deeper into the functioning of this protein to parse the role it plays in the AM symbiosis and the downstream signalling involved in the complex interactions. A complementation of the phenotype would be required, along with an assay where the *Rhizophagus irregularis* alpha-tubulin and the *M. truncatula* PT4 transcripts, both specific to the AM symbiosis, are analyzed (Bravo *et al.,* 2016). This would provide a more thorough analysis of the effect of the EDS1 gene on the AM symbiosis. Results of this screening would provide valuable insight into the signalling that occurs downstream of AM fungal recognition. In addition to screening this EDS1-like gene, it would be interesting to screen an NDR1 gene, a similar signalling protein to EDS1 that functions downstream of CC-NB-LRRs (Aarts *et al*., 1998).

Overall, no solid conclusions can be drawn from the screening of the EDS1-like gene in *M. truncatula* due to the reliability of the data in the second symbiosis assay being affected by rhizobia contamination. Current work not in the scope of this thesis will provide greater insight to the role, if any, that EDS1 plays in the AM symbiosis.

4.3. LRR-Kinases in the arbuscular mycorrhizal symbiosis

One LRR-kinase (Medtr8g087420) upregulated during AM symbiosis was screened for involvement in the mutualism by undergraduate student Monique Power under my supervision. This assay displayed a statistically significant decrease in the mean root length colonized at 3 wpp (**Figure 3.3.10**), aligning with our prediction that a decrease in mean root length colonized indicates that the gene of interest is downregulating defence signalling in response to AM fungi.

At 4 wpp, there was no phenotype, and the mean root length colonized was almost identical between the two treatments. Since LRR-kinases are involved in early (and sometimes transient) responses (Zipfel, 2010), it is possible that the LRR-kinase knockout delays the entry of the AM fungi into the root at 3 wpp.

In conclusion, these preliminary results fit with the prediction that this LRR-kinase is involved in suppressing defence signals to allow colonization by AM fungi. However, more work is required to follow-up with these results and confirm the hypothesis. Another assay has been planned for the near future, and to determine if there is a difference in successful root entry between the *Tnt1* mutants and segregating WT, the number of successful appressorium will also be screened similar to the work done by Feddermann *et al.,* (2008).

4.4. General discussion

Screening the many thousands of genes involved in the arbuscular mycorrhizal symbiosis is a difficult (MacLean, 2017) but necessary task that allows us to determine which genes influence this globally important mutualism. We identified four defence genes of interest and screened their involvement in the AM symbiosis. Results from these symbiosis assays were varied, with the two TIR-NB-LRRs showing an inconsistent phenotype of increased colonization (**Figures 3.3.1-3.3.6**), while the EDS1 (**3.3.7**) and LRR-kinase (**3.3.10**) showed a clear phenotype of reduced AM fungal colonization and require follow-up experiments to confirm the observations.

Implications of this study could be very important for understanding how the two partners in this ecologically important relationship interact with each other, depending on the outcome of follow-up experiments that are not within the scope of this thesis. In this section, I

discuss some reasons for variability within and between the symbiosis assays, go over the potential for redundant involvement of these genes, and consider how the AM fungal species used in these experiments may have affected the outcome.

4.4.1. Variability within and between symbiosis assays

There are two distinct observations of variability in the symbiosis assays. The first being the variability within biological replicates depicted by the standard error bars. The second is the variability between technical replicates where there were sometimes large differences in mean root length colonized despite harvesting occurring at the same wpp. There were great efforts made to ensure all biological and technical replicates were placed in the exact same treatment conditions. Variability could be random, or it could be caused by the position of each replicate in the growth chamber causing small differences in light availability, or by differences in the nutrient status of the plants (Ji & Bever, 2016).

4.4.1.1. Variability within biological replicates

Although there are large error bars (around 10% SE) within biological replicates in the majority of the symbiosis assays (**Figures 3.3.1, 3.3.3, 3.3.4, 3.3.5, 3.3.7, 3.3.10**), this appears to be typical of the mutant lines, as Bravo *et al.,* (2016) and An *et al.,* (2019) depict a similar standard error in their symbiosis experiments using NF mutants from the Noble Foundation. Some of the assays in this experiment had data points which were far above or below the mean root length colonized but were not considered outliers. A larger sample size may have accounted for the presence of such data points, however, Bravo *et al.,* (2016) had a sample size of 4 for all of their assays, and still observed a phenotype of reduced colonization in six of the seven

mutants they screened. For the genes screened in this thesis, a larger sample size (>8) may be required to effectively observe the role these genes play in the symbiosis, especially if there is some redundancy.

4.4.1.2. Variability between technical replicates

There are also some large differences in the level of colonization between the segregating WT and mutant lines in different assays, which also appears to be a standard trend based on the data shown by Bravo *et al.,* (2016) where at 5 wpp some segregating WT lines had around 40% colonization (FatM – WT, KIN2 – WT) and others had as little as 17% colonization (KIN3 – WT). This was observed in the experiments in this thesis apart from the rhizobia contamination that may have caused a decrease in colonization in the second assay screening the EDS1-like gene (Section 4.2). Since the comparisons made in these experiments are within each individual assay between the *Tnt1* mutants and their segregating WT counterparts, these differences in colonization levels between technical replicates should theoretically bear little effect on our results and conclusions. If a gene of interest plays a role in the symbiosis under these experimental conditions, it should be consistently apparent despite differences in colonization between assays.

4.4.2. Potential for redundant involvement

It is possible that the genes we've screened are either fully or partially functionally redundant. There are some examples in previous research that describe genes that are partially redundant in the AM symbiosis but are still involved (An *et al.,* 2019; Maillet *et al.,* 2011). The MtNFP gene codes for a LysM receptor-like kinase upstream of the common SYM pathway that

is partially involved in the host response to lipochitooligosaccharides (Maillet *et al.,* 2011). However, the phenotype of the *mtnfp* mutant is WT (Ben Armor *et al.,* 2003). Furthermore, the MtSWEET1b glucose transporter has increased expression in cells containing arbuscules, and is located near the peri-arbuscular membrane in cortical cells but mutant knockouts of this gene did not produce a statistically significant difference in colonization or arbuscule abundance of *M. truncatula* inoculated by *R. irregularis* at 5 wpp (An *et al.,* 2019). However, when MtSWEET was over-expressed, the maintenance of arbuscules was reduced causing enhanced collapse of the structures. The overall conclusions of this study were that the MtSWEET gene had a redundant role in glucose transport (An *et al.,* 2019).

As suggested by An *et al.,* (2019) the knockout of a gene may not be sufficient to show a statistically significant phenotype of reduced colonization. In addition to an over-expression experiment, a double mutant knockout of two potentially redundant defence genes, or of genes that encode proteins functioning upstream/downstream of one another (such as a TIR-NB-LRR and an EDS1-like protein) could provide interesting insights.

4.4.3. Impact of AM fungal species on the symbiotic machinery

AM fungi can cheat host plant out of carbon resources by failing to provide the level of nutrients required to make the mutualism beneficial for the plant. As a 450 million year old relationship, persistent cheaters through time would de-stabilize the plant-AM fungi mutualism (Grman, 2012). To prevent this, host plants distinguish between high-quality and low-quality AM fungal partners and allocate carbon to the individuals providing greater benefits (Grman, 2012). Although the mechanism used to identify the quality of the AM fungal partners in this

context is unknown, the fitness of the individuals that are more beneficial to the plant increases, while the fitness of "cheaters" decreases, thereby stabilizing the mutualism (Grman, 2012).

R. irregularis is considered a high-quality partner (Kiers *et al.,* 2011) and therefore may be subject to a different set of defence mechanisms than lower-quality counterparts (Feddermann *et al.,* 2008). When Feddermann *et al.,* (2008) inoculated *M. truncatula* with three different fungal species, *R. irregularis* colonized 60% of the root, while *Glomus mossaeae* colonized 30% and *Scutellospora castanea* colonized a mere 10% at 10 wpp. Importantly, there was a specific sub-set of genes that were exclusively induced during colonization by *S. castanea*. *R. irregularis* was also the most successful at entering the roots based on counts of appressoria per successful root entry (i.e. appressoria were successful most often) (Feddermann *et al.,* 2008).

Gao *et al.,* (2004) showed that in tomato, the upregulation of the rmc defence gene, involved in AM fungal root penetration, differed based on the species of fungi used. Furthermore, there was a statistically significant difference in the total root length colonized by *Scutellospora calospora* compared to *R. irregularis* in the *rmc* mutant. This indicates that there is potential for one defence gene to differentially impact different AM fungal species. Therefore, some of the defence genes we screened, although induced during colonization with *Rhizophagus irregularis*, may not be necessary for the WT colonization of this particular species. Since AM fungi are functionally diverse, it is conceivable that the species quality affects not only the gene expression, but the post-transcriptional (Mallory and Vaucheret, 2006) and post-translational regulation of defence proteins (Cheng and Li, 2012; Trujillo *et al.,* 2008).

In addition to screening the involvement of defence genes using different AM fungal species, it would also be interesting to see how nutrient concentrations affect the outcomes. In these symbiosis experiments, we used a relatively low concentration of phosphorus and nitrogen.

Under these conditions, AM fungi are more likely to form the mutualism, as there is a lower-abundance of nutrients (Balzergue *et al*., 2013). Increasing the nutrient concentrations may influence the functioning of the proteins encoded by the GOIs as the requirement for the AM fungi is reduced (Ji & Bever, 2016; Balzergue *et al*., 2013; Blanke *et al*., 2005).

4.4.4. Conclusions

Plants and AM fungi have an ancient intimate relationship that likely enabled the terrestrialization of the first land plants (Harrison, 2005). The nutrients transferred to plants by AM fungi fill a necessary gap in global plant nourishment, making the AM symbiosis an important component of global plant health, and therefore agriculture (Zhang *et al.,* 2016; Koide & Elliott, 1989). There are many gaps in the knowledge regarding the set of genes that are involved in the mutualism. In particular, it is unclear how plants identify AM fungi and allow them to colonize their roots in order to gain the nutritional benefits they provide. To help fill these gaps, we tested four *Medicago truncatula* defence genes for their involvement in the mean root length colonized by AM fungi *Rhizophagus irregularis*. The results of these experiments showed that the two TIR-NB-LRRs screened were not essential for the AM symbiosis. The EDS1-like gene showed a phenotype of reduced colonization in one assay, while a second assay had root nodules indicating rhizobia contamination. Current work is in progress to identify whether this gene plays a role in this mutualism. One LRR-kinase also showed a statistically significant reduction in colonization and another assay has been planned for the near future. Importantly, the promoter activity of three of the four genes (TIR-NB-LRR's and LRR-kinase) will be determined, which will provide valuable insights into the functioning of these genes. Overall, although no clear conclusions could be made from the results in this thesis, there are

promising preliminary results to follow-up on that could provide novel information regarding the plant receptors involved in AM fungal recognition.

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Supplemental information:
S1. Background insertions in *Tnt1* mutant lines

Supplemental Table 1.1. Background insertions in the *Medicago truncatula* NF4789 mutant line from the Noble Foundation. This table displays the insertions that potentially truncate off-target proteins, the insertion number, protein type, molecular function and the biological process. Information obtained from uniprot.org. All other insertions did not return a NCBI blast hit

Insertion Number	Protein type	Protein Function	Biological Process
2	Serine/threonine-protein kinase tricorner[1]	Protein serine/threonine kinase activity	Signal transduction
6	Putative UDP-glucose glucosyltransferase[2]	Glycotransferase	Polysaccharide biosynthesis
7	Berberine bridge enzyme-like[3]	Catalyzes oxidation	Oxioreductase activity
9	AIG2-like protein[4]	Catalyses acyl group transfer	transferase activity
10	Subtilisin-like protease SBT1.5[5]	Catalysis of hydrolysis of internal peptide bonds	Protein Maturation
19	1-aminocyclopropane-1-carboxylate synthase 7[6]	Catalyzes precursor of ethylene	Ethylene biosynthesis
20	Histone-lysine N-methyltransferase ATX3[7]	DNA binding	Positive regulation of histone H3-K4 methylation
26	3-oxoacyl-[acyl-carrier-protein] synthase I, chloroplastic[8]	Catalyses condensation of fatty acid synthesis	Fatty acid biosynthesis
27	Alpha carbonic anhydrase 7[9]	Interaction with Zinc	Hydration of carbon dioxide
29	Pre-mRNA-processing-splicing factor 8A[10]	Pre-RNA intronic binding	Alternative mRNA splicing
31	COP1-interactive protein 1	Actin binding	Abiotic stress response
32	26S proteasome non-ATPase regulatory subunit 3[12]	Enzyme regulator activity	Removing misfolded or damaged proteins
23	Histidine kinase 113	Cytokinin receptor activity	Many cellular responses such as cold response

Supplemental Table 1.2. Background insertions in the *Medicago truncatula* NF15831 mutant line from the Noble Foundation. This table displays the insertions that potentially truncate off-target proteins, the insertion number, protein type, molecular function and the biological process it is involved in were obtained from uniprot.org. All other insertions did not return a NCBI blast hit.

Insertion Number	Protein type	Molecular Function	Biological Process
1	Cytochrome P450 93A3 [14]	Iron Ion Binding	Various
3	Serine/arginine-rich splicing factor SR30 [15]	RNA binding	mRNA splicing
12	B-box zinc finger protein 20 [16]	DNA-binding transcription factor	Photoprotection in natural environments
20	Cysteine-rich receptor-like protein kinase 10 [17]	Protein serine/threonine kinase activity	Defence response to bacterium
29	Probable calcium-binding protein CML21[18]	Calcium ion binding	Calcium-mediating signal
30	Pectinesterase inhibitor 3[19]	Pectinasterase inhibitor	Response to fungus, bacteria and nematode trying to enter cell wall
33	Receptor-like protein 9DC3[20]	Binds to effectors	Defence response to fungal pathogen *C. fulvum*
38	UDP-glycosyltransferase TURAN (69% identities)[21]	Mannosyltransferase activity	Pollen tube development

Supplemental Table 1.3. Background insertions in the *Medicago truncatula* NF16380 mutant line from the Noble Foundation. This table displays the insertions that potentially truncate off-target proteins, the insertion number, protein type, molecular function and biological process. Information obtained from uniprot.org. All other insertions did not return a NCBI blast hit.

Insertion number	Protein Name	Molecular Function	Biological Process
3	Cation/H(+) symporter 13[22]	Potassium Importer	Plays a role in potassium acquisition
5	Exocyst complex component	Docking of exocytic vesicles	Golgi to plasma membrane transport
12	DETOXIFICATION 49	Transmembrane transporter activity	Transport
13	Glutathione S-transferase F9	Copper ion binding	Defence response to bacterium
17	Disease resistance protein RML1A	ATP binding	Defence response to fungal pathogen
19	Cyclin-A3-2	Kinase regulator activity	Cell division
21	Heat shock cognate 70 kDa protein	ATPase activity	Cellular response to heat
23	Transcription repressor OFP5	Regulates transcription factors	Embryo development
28	Transcription factor MYB48	Transcription factor activity	Response to salicylic acid
31	Probable serine/threonine-protein kinase	ATP binding	Defense response
32	Type I inositol polyphosphate 5-phosphatase 5	Phosphatase activity	Inositol phosphate dephosphorylation
35	Squamosa promoter-binding-like protein 2	DNA-binding TF activity	Anther development
36	Disease resistance-like protein DSC1	ATP binding	Defence response to bacterium
40	Putative GEM-like protein 8	N/A	N/A

Supplemental Table 1.4. Background insertions in the *Medicago truncatula* NF12940 mutant line from the Noble Foundation. This table displays the insertions that potentially truncate off-target proteins, the insertion number, protein type, molecular function and biological process. Information obtained from uniprot.org. All other insertions did not return a NCBI blast hit.

Insertion number	Protein Name	Molecular Function	Biological Process
3	*Medicago truncatula* NADPH-dependent pterin aldehyde reductase	Oxidioreducatase* (involved in symbiosis)	Folic acid-containing compound metabolic process
4	Galactinol-raffinose galactosyltransferase	Raffinose alpha-galactosidase activity	Transferase activity, transferring glycosyl groups

Supplemental Table 1.5. Background insertions in the *Medicago truncatula* NF20332 mutant line from the Noble Foundation. This table displays the insertions that potentially truncate off-target proteins, the insertion number, protein type, molecular function and the biological process. Information obtained from uniprot.org. All other insertions did not return a NCBI blast hit.

Insertion number	Protein Name	Molecular Function	Biological Process
2	Kinesin-like protein KIN-14E	Actin filament binding	Cortical microtubule organization

S2. Bacterial strains and plasmids used.

Supplemental Table 2.1. Bacterial strains and plasmid used in this thesis.

Strain or plasmid	Description – what is it used for, what genes are in it	Source/reference
(SPT, STR) Medtr5g031270: GUSNosT/pKm43GWRR	Used to determine promoter activity of the Medtr5g031270 TIR-NB-LRR. Includes the Medtr5g031270 promoter, the GUS gene, a NosT terminator and the pKm43GWRR backbone	Plasmid created by Mina Nasr-Sharif using GateWay™ technology. pKm43GWRR backbone obtained from Dr. Maria Harrison's lab at Cornell University
(SPT, STR) 4g023060: GUS-NosT/pkm43GWRR	Used to determine promoter activity of the Medtr4g023060 TIR-NB-LRR. Includes the Medtr4g023060 promoter, the GUS gene, a NosT terminator and the pKm43GWRR backbone	Created by Mina Nasr-Sharif using GateWay™ technology. pKm43GWRR backbone obtained from Dr. Maria Harrison's lab at Cornell University
(SPT, STR) 8g087420: GUS-NosT/pkm43GWRR	Used to determine promoter activity of the Medtr8g087420 LRR-kinase. Includes the Medtr8g087420 promoter, the GUS gene, a NosT terminator and the pKm43GWRR backbone	Created by Mina Nasr-Sharif using GateWay™ technology. pKm43GWRR backbone obtained from Dr. Maria Harrison's lab at Cornell University
(SPT, STR) BCPp: NLSGFPGUS/pkm43GWRR	Used as a control for the promoter activity experiments. Includes the *Medicago truncatula* BCPp promoter region, an NLS sequence, GFP gene, GUS gene and the pKm43GWRR backbone	Full plasmid obtained from Dr. Maria Harrison's lab at Cornell University
(SPT, STR) AtUBQ: GUS-NosT/pkm43GWRR	Used as a control for the promoter activity experiments. Includes the *Arabidopsis thaliana* UBQ10 promoter region, an NLS sequence, GFP gene, GUS gene and the pKm43GWRR backbone	Created by Mina Nasr-Sharif using GateWay™ technology. The UBQ10 gene, and the pKm43GWRR backbone obtained from Dr. Maria Harrison's lab at Cornell University

S3. Primers used in this thesis

Supplemental Table 3.1. Primers used for isolating DNA from *Medicago truncatula* tissues

Primer name	Description	Sequence (5'-3')
NF15831_Fw	Gene specific primers for NB-LRR gene in NF15831 mutant line	GGATCATCTCTATCACTCTCTTCTCC
NF15831_Rv	Gene specific primers for NB-LRR gene in NF15831 mutant line	GTGTCTGACACTCCTATGACACTC
NF4789_Fw2	Gene specific primers for NB-LRR gene in NF4789 mutant line	CATGTACAAATCCAGAATGGGACTTCG
NF4789_Rv	Gene specific primers for NB-LRR gene in NF4789 mutant line	AGTCTGGAATAGCAATATTAGTCAGAACAG
NF12940_Fw	Gene specific primers for EDS1 gene in NF12940 mutant line	GTTGGGACTGAAAAGTGAGTATAGG
NF12940_Rv	Gene specific primers for EDS1 gene in NF12940 mutant line	GTTGAAGCGATTCTTCCAAGACTCG
NF20332_Fw	Gene specific primers for LRR-kinase gene in NF20332 mutant line	GAATGCACAATCACAGTCTCATAACACC
NF20332_Rv	Gene specific primers for LRR-kinase gene in NF20332 mutant line	CCAATCCAACTCACAATGTCTACCTG
NF16380_Fw	Gene specific primers for NB-LRR gene in NF16380 mutant line	GTACAGGTAATTGAATACGTGTCTTTCAG
NF16380_Rv	Gene specific primers for NB-LRR gene in NF16380 mutant line	GTAATATTCAACGTAATCATGTTCCAGTGC

*Tnt1*_F2	Primers for *Tnt1* mutants corresponding to *Tnt1* insertion sites	TCTTGTTAACCGTATCTCGGTGCTACA
*Tnt1*_Rv	Primers for *Tnt1* mutants corresponding to *Tnt1* insertion sites	AGTTGGCTACCAATCCAAGGA
5g031270_attB4For	To isolate promoter region of *M. truncatula* 5g031270 gene	GGGGACAACTTTGTATAGAAAAGTTGTAATGATGGCGATGTAGCATCC
5g031270_attB1Rev	To isolate promoter region of *M. truncatula* 5g031270 gene	GGGGACTGCTTTTTTGTACAAACTTGCTGTCACAGGTTAATAGTGACAAAATAC
8g087420_attB4For	To isolate promoter region of *M. truncatula* 8g087420 gene	GGGGACAACTTTGTATAGAAAAGTTGCCTCGTGAACTTAGTTCAATTGG
8g087420_attB1Rev	To isolate promoter region of *M. truncatula* 8g087420 gene	GGGGACTGCTTTTTTGTACAAACTTGCTTCTCTTTCATGAAACATGATTAGAG
4g023060_attB4For	To isolate promoter region of *M. truncatula* 4g023060 gene	GGGGACAACTTTGTATAGAAAAGTTGTGCTCGTGGTGTAGTTGGTTTGG