Identifying *NPF* Genes Involved in Arbuscular Mycorrhizal Symbiosis

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi are a group of fungi that are able to establish a symbiotic relationship with the root system of many land plants. This symbiosis improves plant fitness by increasing the uptake of crucial mineral nutrients, particularly phosphorus and nitrogen. In return, the fungi receive organic carbon from the plant host in the form of sugars and lipids. The objective of my research is to assess whether the Nitrate and Peptide Transporter Family (*NPF*) of transport proteins play a role in mediating AM symbiosis. Firstly, we explored the involvement of NPF genes *NPF1B* and *NPF4.12* by examining the phenotype of *Medicago truncatula* mutants. Secondly, we employed a modified yeast two-hybrid system to determine the phytohormone import capabilities of these *NPF* transport systems. Lastly, we ~~are~~ employed reporter gene fusions to assess the spatial and temporal expression profiles of these *NPF* genes. The results of our research do not support our hypothesis that these *NPF* genes play a role in mediating AMF symbiosis. The results of the modified yeast-two hybrid tests revealed abscisic acid (ABA) and gibberellic acid (GA_3) import capabilities of the transport system encoded by the gene *NPF4.12*. Future study of the diverse mechanisms that underpin AM symbiosis will nonetheless be useful to the agricultural industry by reducing farmer's reliance on chemical fertilizers.

RESUMÉ

La symbiose mycorhizienne arbusculaire (AM) sont un groupe de champignons capables d'établir une relation symbiotique avec le système racinaire de nombreuses plantes terrestres. Cette symbiose améliore la condition physique des plantes en augmentant l'absorption de nutriments minéraux essentiels, en particulier le phosphore et l'azote. En retour, les champignons reçoivent du carbone organique de la plante hôte sous forme de sucres et de lipides. L'objectif de ma recherche est d'évaluer si la famille des protéines de transport, la famille des transporteurs de nitrates et peptides (NPF) joue un rôle dans la médiation de la symbiose AM. Tout d'abord, nous avons exploré l'implication des gènes NPF, NPF1B et NPF4.12 en examinant le phénotype des mutants de Medicago truncatula. Deuxièmement, nous avons utilisé un système à deux hybrides de levure modifié pour déterminer les capacités d'importation de phytohormones de ces systèmes de transport NPF. Enfin, nous utilisons des fusions de gènes rapporteurs pour évaluer les profils d'expression spatiale et temporelle de ces gènes NPF. Les résultats de nos recherches n'ont pas soutenu notre hypothèse selon laquelle ces gènes NPF jouent un rôle dans la médiation de la symbiose AMF. Les résultats des tests hybrides levure-deux modifiés ont révélé les capacités d'importation d'acide abscissique (ABA) et d'acide gibbérellique (GA) du système de transport codé par le gène NPF4.12. L'étude future des divers mécanismes qui sous-tendent la symbiose AM sera néanmoins utile à l'industrie agricole en réduisant la dépendance des agriculteurs aux engrais chimiques.

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ABBREVIATIONS

ABA = abscisic acid

AD = activating domain

AM = arbuscular mycorrhizal

AMF = arbuscular mycorrhizal fungi

DBD = DNA-binding domain

Dpp = Days post planting

DO = dropout

GA = gibberellic acid

GUS = β-glucuronidase

IAA = indole-3-acetic-acid

KOH = potassium hydroxide

LCO = lipochitooligosaccharides

MT = mutant

NPF = nitrate and peptide transporter family

PBS = phosphate buffered saline

PPA = pre-penetration apparatus

SWT = segregating wild type

WGA = wheat germ agglutinin

Wpp = Weeks post planting

WT = wild type

Y2H = yeast-two hybrid

YPD = yeast extract peptone dextrose

INTRODUCTION

Chapter 1: Overview of Arbuscular Mycorrhizal Fungi

1.1 Plant-microbe interactions

Plants associate with a wide range of microorganisms that facilitate the acquisition of nutrients and protect against biotic and abiotic stresses. Over hundreds of millions of years, these associations between plants and their microscopic counterparts has led to an array of complex relationships and interactions. These relationships can take place in natural and agricultural environments and include mainly partnerships with bacteria and fungi (Wille et al., 2017). Mutualistic plant-microbe relationships can benefit the plant by improving plant health and growth, enhancing disease resistance, and facilitating nutrient uptake (Lugtenberg et al., 2002; Morrissey et al., 2004). However, not all plant-microbe interactions are beneficial or initiated on the part of the plant (Lugtenberg et al., 2002). Rust is a fungal plant pathogen that forms a parasitic relationship with plants and can reduce photosynthesis by 50% due to its ability to alter leaf physiology (Gortari et al., 2018). Based on the rapidly expanding field of research focused on plant-microbe interactions, microbial inoculants are being developed for agricultural use as biofertilizers, phytostimulators, and biopesticides (Berg., 2009).

1.2 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) are a group of fungi within *Glomeromycota* that establish symbiotic and mutually beneficial relationships with the root systems of many plants. Eighty percent of land plant species have the ability to establish this mycorrhizal symbiosis (Wang & Qiu, 2006), making this one of the most widespread and significant partnerships in the world. Fossils containing fungal structures have been found dating back >400 million years in

the early Devonian land plant *Aglaophyton major* (Remy et al., 1994). It is thought that the initial appearance of AMF coincides with origins of the first established land plants such as liverworts (Wang & Qiu, 2006). This mutualistic partnership equipped the plants, which were poorly suited to the new terrestrial environment, to combat issues such as desiccation and starvation of essential nutrients (Pirozynski & Malloch, 1975).

AMF are obligate biotrophs that require a mutualistic association with plant roots to produce spores and complete their life cycle (Rosendahl, 2008). These characteristics make this symbiont difficult to study because the fungus cannot be genetically transformed (Harrier & Millam, 2001), nor cultivated in the absence of its host. It has also been difficult to classify AMF into specific species groups because fungal taxonomy does not follow the same process as plants or animals (Rosendahl, 2008). The distinction between a population and individuals is clouded due to the clonal nature seen in AMF populations (Rosendahl, 2008). Their multinucleate nature leads to them carrying thousands of nuclei in their cells (Kokkoris et al., 2020). The shape and organization of the nuclei can vary within and among different AMF species (Kokkoris et al., 2020). AMF are also fatty acid auxotrophs and lack the fatty acid synthase (FAS) gene which allows organisms to synthesize fatty acids (Kobayashi et al., 2018). Accordingly, the host plant synthesizes its own fatty acids and delivers these to the fungal symbiont (Luginbuehl et al., 2017). This is a major source of organic carbon for the fungus as it allows them to produce their own essential fungal lipids (Luginbuehl et al., 2017).

1.3 Pre-contact signalling between AMF and plants

The mutualistic association between AMF and the plant roots will be initiated when conditions are conducive to symbioses, for example when there is a lack of phosphorus readily

available in the soil (Yoneyama et al., 2007). AMF spores lie dormant in the soil until plant root exudates are sensed in the surrounding substrate (Bonfante & Genre, 2010). One example of these exudates are strigolactones, phytohormones secreted by the plant that also act to stimulate spore germination and subsequent branching of the fungal hyphae, mediating an initiation of symbiosis at low strigolactone concentrations (Besserer et al., 2006). Both hyphopodium formation and expansion of root colonization have been shown to be suppressed in *Oryza sativa* mutants that lack strigolactone biosynthesis genes (Kobae et al., 2018).

On the AMF side, so-called "myc" factors secreted by the fungus are recognized by the host plant and initiate a symbiotic accommodation program (Luginbuehl & Oldroyd, 2013). One class of these myc factors, lipochitooligosaccharides (LCOs), stimulate root growth and branching in the model legume *Medicago truncatula* through the common symbiosis signalling pathway (Maillet et al., 2011). *dmi1*, *dmi2*, and *dmi3* plant mutants are unable to establish a symbiotic association with endomycorrhizal fungi (Catoira et al., 2000). The three associated genes also share central roles in the rhizobial Nod factor-activated signal transduction pathway (Catoira et al., 2000). This demonstrates that AM and rhizobial symbioses share common components within their distinct pathways. When LCOs activate the common symbiosis signalling pathway, there is a resulting pattern of oscillation in calcium levels that may be observed in root epidermal cells of *M. truncatula* (Sun et al., 2015). The nature of the calcium oscillations appears to be similar to that triggered by LCOs produced by rhizobial bacteria, however, mycorrhizal LCOs activate a distinct gene expression profile (Sun et al., 2015). DMI1 and DMI2 are both essential for these mycorrhizal-induced calcium oscillations (Kosuta et al., 2008).

1.4 Post-contact reorganization

Once the pre-contact signalling has been established between the host plant and its mycorrhizal partner, physical changes to the structures and cellular organizations of both organisms occur. The branching hyphae of the fungus make contact along the root surface to find a preferable location to differentiate into hyphopodia (Genre et al., 2005) and subsequently penetrate the epidermis (Bonfante & Genre, 2010). Hyphopodia are fungal structures that allow the fungus to tightly adhere to the root epidermis. Several host genes involved in signal transduction, defence response and cell wall modification are significantly up-regulated during the early stages of AM contact with the host plant's roots (Siciliano et al., 2007). At this point, epidermal cells will reorganize their cytoplasm to produce a structure called the pre-penetration apparatus (PPA), that is necessary for successful fungal penetration (Genre et al., 2005). The fungus will develop a thick column at a site of initial cytoplasmic aggregation and move in across the cell (Genre et al., 2005). The tip of the hyphae will subsequently move through the epidermal cell wall along the track of the PPA (Genre et al., 2005).

Once the root epidermal cell layer has been penetrated, the fungal hyphae will spread throughout the plant root, reaching the inner cortex of the root (Luginbuehl & Oldroyd, 2017). At this point, the hyphae differentiate within the cortical cells into highly branched arbuscules (MacLean et al., 2017). Extensive reorganization of the cortical cells will occur, which includes the deposition of the periarbuscular membrane around the arbuscule, resulting in a membrane-bound apoplastic compartment (MacLean et al., 2017). Nutrient transfer between the plant and fungus is conducted across this common apoplast (MacLean et al., 2017). Periarbuscular membrane-localised proteins, such as phosphate transporters, are incorporated in the new

membrane to achieve the specialised membrane composition required for nutrient exchange (Luginbuehl & Oldroyd, 2017). Figure 1 is a schematic depiction of the colonization process.

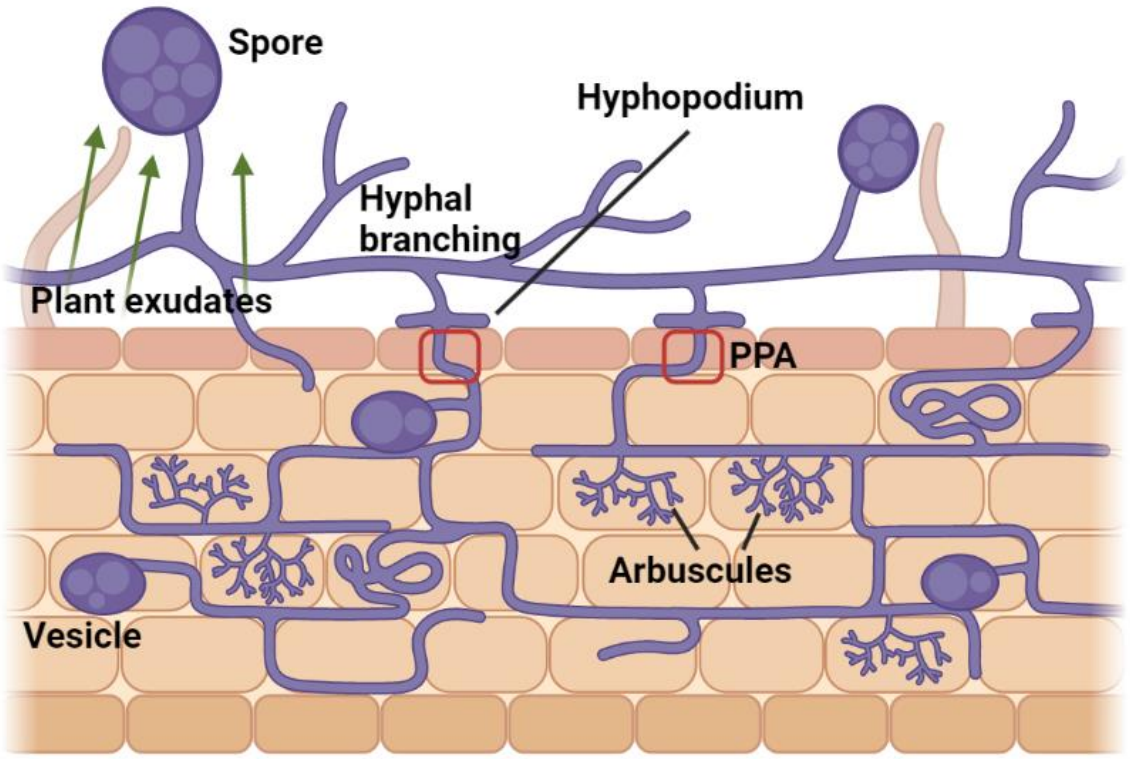

Figure 1. Colonization of a plant root by arbuscular mycorrhizal fungi. Dormant AMF spores in the soil detect plant exudates such as strigolactones. Hyphal branching begins in the direction of the plant root until contact is made. The hyphae differentiate into the hyphopodium, then the pre-penetration apparatus (PPA) forms to allow fungal hyphae to penetrate the root epidermis. The hyphae enter root cortical cells and differentiate into arbuscules to facilitate nutrient exchange between the plant and AMF. Figure adapted from Bonfante & Genre., 2010.

1.5 Mutualistic benefits

Mycorrhizal symbiosis can improve plant fitness by increasing the uptake of crucial nutrients, particularly phosphorus (Kiers et al., 2011). In many ways, the fungal symbiont can be seen as an extension of the plant's root system. The extraradical fungal mycelium in the surrounding substrate can access and absorb soil minerals that are not directly accessible to plant roots (Smith & Smith, 2011). This is due in part to the presence of high-affinity nitrate and phosphate transporters that have been identified to be expressed in the extraradical mycelium (Luginbuehl & Oldroyd, 2017; Benedetto et al., 2005). The very large surface area to volume ratio of fungal hyphae in the soil also greatly improves nutrient absorption (Schnepf et al., 2008). It is not yet known whether the release of nutrients from the fungal symbiont into the periarbuscular space is a passive process or undertaken by fungal transporters that are unknown (Luginbuehl & Oldroyd, 2017)

The increased quantity of nutrients available to the plant allows it to allocate more resources towards improving disease resistance (Chen et al., 2019). The colonization of the invasive plant *Wedelia trilobata* by the model AMF species *Rhizophagus irregularis* was shown to significantly reduce the infection area of the pathogenic fungus *Rhizoctonia solania* (Chen et al., 2019). Greenhouse studies have revealed that AMF significantly reduce the negative effects that accompany drought (Li et al., 2019). An *in situ* rainfall exclusion experiment was also carried out to evaluate the effects of indigenous AMF on plant drought resistance. AMF significantly reduced the negative effects on plant growth in terms of plant biomass, photosynthetic rate, stomatal conductance, and intrinsic water use efficiency (Li et al., 2019). This confirmed the results of other greenhouse studies (Abbaspour et al., 2012) and demonstrated that AMF confer benefits that can be applied to real-world scenarios. In return, the

fungi receive organic carbon in the form of sugars and lipids (Luginbuehl et al., 2017). The host plant will direct 4% to 20% of its photoassimilates to the accompanying mycorrhizal systems (Bago et al., 2000).

1.6 Role of phytohormones in AM symbiosis

AM symbiosis requires specific conditions to be met in order to establish and maintain a successful relationship between the plant host and the fungal symbiont. Phytohormones play a significant role in regulating the symbiosis, both positively and negatively. Gibberellic acid (GA) has been shown to inhibit AM symbiosis (Foo et al., 2013). Foo et al., 2013 showed that AMF colonization rates of pea roots were substantially increased in gibberellin-deficient mutants compared to their wild type counterparts. Interestingly, Takeda et al., 2015 showed that GA can also promote fungal colonization of the host root. It was demonstrated that low-GA conditions suppressed arbuscular mycorrhiza-induced subtilisin-like serine protease1 (*SbtM1*) expression that is associated with AMF colonization, leading to reduced hyphal branching in the host root (Takeda et al., 2015). Inhibition of Abscisic acid (ABA) biosynthesis negatively altered parameters of AMF colonization in tomato plants such as frequency of colonization, intensity of colonization, and arbuscule abundance (Martın-Rodrıguez et al., 2011). An antagonistic interaction between ABA and ethylene during AM formation (Martın-Rodrıguez et al., 2011) shows that there is a complex interaction between AM symbiosis and many phytohormones.

1.7 Impact of chemical fertilizers

The agricultural revolution had a world-wide impact on the way food is produced and consumed. Nitrogen, in the form of nitrates, is a limiting nutrient required by plants in the largest

quantity in many soils (Patterson et al. 2010). In most agricultural settings, nitrate is the primary form of inorganic nitrogen provided (Falkengren-Grerup., 1995). It has been estimated that 85-90 million tons of nitrogen fertilizers are added to soil around the world annually, which has increased substantially from the 1.3 million tons used in 1930 (Good et al., 2004). The nitrogen fertilizer uptake efficiency in cereal crops has been estimated to be below 50% (Jayasundara et al., 2007).

With the large amounts of fertilizer required to support modern agriculture and a low efficiency of fertilizer uptake from soil by many plants, a problem has arisen. The excess fertilizer that is not absorbed by agricultural crops will leach into surrounding environments, contaminating habitats (Chakraborty et al., 2017). Fertilizer runoff into waterways can cause an excess of algae growth, resulting in algal blooms (Chakraborty et al., 2017). Algal blooms deplete dissolved oxygen from the water (Malone et al., 1986) and produce noxious substances that are detrimental to the health of aquatic life (Sand-Jensen & Borum., 1991). Increased levels of eutrophication can also lower the level of light reaching the bottom-dwelling plants residing in bodies of water, causing them to die off (Sand-Jensen & Borum., 1991). We anticipate that as our understanding of the mutualistic relationship between plants and AMF advances, the widespread application of potentially detrimental fertilizers can be phased out in favour of safer alternatives.

Chapter 2: Nitrate and Peptide Transporter Family

2.1 Nitrate and Peptide Transporter Family

The Nitrate and Peptide Transporter Family (NPF) is a family of transporters within *Plantae*. Its members transport a variety of substrates such as nitrates, peptides, amino acids, dicarboxylates, glucosinolates, and phytohormones such as indole-3-acetic-acid (IAA), and ABA (Léran et al., 2014). A unified nomenclature for the NPF family was collectively agreed upon in Léran et al., 2014. Originally, these transporters were classified as a part of the NRT1/PTR family, named after the substrate which is transported: NRT1 referring to a nitrate transporter and PTR, a peptide transporter. As newer members were subsequently found to transport more than one substrate, a different nomenclature was then established. *NPF4.1* is a known importer of both ABA and GA (Chiba et al., 2015). NRT1/CL1 was the first member of this family to be characterized by Tsay et al., 1993. It would later be renamed *NPF6.3*, and this protein was found to be a part of NPF subfamily 6 (Corratgé-Faillie & Lacombe., 2017). *NPF6.3* is an interesting case because it is the only transporter within the family that has exhibited dual levels of affinity for its substrates (Prabhala et al., 2021). It functions as a high-affinity nitrate transporter, but in its phosphorylated state it functions as a low-affinity nitrate transporter (Liu & Tsay., 2003). This allows the plant to rapidly adapt to the nutrient availability in its environment, which is crucial when competing for limited nitrogen (Liu & Tsay., 2003).

The amino acid sequences of all NPF members were analyzed using the BigPlant phylogenomic pipeline in Lee et al., 2011. These proteins were queried against each other, using BLAST, to determine the level of homology (Léran et al., 2014). OrthoMCL, a program that groups orthologous protein sequences, used a clustering algorithm to classify all known members of the NPF family (Léran et al., 2014). Using this approach, NPF was determined to contain 8

distinct subfamilies, which have been organized based on a parsimony analysis of the 2398 sequences from 33 fully sequenced genomes (Léran et al., 2014).

The NPF transporter proteins use an electrochemical proton gradient to uptake their given substrate (Jorgensen et al., 2017). Many NPF transporters, such as *NPF6.*3, *NPF*6*.4* and *NPF6.6* are localized to the plasma membrane (Wen et al., 2017), but they also appear to be localized to other locations such as the tonoplast (He et al., 2017). In terms of structural information, two *AtNPFs* have exhibited a canonical overfold with twelve transmembrane helices interconnected by a loop through their crystal structure (Prabhala et al., 2021).

Table 1 summarizes known NPF substrates for each of the 8 subfamilies in *Arabidopsis thaliana* and demonstrates how exceptionally broad the substrate range is within this family of proteins. Most NPF members are still uncharacterized and phylogenetically close family members may have different functions, so it is not reliable to consider phylogeny alone when determining transporter function (Harris & Dickstein., 2010). Also, not much is known about the evolution of transport substrate specificities within the NPF family (Jorgensen et al., 2017).

Table 1. Summary of known NPF substrates in *Arabidopsis* by NPF subfamily. Data summarized from Corratgé-Faillie & Lacombe., 2017.

NPF Subfamily	Known Substrates
NPF1	Nitrate (NO_3-), Abscisic acid, Gibberellic acid, Jasmonic acid-isoleucine
NPF2	Nitrate (NO_3-), Abscisic acid, Gibberellic acid, Jasmonic acid-isoleucine, glucosinolates, chloride
NPF3	Nitrate (NO_3-), Abscisic acid, Gibberellic acid, Jasmonic acid-isoleucine, Nitrogen dioxide (NO_2)
NPF4	Nitrate (NO_3-), Abscisic acid, Gibberellic acid, Jasmonic acid-isoleucine
NPF5	Nitrate (NO_3-), Abscisic acid, Gibberellic acid, Jasmonic acid-isoleucine, di-peptides
NPF6	Nitrate (NO_3-), indole-3-acetic-acid
NPF7	Nitrate (NO_3-)
NPF8	Nitrate (NO_3-), Abscisic acid, Gibberellic acid, Jasmonic acid-isoleucine, di-peptides, histidine

2.2 Nitrate and Peptide Transporter Family and Medicago truncatula

Medicago truncatula has emerged as an excellent model for legumes, having a small, diploid genome, short generation time, and high transformation efficiency (Bell et al., 2001). The diploid genome and autogamous nature makes *M. truncatula* a more attractive model organism due to ease of transformation (Rose., 2008). The regenerative ability of this model legume is essential for its ability to be transformed (Rose., 2008). *M. truncatula* is also able to establish symbiotic relationships with AMF and nitrogen-fixing rhizobia within its roots (Bell et al., 2001). Moreover, it is one of the plant species in which the common symbiosis signalling pathway is found (Maillet et al., 2011).

The NPF family has been widely studied in *M. truncatula*. To this point, the sequenced genome of *M. truncatula* has enabled an identification of 80 genes encoding putative *NPFs* (Léran et al., 2014). Several of the identified NPF members in *M. truncatula* have been revealed to have a wide range of roles in nitrate transport (Pellizzaro et al., 2017). *Medicago truncatula NPF6.8* acts as a nitrate sensor during the cell elongation of the primary root (Pellizzaro et al., 2017). Certain *M. truncatula NPF* (*MtNPF*) genes also have a role in establishing symbiotic relationships with microorganisms. Our collaborators at the University of Vermont found that *MtLATD/NIP* (*MtNPF1*.7) is a gene essential for developing lateral and primary roots, as well as the development of nodule meristems and rhizobial invasion of nodules (Harris & Dickstein., 2010). ABA was hypothesized to be a potential substrate for this *MtNPF* gene due to the observation that the lateral and primary root defects exhibited by *MtLATD/NIP* mutants can be rescued by exogenous application of ABA (Harris & Dickstein., 2010).

2.3 Genes of interest

NPF transporters within *NPF1* and *NPF4* subfamilies are the focus of this research project. The *NPF1* subfamily contains 5 different clades based on their evolutionary relationships, one of which is *NPF1B*. Expression of *NPF1B* and *NPF4.12* genes is highly up-regulated during AM symbiosis, making these interesting candidates to assess for a potential role in the mediation of this beneficial symbiosis. These are 2 of the 657 genes in *M. truncatula* which show increased gene expression through RT-PCR in roots colonized with AMF (Gomez et al., 2009). The *Medicago truncatula* Gene Expression Atlas, which is hosted by the Noble Research Institute, is a powerful resource for visualizing levels of gene expression in all major organ systems of the species (Benedetto et al., 2008). Most relevant to this research project, the expression of many *M. truncatula* genes was assessed under conditions such as AM symbiosis and rhizobial symbiosis (Benedetto et al., 2008). The gene expression atlases for *NPF1B* and *NPF4.12* candidate genes are demonstrated in Figure 2. *NPF1B* is particularly interesting because a duplication event gave rise to *NPF1B* early in flowering plants and this duplicated gene has been maintained throughout the angiosperm lineage exclusively in plants that have the capacity to engage in AM symbiosis (Sassi., 2019). One hypothesis is that *NPF1B* has an ancestral role in initiating and/or maintaining AM symbiosis, which is why this gene has been retained in so many mycorrhizal plant species since the duplication event.

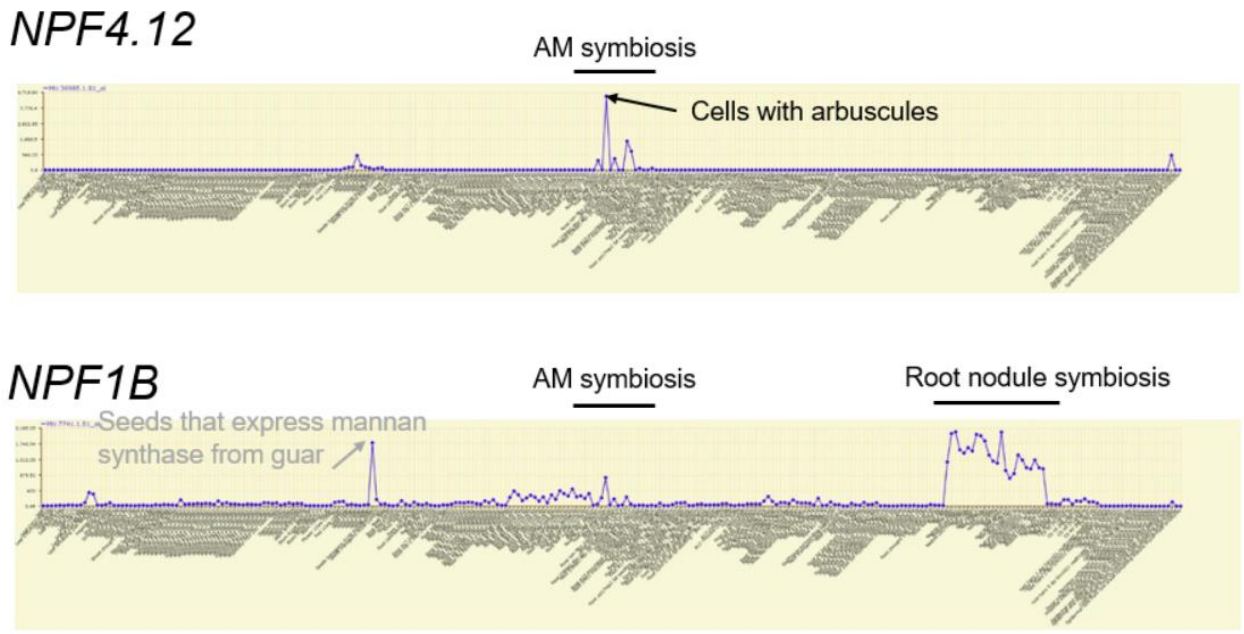

Figure 2. Gene Expression Atlas of *NPF4.12* and *NPF1B* under various conditions.

Information obtained from the Noble Research Institute (noble.org). *NPF4.12* exhibits up-regulation of gene expression during AM symbiosis. *NPF1B* exhibits up-regulation of gene expression during AM and root nodule symbiosis and is highly expressed in seeds.

Chapter 3: Modified Yeast-Two Hybrid Assays

In the past, a variety of techniques have been used to identify and characterize NPF family members. Functional characterization has been performed upon transformed *Xenopus* oocytes using electrophysiological approaches and substrate quantification with radioactive isotopes, non-radioactive isotopes, and fluorescent-tagged molecules as labelled substrates (Corratgé-Faillie & Lacombe., 2017). An alternate approach involving *Saccharomyces cerevisiae* included a functional complementation of mutant yeast strains which were deficient for peptide transport (Steiner et al. 1994).

Yeast two-hybrid (Y2H) screening is a technique originally designed to study protein-protein interactions *in vivo* (Brückner et al., 2009). It is based on the activation of downstream reporter genes as a reconstituted transcription factor binds to the upstream activation site (UAS) (Brückner et al., 2009). In these assays, the Gal4 transcription factor has been separated into distinct modules comprising the activating domain (AD) and DNA-binding domain (DBD) (Brückner et al., 2009). The DBD binds to the UAS and the AD activates transcription via association with the RNA polymerase (Van Criekinge & Beyaert., 1999). In Y2H assays, a protein of interest is fused to the AD, while the other protein of interest is fused to the DBD (Van Criekinge & Beyaert., 1999). If two candidate proteins interact with one another, the Gal4 transcription factor will be brought together, allowing the AD and DBD to interact with one another, and with the UAS (Van Criekinge & Beyaert., 1999). At this point, transcription of the downstream reporter gene will occur, allowing the yeast strain to grow on selective media (Van Criekinge & Beyaert., 1999).

This system was modified by Kanno, et al., 2012 to investigate the role that plant transport systems have in importing phytohormones. The premise of the screen is that a receptor and interacting co-receptor relevant to a given phytohormone will only associate together in the presence of the particular phytohormone in question, which can be supplemented into the growth media. For example, the proteins PYR1 and ABI1 will associate together in the presence of the phytohormone ABA. In this system, PYR1 is fused to the GAL4 DNA-binding domain (DBD) and ABI1 is fused to the GAL4 activating domain (AD) (Chiba et al., 2015), allowing GAL4 domains to re-form upon interaction of PYR1 and ABI1, which should only occur in the presence of ABA. A third plasmid is then introduced to the yeast strain, encoding a candidate transport gene, in our case an *NPF* gene. If the transport system encoded by the *NPF* gene, imports ABA into the yeast cell (for example), there will be a phytohormone-dependent formation of a receptor complex resulting in the growth of the yeast (Chiba et al., 2015) in selective media supplemented with ABA. However, if the *NPF* gene product does not import ABA, the yeast will not grow upon this medium. Figure 3 demonstrates the modification of the yeast-two hybrid test to investigate phytohormone import capabilities of candidate *NPF* genes.

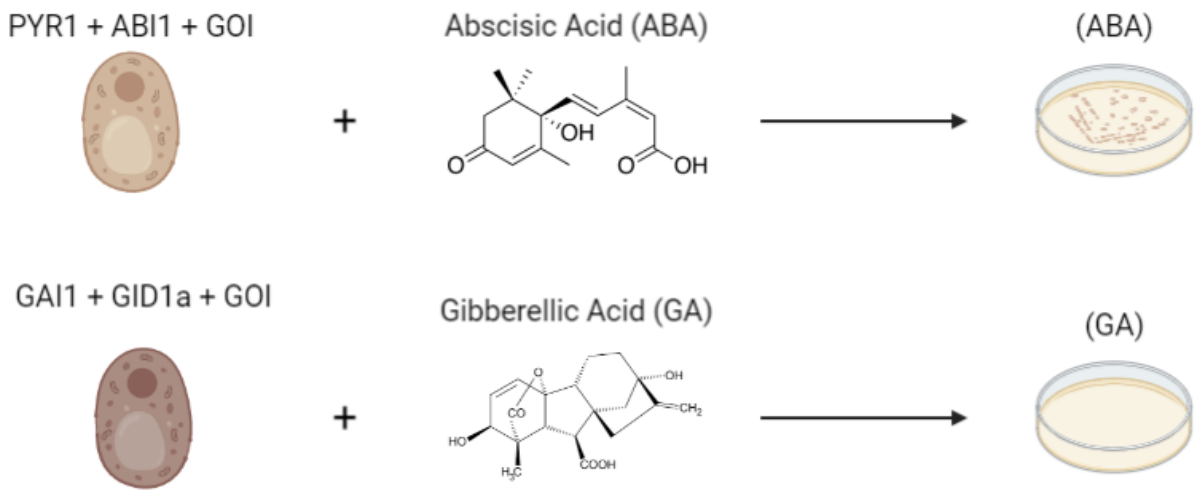

Figure 3. Modification of yeast two-hybrid assays to assess the candidate NPF import capabilities. *Saccharomyces cerevisiae* cells are transformed with plasmids encoding PYR1 + ABI1 + *NPF4.6* or GAI1 + GID1a + *NPF4.6* respectively. Defined minimal media upon which the transformed *S. cerevisiae* are grown will be supplemented with ABA or GA, respectively. In this hypothetical example, our gene of interest (GOI) can import ABA, but not GA.

Chapter 4: Research Hypotheses and Goals

4.1 Hypotheses

We hypothesize that *NPF1B* and *NPF4.12* encode transport systems that are involved in mediating AM symbiosis. This hypothesis has been proposed based upon data demonstrating that expression of these genes is strongly up-regulated during AM symbiosis.

4.2 Research goals

Our overarching research goal is to assess whether these genes are required or are involved in AM symbiosis. We will use the following methods to achieve this goal. The involvement of *NPF1B* and *NPF4.12* in mediating symbiosis will be directly assessed through the use of symbiosis assays. Root phenotypic assessments will be completed to ascertain whether *NPF4.12* contributes to the development of *M. truncatula* roots. We will also assess whether these transport systems mediate the transport of select phytohormones using modified yeast-two hybrid tests. Lastly, we will use a promoter/GUS fusion to visualize the expression of *NPF1B* in roots colonized by AMF.

MATERIALS AND METHODS

1.0 Symbiosis assays

Symbiosis assays were performed to determine if *M. truncatula Tnt1* mutants exhibit an altered symbiotic phenotype when inoculated with the AMF species *Rhizophagus irregularis*, when compared against wild type and segregating wild type plants. To perform these assays, two seedlings of the same genotype were planted into each cone-tainer. We included 7-12 replicates for each group, representing wild type, segregating wild type, and homozygous Tnt1 mutant lines collected at 1-2 different timepoints. Germination rates of sterilized seeds and survival of seedlings determined the number of replicates for the assay. All plants consisted of the R108 ecotype genetic background. The growth chamber where the plants were grown had a temperature of 25℃, light intensity of 160µM/m^2s, humidity of 60% and a photoperiod of 16 hours of light and 8 hours of dark.

1.1 Tnt1 mutant seeds

The *M. truncatula NPF1B* seeds used in this project were sent to us by Dr. Jeanne Harris from the Biology Department at the University of Vermont. The *M. truncatula* seeds from the NF9804 lines were received from the *Tnt1* mutant library at the Noble Research Institute. Homozygous mutant and segregating lines were generated by Dominique Daniels prior to this research project.

1.2 Seed sterilization

M. truncatula seeds were removed from their seed pods and counted in order to obtain a sufficient quantity for the assay (typically requiring 100 seeds per assay). The seeds were placed

in different Falcon tubes according to their genetic line and covered in concentrated sulfuric acid for 9 minutes to scarify the seed coats. The acid was removed and the seeds were rinsed five times with 50mL of sterile dH_2O. Next, 20mL of a 10% bleach and 0.1% tween solution was added to the seeds and put on a shaker with gentle agitation for 9 minutes to sterilize any microbial contamination. The seeds were rinsed again five times with 50mL of sterile dH_2O in the laminar flowhood. Next, the seeds were placed on sterile filter paper in Petri dishes. The seeds would be stored at 4℃ for 3 days, before transfer to a dark drawer at room temperature for 1 day, and finally placed by a window for 1-2 days until the germinated seedlings were ready to plant.

1.3 Cone-tainer preparation

An autoclaved 1 inch marble was placed on the bottom of each cone-tainer to prevent substrate from falling out of the holes in the bottom. Autoclaved Turface MVP which was soaked in ½ strength Hoaglands solution for 20 minutes was place in the bottom ¼ of the conetainer. Next a 50/50 mixture with the same Turface and autoclaved play sand filled up the conetainer to three-quarters. At this point, 200 spores (0.25g of *Rhizophagus irregularis*) were placed on top of the substrate. The mycorrhizal inoculant was obtained from MYKE® PRO (Lot#:15545433). The rest of the cone is then filled with the 1:1 mixture of sand and Turface. Lastly, the two seedlings of the same genotype were planted into the sand and Turface mix.

1.5 Harvesting of plants

Plant roots were harvested at their given timepoints of 3-6 weeks post planting. The roots were cut from the bottom of the stem, and surrounding sand and Turface substrate was washed off with water. Roots were then placed in a Falcon tube and covered in 50% ethanol for 20

minutes. Next, the ethanol was removed and the roots were covered in 20% potassium hydroxide (KOH) and incubated overnight at 65℃ to clear the roots of lignin to allow easier visualization. The next day, the KOH was removed and the roots were rinsed with phosphate buffer saline (1xPBS) and put on a gentle shaker three times to neutralize the KOH. After the last rinse, 10mL of PBS was added to the Falcon tube with the roots, which were stained with 10µL of wheat germ agglutinin (WGA) (200ng/µL) conjugated with Alexa-488 fluorophore. WGA is a protein that binds with high affinity to chitin, causing chitin to fluoresce under the microscope when excited by a laser at 488nm. The roots were stored in the fridge at 4℃, covered in aluminum foil to prevent bleaching of the WGA fluorophore.

1.5 Quantification of root colonization

The WGA-stained roots from each genetic line were spread over a plate with a grid (total plate size; 8cm x 8cm). A Zeiss Axiozoom Stereoscope was used to magnify the roots and to visualize fluorescence. Each root that crossed a line on the grid was scored as either "colonized" or "uncolonized" based upon the presence or absence of fungi, respectively, in that portion of the root. At the end, the quantity of "colonized" roots was divided by the total number of roots assessed, to obtain a percentage of mean root length colonization.

Mean root length colonized = ((Number of colonized roots)/(Number of total roots examined)) x 100%

1.6 Statistical analysis

Statistical analyses were performed upon colonization data obtained for each symbiosis assay that was performed. The colonization rates observed for each of the Tnt1 mutants (NF9804 and NF11778) were compared to those obtained for the wild type and segregating wild type

controls, and a one-way ANOVA was performed to determine the statistical significance ($p < 0.05$) of the data obtained for each of the different genotypes. A one-way ANOVA was likewise performed upon data collected from the phenotypic root assessments of the mutants and wild-type control plants (Section 4.0).

Table 2. List of genes of interest and relevant transgenic lines. WT represents the wild type line name, SWT represents the segregating wild type line name, and MT represents the mutant line name.

Gene of Interest	NPF Name	WT	SWT	MT
Medtr8g103233	*NPF1B*	R108	NF11778-1	NF11778-6
Medtr2g017750	*NPF4.12*	R108	NF9804-4	NF9804-1

2.0 Genomic DNA extraction

~2 leaves from each *M. truncatula* plant were removed and placed in 1.5mL Eppendorf tubes, which were immediately frozen in liquid nitrogen for the genomic DNA extraction. The plant matter in the tubes was crushed in liquid nitrogen using a plastic pestle. Next, 400µL of DNA extraction buffer (0.2M TRIS, 0.25M NaCl, 0.025M EDTA, 20% SDS) was added and the tubes were centrifuged at 12,000 xg for 5 minutes. 300µL of the supernatant was removed and transferred to a new Eppendorf tube, along with 300µL of 100% isopropanol. The tubes were centrifuged at 12,000 xg for 10 minutes to pellet the genomic DNA, and the resulting supernatant was decanted. Next, 500µL of 70% ethanol was added to wash the DNA pellet and the tubes were centrifuged as per the previous step. The ethanol was decanted, and the tubes were left to dry in a Flowhood for ~2 minutes to allow the rest of the ethanol to evaporate. 100µL of dH_2O was pipetted over the DNA pellet and the tube was gently vortexed to allow for resuspension. The samples were stored at -20°C.

3.0 Genotyping

The genotypes of the *Tnt1* mutant plants used in all symbiosis assays were verified to confirm the presence of the appropriate *Tnt1* insertion (Figure 6). Wild type (R108) and segregating wild type plants were also genotyped to confirm the presence of two wild type copies of the appropriate gene of interest.

3.1 Polymerase chain reaction (PCR)

Four different primer combinations were used to genotype plants from the symbiosis assays: 1) GS_F + Tnt1_F2, 2) GS_F + Tnt1_R2, 3) GS_R + Tnt1_R2, 4) GS_R + Tnt1_F2 (Nucleotide sequences for each primer included in Table 3). To genotype wild type and

segregating wild type plants, the appropriate gene-specific forward and reverse primers were used. Homozygous *Tnt1* mutants were included as a control when genotyping wild type and segregating wild type plants with gene-specific primers. R108 wild type plants were included as a control when genotyping *Tnt1* mutants with *Tnt1* primers. PCR products were visualized by electrophoresis on a 0.8% agarose gel containing RedSafe Nucleic Acid Staining Solution (20,000x) at 100 volts for 30 minutes.

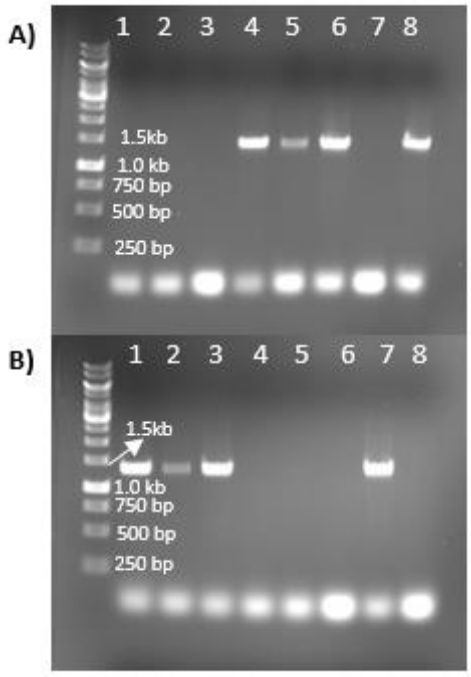

Figure 4. Representative photo of genotyping plant line NF11778 (*Medtr8g103233*) via PCR. Panel A primers: NF11778_F + NF11778_R. Panel B primers: NF11778_F + Tnt1_R. Lanes 4, 5, and 6 depict a PCR completed with DNA from segregating wild type plants at the gene of interest. Lanes 1, 2, and 3 depict a PCR completed on Tnt1 homozygous plants. Lane 7 is the NF11778 Tnt1 homozygous positive control. Lane 8 is the R108 wild type control.

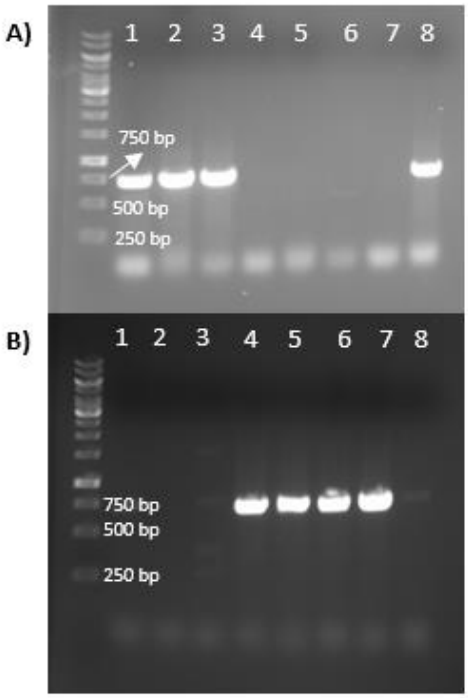

Figure 5. Representative photo of genotyping plant line NF9804 (*Medtr2g017750*) via PCR. Panel A primers: NF9804_F + NF9804_R. Panel B primers: NF9804_R + Tnt1_R. Lanes 1, 2, and 3 depict a PCR completed with DNA from segregating wild type plants at the gene of interest. Lanes 4, 5, and 6 depict a PCR completed on Tnt1 homozygous plants. Lane 7 is the NF9804 Tnt1 homozygous positive control. Lane 8 is the R108 wild type control.

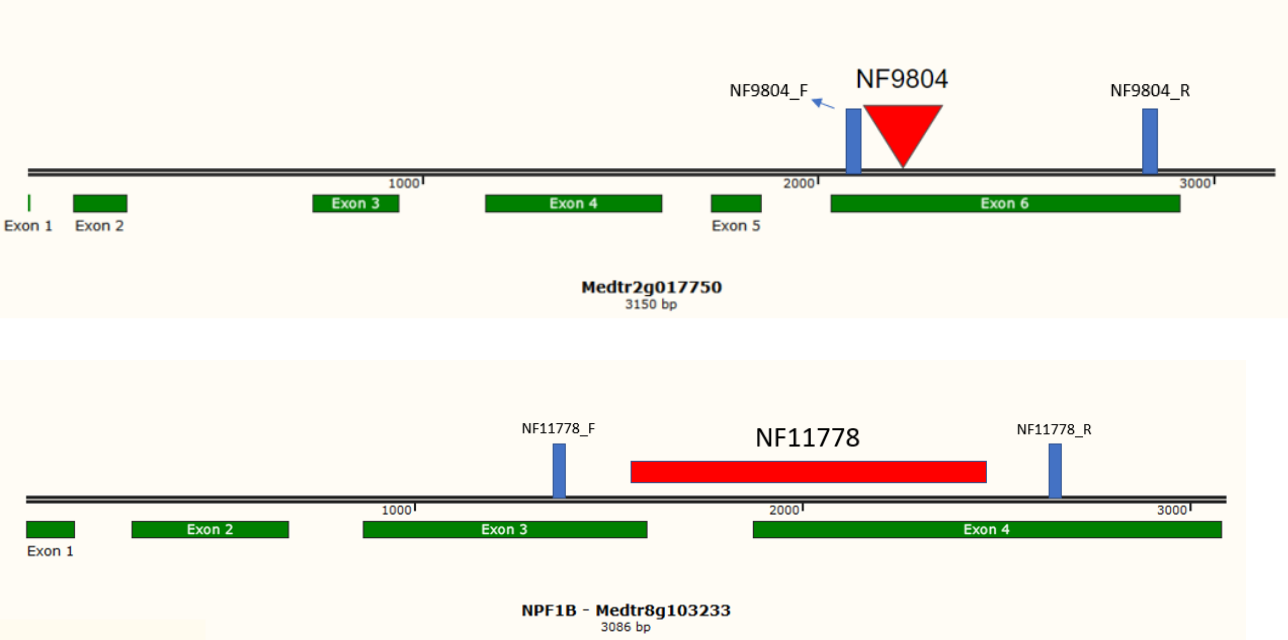

Figure 6. Relative insertion sites of two *Tnt1* insertions within their corresponding gene of interest. Green boxes indicate predicted exons. A red triangle indicates the known insertion site for NF9804 mutant plants within the gene *Medtr2g017750* as determined by DNA sequencing (Daniels & MacLean, unpublished data). The red bar represents the relative location of the insertion site for NF11778 mutants, within the gene *Medtr8g103233*. Blue bars represent the relative location of the forward and reverse primers used in genotyping.

Table 3. Nucleotide sequences for primers used in genotyping. NF9804_F and NF11778_F are the gene-specific forward primers. NF9804_R and NF11778_R are gene-specific reverse primers.

Primer Name	**Nucleotide Sequence (5' to 3')**
Tnt1_F2	GTTGATCAAGCTCACAATGGAAAG
Tnt1_R2	AGTTGGCTACCAATCCAAGGA
NF9804_F	AGGCACCAAGTACAAGTCCATGG
NF9804_R	GTTGATCAAGCTCACAATGGAAAGG
NF11778_F	CTCTTCACTTGCTCTAATGGC
NF11778_R	CATAGTGCCCTTGATTGATATTTGC

4.0 Root phenotypic assessment

M. truncatula plants (N = 18-22, for each genetic line depending on the experiment) corresponding to wild type, segregating wild type, and mutant genetic lines were evaluated to assess root development via physiological measurements. The goal for these assays was to compare the phenotype of the wild type plants to the segregating wild type and mutant lines, as a means of determining whether the disruption of *NPF4.12* gene expression in the NF9804 mutants has an impact on the root development of the plant. The assessment was guided by a protocol developed by our collaborators at the University of Vermont who already performed phenotypic assessments of the *NPF1B* gene in *Lotus japonicus*.

4.1 Potting and preparation of plants

M. truncatula seeds were sterilized as per the method used in the symbiosis assays (Section 1.2). *M. truncatula* plants were grown in pots with 100% Turface, supplied by Ritchie Feed & Seed Inc., as the substrate. Four seedlings were placed in each pot (13.5cm x 13.5cm x 13cm). The plants were watered 3 times a week and fertilized twice a week with 20mL of ½ strength Hoagland's solution after the first week of growth.

4.2 Root phenotypic assessment

Plants were harvested at 14 and 21 days post transplantation. The pots were placed in water to allow the substrate to fall away gently, allowing the roots to be collected in an undamaged state. Photos of each plant's roots were taken next to a ruler as a size reference for later analysis in ImageJ. Fresh masses of the shoot and root were weighed immediately upon collection. Next, the plants were placed in paper bags and dried in an oven at 65℃ for five days, at which point the dry masses of the shoot and root were weighed.

4.3 Image J assessments

The photos of the harvested plants beside the ruler were analyzed in ImageJ to calculate measurements of longest root length, total root length, and total number of roots. The “Set Scale” tool was used on the ruler to calibrate the other measuring tools. The “Measure” tool was used to draw lines on the roots in the image to obtain an accurate length in millimeters. Statistical analyses were performed as described above (Section 1.6).

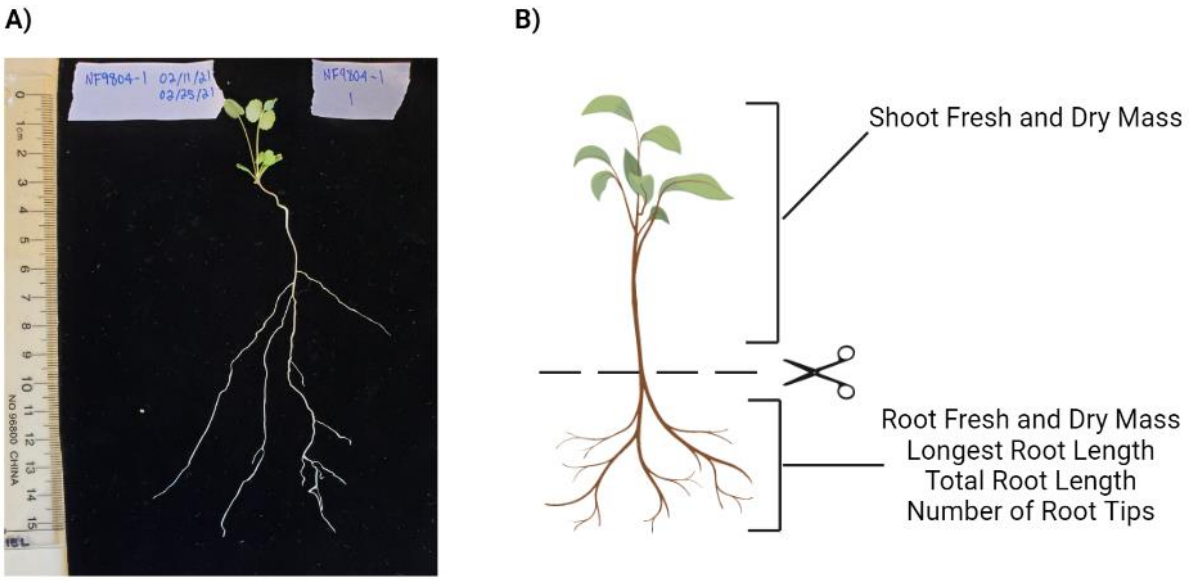

Figure 7. Demonstration and visualization of the phenotypic assessment. A) Photo of *M. truncatula* plant positioned alongside a ruler to use as a scale for the ImageJ analysis. Longest root length, total root length, and number of root tips were quantified in ImageJ. B) Visual representation of how the plants were assessed. The root system was cut with scissors after the photography to determine the weight of the shoot and root separately.

5.0 Yeast transformation

Purified plasmid DNA was transformed into yeast cells using a high-efficiency yeast transformation protocol (Geitz et al, 2007). MaV203 *Saccharomyces cerevisiae* was inoculated into 5mL of liquid 2x yeast extract peptone dextrose (YPD) media. Next, the culture was incubated overnight on a rotary shaker at 28°C. A flask with 50mL of 2x YPD was also incubated on the same shaker overnight to pre-warm the broth. The next morning, 40µL of the overnight MaV203 culture was pipetted into 40mL of dH_2O to measure the OD_{600} with a spectrophotometer. A suspension containing $1x10^6$ cells/mL was used.

$$\text{Number of cells/mL} = ((1x10^6)(\ OD_{600})(100))/(0.1)$$

Next, $2.5x10^8$ cells were added to the pre-warmed flask with 50mL of 2x YPD.

$$\text{Volume of cell suspension to add} = (2.5x10^8 \text{ cells})/(\text{Number of cells/mL})$$

The flask was placed back on the shaker at 28°C for 3-5 hours until the number of cells/mL reached at least $2.7x10^7$. The inoculated media in the flask was poured into a Falcon tube and centrifuged at 3,000 xg for 5 minutes. The resulting pellet of yeast cells was washed and centrifuged twice in 25mL of sterile dH_2O and resuspended in 1mL of sterile dH_2O. The cell suspension was transferred to a 1.5mL Eppendorf tube and centrifuged at 12,000 xg for 30 seconds. The supernatant was discarded, and the pellet was resuspended in 1mL of sterile dH_2O. 100µL of the cell suspension was pipetted to one Eppendorf tube for each transformation. Next, cells were centrifuged at 12,000 xg for 30 seconds and the supernatant was removed. 360µL of Transformation Mix, containing 240µL of polyethylene glycol 3350 (50% w/v), 50µL of boiled salmon sperm carrier DNA (2.0mg/mL), 36µL of 1.0M lithium acetate, 31-33µL of sterile dH_2O,

100-300µg of plasmid DNA, was added to each Eppendorf tube. The yeast pellet was resuspended in the Transformation Mix and incubated in a 42℃ water bath for 40 minutes. Next, the Eppendorf tubes were centrifuged at 12,000 xg for 30 seconds and the Transformation Mix was removed. The pellet in each Eppendorf tube was resuspended in 1mL of sterile dH_2O. Lastly, 100µL of the transformed cell suspension was pipetted onto Petri dishes containing selective media. The plates were incubated at 28℃ for 5 days. The yeast media for the plates contained agar (20g/L), minimal SD base (26.7g/L), and DO supplement (0.62g/L). The DO supplement lacked leucine, tryptophan, and uracil. The minimal SD base (Cat#630411) and the -LTUH DO supplement (Cat#630426) were ordered from Takara Bio USA.

6.0 Modified yeast-two hybrid assays

From each different transformation, one colony of the transformed yeast was picked and diluted in 300µL of 0.9% saline sterile dH_2O. The same was performed for untransformed MaV203 as a negative control. We used 3 replicates from each different transformation. Serial dilutions (undiluted, 10-fold diluted, and 100-fold diluted) of suspended, transformed yeast cells were pipetted into three spots on selective yeast media.

The yeast media for the plates contained agar (20g/L), minimal SD base (26.7g/L), DO supplement (0.60g/L), 25mM 3-Amino-1,2,4-triazole (2.102g/L), sterile dH_2O, and the corresponding amount of phytohormone stock. The DO supplement lacked leucine, tryptophan, uracil, and histidine. 1µL of the 1mM phytohormone stocks was added for every 1mL of yeast media to get a 1µM phytohormone concentration. 5µL of the 1mM phytohormone stocks was added for every 10mL of yeast media to get a 0.5µM phytohormone concentration. 1µL of the 1mM phytohormone stocks was added for every 10mL of yeast media to get a 0.1µM

phytohormone concentration. Three control plates were also prepared in the same way, to which no phytohormones were added. Another three plates were made the same way with DO supplement (lacking leucine, tryptophan, uracil) as a positive control to ensure the yeast cells were alive and able to grow properly. All plates were incubated at 28℃ for 5 days.

ABA (catalogue #: 90769) and GA_3 (catalogue #: G7645-500MG) were ordered from Sigma-Aldrich. A 1mM stock solution of GA_3 was made by dissolving 0.0035g of GA_3 in 10mL of 95% ethanol. A 1mM stock solution of ABA was made by dissolving 0.0026g of ABA in 10µL of 1M NaOH, then topped up to 10mL with sterile dH_2O.

7.0 Cloning NPF4.12 *into a yeast expression vector*

Primers complementary to nucleotide sequences spanning the gene *Medtr2g017750,* and containing *attB2* and *attB1* sites encoded at the 5' and 3' ends were designed in SnapGene to be complementary to the recipient pDONR vectors (pDONR2,1). The resulting PCR product (corresponding to the *Medtr2g017750* coding sequence) was visualized on a 0.8% agarose gel via electrophoresis at 100 Volts for one hour. The DNA product of interest was excised from the gel, and the DNA was extracted using the GenElute Gel Extraction Kit. GoTaq DNA polymerase was used to amplify the gene.

The purified gel product was then cloned into pDONR221 through a Gateway BP reaction. The BP reaction contained extracted DNA from the coding sequence of the gene of interest, the pDONR221 plasmid, and the BP Clonase II enzyme. This mixture was incubated overnight at 25℃. The next morning, 1µL of Proteinase K was added and it was incubated for an additional 15 minutes at 37℃. The resulting BP reaction mix was transformed into chemically competent *E. coli*. One colony from the transformation plate was inoculated into LB containing

50 μg/mL of kanamycin for overnight growth, and plasmid DNA was isolated via miniprep using the aforementioned GenElute Plasmid Miniprep protocol.

The coding sequence of *Medtr2g017750* was transferred into the destination vector pYES-DEST52 from pDONR221 via Gateway LR clonase, as per manufacturer's instructions. As with the BP reaction mixture, the LR reaction mixture was transformed into *E. coli*, and a transformant was inoculated into LB containing 50μg/mL of streptomycin, and miniprepped for transformation into yeast strain MaV203. The coding sequence of the gene was verified to be correct via DNA sequencing prior to transfer to the pYES-DEST52 plasmid.

8.0 Medicago root transformation

The protocol employed for the *M. truncatula* root transformations was established by (Floss et al., 2013). In these experiments, we attempted to visualize expression of *NPF1B* promoter in transformed *M. truncatula* roots, with said promoter:*gusA* construct provided to us by the Jeanne Harris lab at University of Vermont. As a positive control for staining, we also included the *MtBCP* promoter known to be expressed during AM symbiosis (Ivanov et al, 2014) cloned upstream of a *gusA* reporter gene, using the RedRoot plasmid pKm43GWRR.

8.1 Agrobacterium rhizogenes transformation

Electrocompetent *A. rhizogenes* cells (strain ARqua1) were transformed with the given destination vector through electroporation. The transformed cells were plated on selective LB media containing the appropriate antibiotic relating to the construct and grown for three days at 28°C. A colony of the transformed *A. rhizogenes* was picked from the LB selection media and resuspended in 50μL of sterile dH_2O. Next, the bacterial suspension in water was spread evenly

across TY plates containing tryptone yeast agar and incubated for two days to allow a thick film of transformed *A. rhizogenes* to grow, which was used in the root transformation.

8.2 Medicago truncatula preparation

A17 ecotype *M. truncatula* seeds were removed from their seed pods and placed in Falcon tubes. Seeds were covered in concentrated sulfuric acid for 10 minutes to scarify the seed coat. The sulfuric acid was removed, and the seeds were rinsed five times with sterile dH_2O. 10mL of a 10% bleach and 0.1% Tween solution was added to the seeds and it was placed on a gentle shaker for 10 minutes to sterilize any microbial contamination. The bleach solution was removed, and the seeds were rinsed five times with sterile dH_2O. The Falcon tube was covered in aluminum foil and left on a shaker for three hours at room temperature to allow the seeds to imbibe. Next, the seeds were incubated at 4℃ in the dark for 26 hours. After that time, the seeds were transferred to sterile Petri dishes and distributed evenly. The plates were inverted to allow the development of straight radicles growing downwards in response to gravity. The plates were wrapped in aluminum foil and incubated at 28℃ for 18 hours.

8.3 Medicago truncatula root inoculation with Agrobacterium rhizogenes

The germinated seedlings had their seed coats removed with sterile forceps and a scalpel. Their radicles were cut approximately 3mm from the root tip. The cut end of the radicle was dipped into the transformed *A. rhizogenes* containing the appropriate construct. Then, seedlings were inserted into grooves made with forceps in Fähraeus media on plates containing the antibiotic Kanamycin as appropriate to select for transformed roots. The plates were sealed with parafilm with several incisions made to allow for gas exchange. Next, the plates were placed in an 18℃ incubator with a 16 hour light/8 hour dark cycle and light intensity of 16$\mu m/m^2s$. After 5

days, the plates were moved to an incubator with the same light cycle. The temperature of the incubator was 25℃ during the light part of the cycle and 22℃ during the dark part of the cycle. The light intensity of the growth chamber at this phase was 45μm/m^2s.

8.4 Root transformation and WGA/GUS staining

Plant roots were screened under a Zeiss Axiozoom Stereoscope to determine whether the transformation was successful. The pKm43GWRR plasmid contains a DsRED gene, allowing the transformed portions of the roots to fluoresce 'red' when excited under the 540nm wavelength. Successfully transformed roots were marked to be included in the subsequent assay.

The transformed plants were set up in cone-tainers and inoculated similarly to the methods described for symbiosis assays. In this case, 24 plants were inoculated with 200 spores of *R. irregularis*, 15 plants were inoculated with the same amount of *R. irregularis* and *Sinorhizobium medicae* strain WSM419, and 5 plants were inoculated solely with *Sinorhizobium medicae* strain WSM419. Plants inoculated with WSM419 were included as a positive control to confirm that the transformation with the *NPF1B* promoter/*gusA* fusion was functional because root nodules from these plants should have shown staining according to past work by our collaborators at the University of Vermont. WSM419 was prepared by inoculation of a single colony into 5mL of TY media grown in a 28℃ overnight. The next day, 2mL of the culture was pelleted by centrifuging at 12,000 xg for 30 seconds. The cells were then resuspended in 2mL of 0.9% saline sterile dH_2O. The solution was centrifuged once again for 30 seconds and resuspended in 10mL of sterile dH_2O. The appropriate number of plants were inoculated with 0.5mL of the bacterial water solution.

At the appropriate time-point of 3-6 weeks, plant roots were harvested in ice cold dH_2O. Next, the roots were incubated in 90% acetone at -20℃ for 20 minutes. The acetone was

removed, and the roots were washed in 10mM phosphate buffer for 10 minutes. At this point, the roots were vacuum-infiltrated with β-glucuronidase (GUS) staining solution (1M Phosphate buffer, 0.5M EDTA, 0.1M K ferricyanide, 0.1M K ferrocyanide) containing 40mg/mL of X-Gluc (Sigma Catalogue#: R0851) for 30 minutes at room temperature to allow the plant material to take up the staining solution. The infiltrated roots were incubated at 37℃ in the GUS staining solution until the appropriate levels of staining were observed, or overnight. Next, the roots were incubated in a fixation buffer overnight at room temperature. Fixation buffer fixes the GUS staining, so it does not degrade as quickly over time. The next day, the fixation buffer was removed, and the roots were rinsed three times with dH_2O. The roots were covered in 20% KOH and incubated overnight at 65℃ to clear the roots of lignin to allow easier visualization. Finally, the roots were rinsed five times with dH_2O and rinsed with phosphate buffer saline (PBS) three times. Lastly, the roots were stained with 10µL of WGA (200ng/µL) that is conjugated with Alexa-488 fluorophore. The roots were stored in the fridge at 4℃ prior to examination.

RESULTS

1.0 Symbiosis assays

Comparisons of AMF root colonization rates between two Tnt1 mutant lines and their respective segregating wild type plants collected at two to three different time points are shown in Figures 8 and 9. These symbiosis assays were completed to assess the involvement of *NPF1B* (*Medtr8g103233)* and *NPF4.12* (*Medtr2g017750)* transport systems for possible roles in establishing and/or maintaining AM symbiosis.

Two additional symbiosis assays were completed, but the data was omitted due to abnormally low colonization rates. The cause was ultimately determined to be low light intensity in the growth chamber.

1.1 NPF1B Assays

The results of 4-week and 6-week timepoints for this symbiosis assay showed no statistically significant differences in colonization rates between the *NPF1B* mutant and wild type control plants. The 4-week timepoint yielded mean colonization rates of 19.7% for the wild type (R108), 23.2% for the segregating wild type (NF11778-1), and 26.6% for the mutant line (NF11778-6). A one-way ANOVA was performed to determine the statistical significance of the data collected from each timepoint. A Post Hoc Tukey Test was performed to compare the differences in colonization rates between the MT and WT, as well as the MT and SWT (as indicated in Table 4).

1.2 NPF4.12 Assays

The results of the 3-week, 4-week, and 6-week timepoints for this symbiosis assay showed no statistically significant difference in colonization rates between *NPF4.12* mutants and

wild-type controls. The 3-week timepoint yielded mean colonization rates of 8.08% for the wild type (R108), 6.80% for the segregating wild type (NF9804-4), and 5.54% for the mutant line (NF9804-1). A one-way ANOVA was performed to determine the statistical significance of the data collected from each timepoint. A Post Hoc Tukey Test was performed to compare the differences in colonization rates between the MT and WT, as well as the MT and SWT (as indicated in Table 5).

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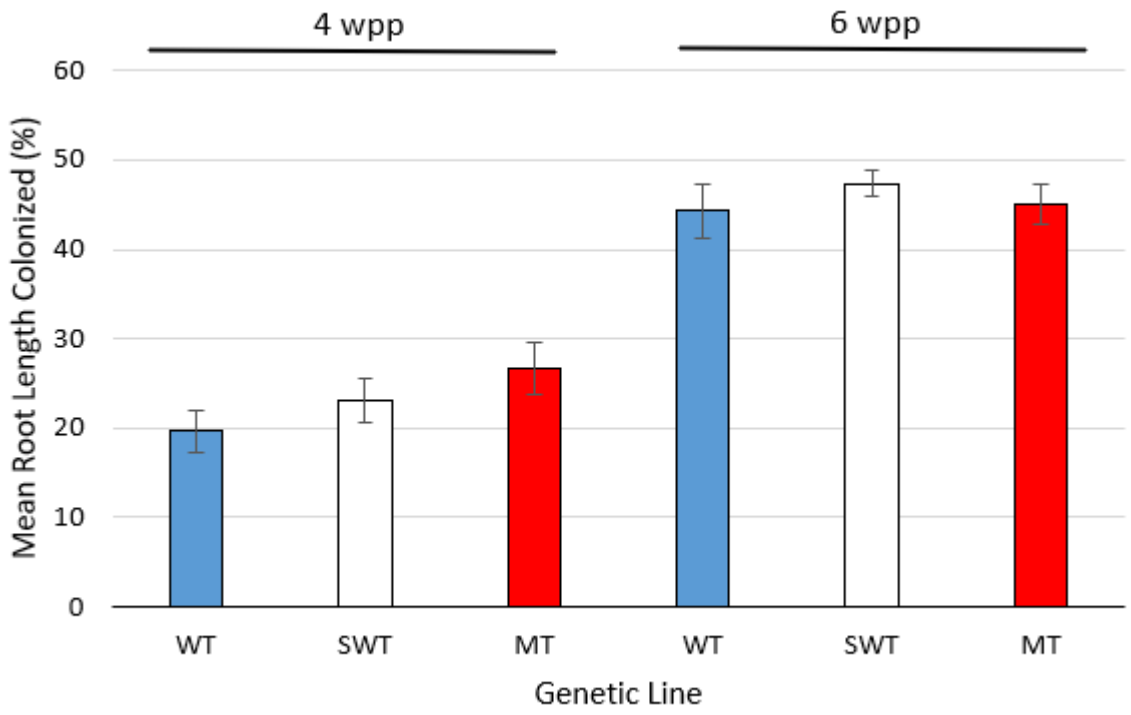

Figure 8. Quantification of arbuscular mycorrhizal fungi colonization of *Medicago truncatula* roots at 4 weeks and 6 weeks post planting. The ecotype of the wild type *M. truncatula* (WT) is R108, the segregating control (SWT) encodes a wild type *NPF1B* gene, corresponding to the genetic line of NF11778-1, and the *NPF1B* mutant (MT) corresponds to the genetic line NF11778-6. Data shown is the mean ± SE using 10 cones with 2 plants each per measurement. Results a Post Hoc Tukey Test indicate that differences in colonization rates are not statistically significant. N=10 cones.

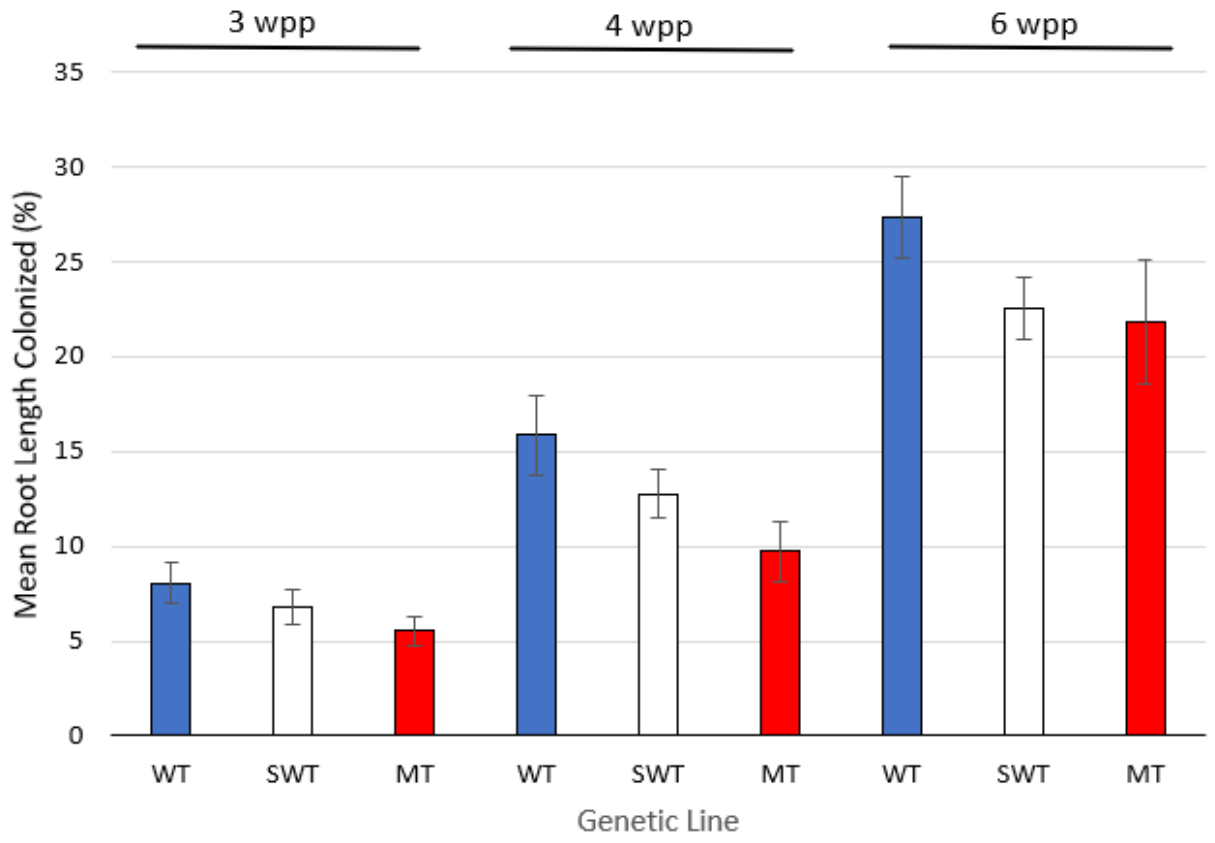

Figure 9. Quantification of arbuscular mycorrhizal fungi colonization of *Medicago truncatula* roots at 3 weeks, 4 weeks, and 6 weeks post planting. The ecotype of the wild type *M. truncatula* (WT) is R108, the segregating control (SWT) encodes a wild type *NPF4.12* gene, corresponding to the genetic line of NF9804-4, and the *NPF4.12* mutant (MT) corresponds to the genetic line of NF9804-1. Data shown is the mean ± SE using 7 cones with 2 plants each per measurement. Results of a Post Hoc Tukey Test indicate that differences in colonization rates are not statistically significant. N=7 cones.

Table 4. Results of the statistical analysis for the NPF1B symbiosis assay. A one-way ANOVA and Post Hoc Tukey Test were performed to complete the statistical analysis for this experiment. The p-value for the results of a one-way ANOVA for the given timepoint is represented by $p\text{-value}_0$. The p-value from comparing the differences of the means of each measurement between the WT (R108) and MT (NF11778-6) is represented by $p\text{-value}_1$. The p-value from comparing the differences of the means of each measurement between the SWT (NF11778-1) and MT is represented by $p\text{-value}_2$.

Timepoint (Wpp)	$p\text{-value}_0$	$p\text{-value}_1$	$p\text{-value}_2$
4 weeks	0.186	0.614	0.618
6 weeks	0.618	0.980	0.622

Table 5. Results of the statistical analysis for the NPF4.12 symbiosis assay. A one-way ANOVA and Post Hoc Tukey Test were performed to complete the statistical analysis for this experiment. The p-value for the results of a one-way ANOVA for the given timepoint is represented by $p\text{-value}_0$. The p-value from comparing the differences of the means of each measurement between the WT (R108) and MT (NF9804-1) is represented by $p\text{-value}_1$. The p-value from comparing the differences of the means of each measurement between the SWT (NF9804-4) and MT is represented by $p\text{-value}_2$.

Timepoint (Wpp)	$p\text{-value}_0$	$p\text{-value}_1$	$p\text{-value}_2$
3 weeks	0.245	0.216	0.669
4 weeks	0.080	0.065	0.467
6 weeks	0.303	0.325	0.977

2.0 Root phenotypic assessments

We assessed the phenotype of roots corresponding to an *NPF4.12* mutant to determine whether this gene was involved in root development. The data comparing various phenotypic measurements of the *M. truncatula* Tnt1 mutant line NF9804 and its respective segregating wild type control, collected at three different time points, are shown in Tables 6 to 8. The root phenotyping assays were completed by assessing the shoot and root fresh masses, shoot and root dry masses, longest root length, total root length, and number of root tips.

The results from Table 6 show a significant increase in all variables measured when comparing the mutant line NF9804-1 (MT) to both the wild type (WT) R108 and segregating wild type (SWT) line NF9804-4. The results of a one-way ANOVA and Post Hoc Tukey Test showed that all comparisons of the results were statistically significant with the exception being the total number of root tips per plant for both genetic line comparisons and the shoot dry mass comparison between the WT and MT.

The results from Table 7 show no consistent differences in all variables measured for both timepoint 1 and 2 when comparing each genotype. The results of a one-way ANOVA and Post Hoc Tukey Test for timepoint 1 showed that statistical significance was confirmed for both genetic line comparisons for longest root length. The comparisons between R108 (WT) and NF9804-2 (MT) showed statistical significance for root fresh mass and total root length. The comparisons between NF9804-11 (SWT) and NF9804-2 showed statistical significance for the number of root tips.

The results of a one-way ANOVA and Post Hoc Tukey Test for timepoint 2 showed that statistical significance was solely confirmed for the comparisons between NF9804-11 (SWT)

and NF9804-2 (MT) for the number of root tips. All other comparisons between genetic lines showed no statistical significance.

The results from Table 8 show no consistent difference in all variables measured for both timepoint 1 and 2 when comparing NF9804-1 (MT) to R108 (WT) and NF980404 (SWT). The results of a one-way ANOVA and Post Hoc Tukey Test showed statistical significance between the differences of the values when comparing the WT and MT for shoot fresh mass, shoot dry mass, root dry mass, longest root length, total root length, and number of root tips. All other comparisons between genetic lines showed no statistical significance.

Table 6. Phenotypic assessment of *Medicago truncatula* wild type (ecotype R108) and mutant lines. Plants were harvested 14 days post planting. Each value represents the mean of 18 samples for the wild type (WT) R108 and 20 samples for the segregating wild type (SWT) NF9804-4 and mutant (MT) NF9804-1 in which *NPF4.12* is disrupted by transposon insertion. The ± value represents standard error of the mean of the measurement data. The p-value from comparing the differences of the means of each measurement between the WT and MT is represented by p-value1. The p-value from comparing the differences of the means of each measurement between the SWT and MT is represented by p-value2.

	Genetic Line				
Measurement	WT	SWT	MT	p-value1	p-value2
Shoot Fresh Mass (mg)	52.3(±3.7)	44.8(±3.8)	68.5(±4.4)	0.016	<0.001
Root Fresh Mass (mg)	34.5(±2.5)	37.5(±4.0)	61.7(±3.3)	<0.001	<0.001
Shoot Dry Mass (mg)	5.3(±0.3)	4.6(±0.4)	6.3(±0.4)	0.224	0.012
Root Dry Mass (mg)	2.14(±0.19)	2.24(±0.22)	3.00(±0.13)	0.005	0.014
Longest Root Length (mm)	120.6(±5.7)	103.8(±7.1)	148.1(±5.4)	0.007	<0.001
Total Root Length (mm)	250.9(±16.4)	262.4(±23.5)	375.2(±19.4)	<0.001	0.001
Number of Root Tips	10.7(±1.0)	10.6(±1.2)	12.2(±0.8)	0.543	0.492

Table 7. Phenotypic assessment of *Medicago truncatula* wild type (ecotype R108) and mutant lines. Plants were harvested 14 days post planting for timepoint 1 and 21 days post planting for timepoint 2. Within timepoint 1, each value represents the mean of 22 samples for the wild type (WT) and 20 samples for the segregating wild type (SWT) and mutant (MT). Within timepoint 2, each value represents the mean of 19 samples for the WT and 20 samples for the SWT and MT. WT corresponds to R108 ecotype, NF9804-11 is the genetic line of the segregating control (SWT) that encodes a wild type *NPF4.12* gene, and NF9804-2 is the MT in which *NPF4.12* is disrupted by transposon insertion. The ± value represents standard error of the mean of the measurement data. "Dpp" refers to days post planting. The p-value from comparing the differences of the means of each measurement between the WT and MT is represented by p-value1. The p-value from comparing the differences of the means of each measurement between the SWT and MT is represented by p-value2.

		Genetic Line				
Timepoint (Dpp)	Measurement	WT	SWT	MT	p-value1	p-value2
14 days	Shoot Fresh Mass (mg)	40.7 (±1.8)	41.0(±1.3)	42.9(±1.7)	0.615	0.689
	Root Fresh Mass (mg)	25.5(±1.1)	29.0(±1.3)	31.2(±1.8)	0.014	0.507
	Shoot Dry Mass (mg)	3.3(±0.2)	3.2(±0.1)	3.6(±0.1)	0.539	0.307
	Root Dry Mass (mg)	1.3(±0.1)	1.4(±0.1)	1.2(±0.1)	0.539	0.434
	Longest Root Length (mm)	93.0(±3.6)	108.2(±3.4)	120.7(±3.9)	<0.001	0.045
	Total Root Length (mm)	192.0(±8.4)	203.31(±8.3)	223.8(±9.8)	0.037	0.240
	Number of Root Tips	6.0(±0.3)	5.0(±0.4)	6.9(±0.4)	0.162	0.001
21 days	Shoot Fresh Mass (mg)	82.6(±5.6)	69.4(±2.5)	72.9(±4.0)	0.236	0.819
	Root Fresh Mass (mg)	50.7(±4.3)	41.1(±2.1)	45.2(±2.7)	0.433	0.624
	Shoot Dry Mass (mg)	7.1(±0.5)	5.9(±0.2)	6.3(±0.4)	0.321	0.619
	Root Dry Mass (mg)	2.6(±0.2)	2.1(±0.1)	2.1(±0.2)	0.894	0.990
	Longest Root Length (mm)	134.6(±6.1)	139.9(±5.5)	151.4(±4.3)	0.073	0.284
	Total Root Length (mm)	381.9(±28.8)	318.7(±16.2)	375.4(±19.2)	0.976	0.168
	Number of Root Tips	13.6(±1.2)	9.9(±0.6)	13.9(±0.9)	0.966	0.007

Table 8. Phenotypic assessment of *Medicago truncatula* wild type (ecotype R108) and mutant lines. Plants were harvested 14 days post planting. Each value represents the mean of 20 samples for the wild type (WT) R108 and 20 samples for the segregating wild type (SWT) NF9804-4 and mutant (MT) NF9804-1 in which *NPF4.12* is disrupted by transposon insertion. The ± value represents standard error of the mean of the measurement data. The p-value from comparing the differences of the means of each measurement between the WT and MT is represented by p-value1. The p-value from comparing the differences of the means of each measurement between the SWT and MT is represented by p-value2.

	Genetic Line				
Measurement	WT	SWT	MT	p-value1	p-value2
Shoot Fresh Mass (mg)	127.2(±6.0)	96.8(±4.8)	95.1(±4.2)	<0.001	0.969
Root Fresh Mass (mg)	98.4(±5.4)	83.7(±4.6)	84.6(±3.6)	0.093	0.991
Shoot Dry Mass (mg)	16.0(±0.7)	12.0(±0.6)	11.3(±0.5)	<0.001	0.643
Root Dry Mass (mg)	6.1(±0.3)	5.4(±0.3)	4.9(±0.2)	0.006	0.292
Longest Root Length (mm)	158.2(±6.2)	176.6(±8.0)	191.6(±5.3)	0.002	0.253
Total Root Length (mm)	739.7(±40.1)	585.1(±33.5)	623.4(±25.5)	0.045	0.700
Number of Root Tips	27.6(±1.3)	21.6(±1.3)	20.5(±1.2)	0.001	0.809

3.0 NPF1B GUS/promoter fusion

We utilized a reporter *GUSA* fusion experiment to visualize the expression of *NPF1B* in *M. truncatula* roots during AM symbiosis. Other plants were transformed with a *BCP1p::GUSA* expressing plasmid (Figure 10) as a positive control. No GUS staining was observed in the plants transformed with *NPF1Bp::GUSA*. The positive control demonstrated GUS staining in cells containing arbuscules.

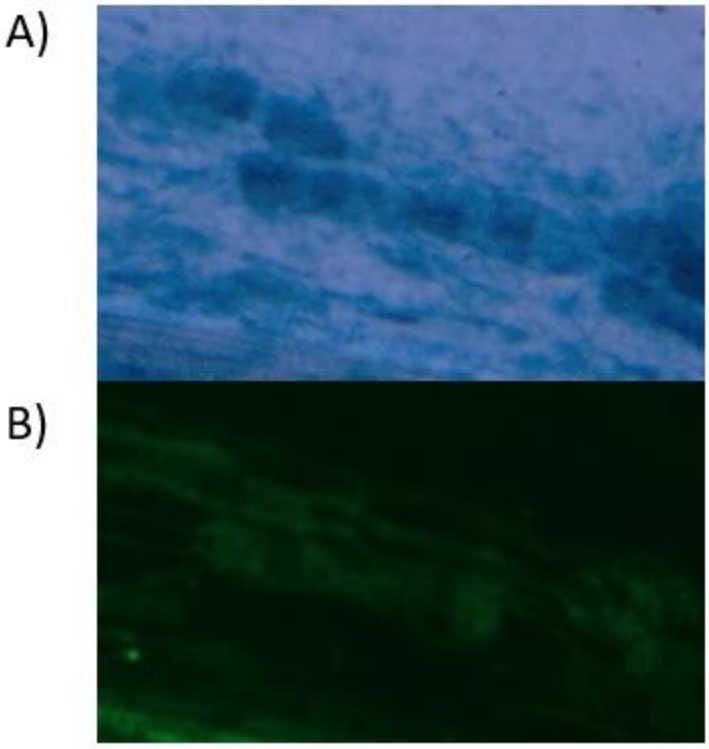

Figure 10. *M. truncatula* roots transformed with the *BCP1p::GUSA* expression plasmid with AMF arbuscules present. A) Visualization of GUS staining. B) Visualization of WGA staining.

4.0 Modified Yeast-Two Hybrid Test

Modified yeast-two hybrid tests were performed using *S. cervisiae* strain MaV203 transformed with replicating plasmids that encode various *NPF* genes of interest. In these assays, the yeast strains were grown on selective media to test each *NPF* gene for the ability to import the phytohormones ABA and GA_3. *NPF4.1* was included as a positive control as it is known to import both ABA and GA_3 (Chiba et al., 2015). *NPF4.6* was included as an additional control, as it is a known importer of ABA, but not GA_3. Yeast cells transformed with an empty vector in lieu of an *NPF* gene were included as a negative control for all assays.

Our assays indicate that *Medicago truncatula NPF4.12* has the capacity to import both GA_3 and ABA as shown in Figures 11 and 12. Assays performed with *Cicer arietinum NPF1C*, *Lotus japonicus NPF1C*, *Populus balsamifera NPF1.14*, and *MtNPF1.7* yielded results demonstrating that these systems do not have the capacity to import either phytohormone. Figures 11 and 12 depict representative data from the assays; the complete data set is provided as supplementary information at the end of this thesis.

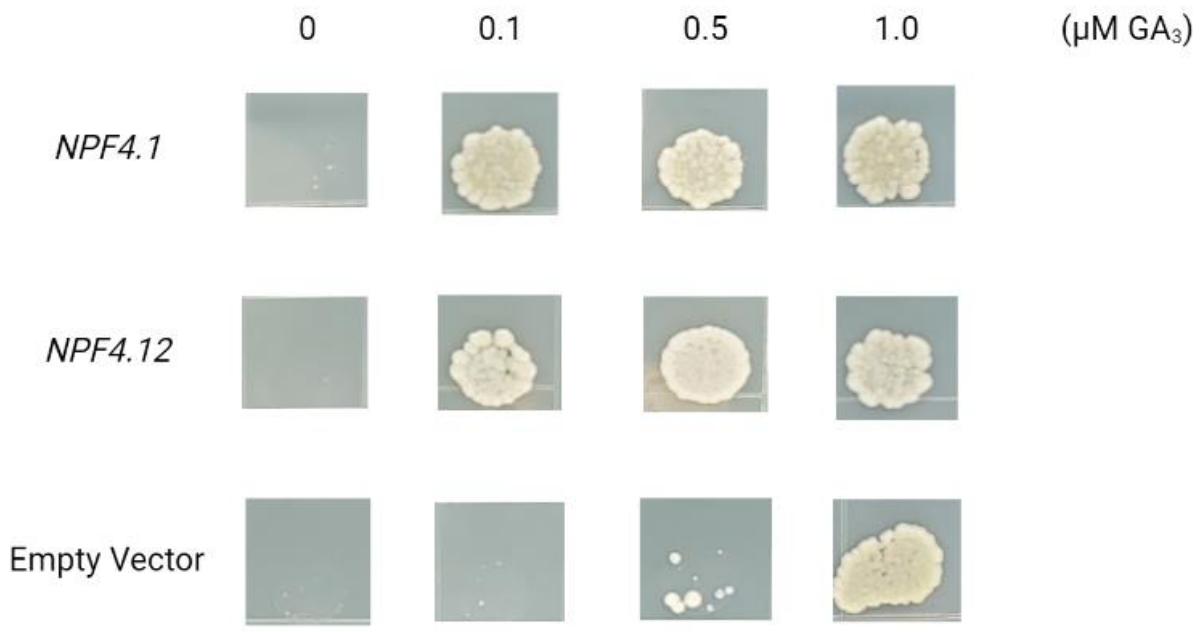

Figure 11. Results of a modified yeast-two hybrid assay to assess ability of NPF4.12 to import GA_3. *S. cerivisiae* strain MaV203 was transformed with pDEST22/GAI and pDEST32/GID1a, along with the candidate *NPF* transporter gene *NPF4.12* to test for capacity to import GA_3. The defined minimal media upon which the yeast was plated lacked leucine, tryptophan, uracil, and histidine. Concentration of GA_3 which is supplemented in the media is denoted in the top row.

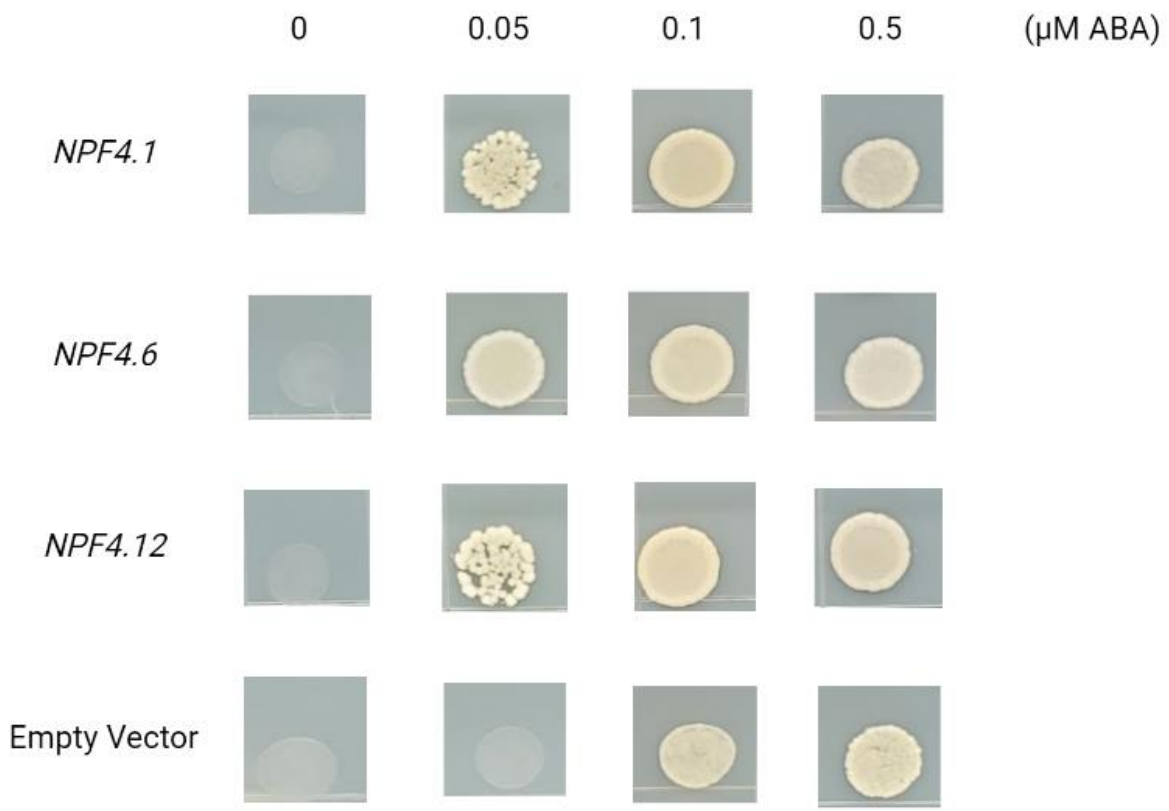

Figure 12. Results of a modified yeast-two hybrid assay to assess ability of NPF4.12 to import ABA. *S. cerivisiae* MaV203 was transformed with pDEST32/PYR1 and pDEST22/ABI1 along with the candidate *NPF* transporter gene *NPF4.12* to test for capacity to import ABA. The defined minimal media upon which the yeast was plated lacked leucine, tryptophan, uracil, and histidine. Concentration of ABA which is supplemented in the media is denoted in the top row.

DISCUSSION

1.0 Symbiosis Assays

1.1 NPF1B Symbiosis Assay

This symbiosis assay was performed to determine whether the *NPF1B* gene has a role in mediating AM symbiosis by assessing differences in mean root colonization rates of *M. truncatula* roots colonized with *Rhizophagus irregularis*. The gene *Medtr8g103233* was disrupted by the insertion of a transposon in the transgenic NF11778-6 mutants. We included the NF11778-1 line as a segregating control, which has the same underlying insertions as NF11778-6, but includes a wild type copy of the gene *Medtr8g103233*. The segregating wild type is a more effective control than R108, which lacks any insertions. Our data revealed no statistically significant difference in mean colonization rates between the different genetic lines. This indicates that this gene is not essential for AM symbiosis, but does not mean that it is not involved in mediating the symbiosis.

The *NPF1B* gene is expressed during AM symbiosis (as seen in Figure 2), but disruption of the gene did not seem to have a significant effect on the mean colonization rates in the mutant line. No obvious effects on the formation of fungal structures in the root cells, such as arbuscules, were observed. This may be due to a redundancy in function that is common to genes in the NPF family (Wulff et al., 2019). There are many NPF transport systems that are responsible for transporting overlapping substrates (Corratgé-Faillie & Lacombe., 2017), especially phytohormones such as GA_3 and ABA that are known to have an effect on AM symbiosis (Foo et al., 2013; Martın-Rodrıguez et al., 2011). Although *NPF1B* is one of the only NPF transport system genes that demonstrates induced expression during AM symbiosis, it is not

expressed exclusively during this symbiosis. The expression levels of *NPF1B* are comparatively much greater during rhizobial symbiotic conditions, specifically in root nodules, as demonstrated in Figure 2 of its gene expression atlas.

1.2 NPF4.12 Symbiosis Assay

This subsequent symbiosis assay was carried out to assess whether *NPF4.12* plays a role in mediating AM symbiosis by determining differences in mean root colonization rates of *M. truncatula* roots colonized with *Rhizophagus irregularis*. The gene *Medtr2g017750* was also disrupted by transposon insertion in the NF9804-1 mutant plants. The NF9804-4 line was used as a segregating control, which had the same underlying insertions as NF9804-1, but had a wild type copy of the gene. Once again, there was no statistically significant difference in mean colonization rates between the different genetic lines.

As with the *NPF1B* gene, the *NPF4.12* gene is expressed during AM symbiosis (as seen in Figure 2), but disruption of the gene did not appear to have a significant effect on the mean colonization rates. It is possible that redundancy within the NPF family (Wulff et al., 2019) is playing a role in the results of this symbiosis assay as well. *NPF4.12* is more intriguing than *NPF1B* due to the fact that it is almost solely expressed during AM symbiosis, especially in host cells that contain fungal arbuscules. Arbuscules are a crucial structure in the symbiosis because these are the primary sites of nutrient exchange between the plant and fungus (Gutjahr et al., 2012).

1.3 Conclusion

Overall, our initial hypothesis stating that *NPF1B* and *NPF4.12* encode transport systems that are involved in mediating AM symbiosis can not be accepted at this time. However, we can

not formally conclude that *NPF1B* and *NPF4.12* do not play any role in regulating AM symbiosis, after only assessing the results of these symbiosis assays. Redundancy has not been ruled out as a factor, and future research with double mutants for *NPF1B* and *NPF4.12* would shed more light on whether these *NPF* genes, which possibly transport the same substrates, are able to complement one another.

The results of the modified yeast-two hybrid experiment, which will be discussed below, show that *NPF4.12* has the capacity to import GA_3 and ABA. These findings have implications for future symbiosis assays because of the role that these phytohormones have been demonstrated to play in mediating AM symbiosis in past studies. GA has the ability to modulate arbuscule formation (Liao et al., 2018). DELLA proteins are also responsible for regulating arbuscule formation in AM symbiosis (Floss et al., 2013). These proteins are negative regulators of GA signalling and inhibit plant growth and development (Floss et al., 2013). Lower concentrations of GA inhibited the formation of arbuscules, while higher concentrations fully suppressed colonization in *Pisum sativum* (El Ghachtouli, et al., 1996). If a certain concentration of GA acts to suppress arbuscule formation, it is possible that we might observe an increase in colonization rates in double mutants lacking *NPF4.12* and a redundant transporter system. In one study, ABA increased the susceptibility of *Lycopersicon esculentum* to AM colonization (Herrera-Medina et al., 2007). It is worth noting that the findings on the roles of GA and ABA in mediating AM symbiosis and arbuscule formation are still not conclusive. The field has still not established whether these phytohormones have a positive or negative role in the process (Liao et al., 2018). Experiments in which multiple *NPF* genes responsible for transporting GA are knocked out simultaneously should be conducted before we are able to make any major conclusions on the role of these genes in AM symbiosis.

2.0 Medicago truncatula root phenotypic assessment

2.1 NPF4.12 phenotypic assessment

The phenotype of roots in NF9804 was assessed to determine whether *NPF4.12* has a role in the development of this organ in *M. truncatula*. As with the *NPF4.12* symbiosis assays, we included both wild-type (R108) and segregating wild-type (NF9804-4 and NF9804-11) genetic lines in our phenotypic assessment. The initial experiment (Table 6) included the NF9804-4 and NF9804-1 (mutant) plants, but the second experiment included NF9804-11 and NF9804-2 (mutant) plants due to the availability of seeds. Since the same insertion within (*Medtr2g017750*) is present in both NF9804-1 and NF9804-11, we would have an expectation of a similar phenotype being observed between these experiments, should the gene of interest influence root development. Once enough seed was available to complete the root phenotyping assays for the plants of the same genetic line, the third experiment was carried out (Table 8).

Variables we assessed were: fresh shoot and fresh root masses, dry shoot and dry root masses, length of longest root, total root length, and total number of root tips. In a phenotypic assessment of *NPF1B* in *Lotus japonicus*, our collaborators at the University of Vermont discovered that this gene plays a role in regulating lateral and primary root growth (Connolly, 2018). *L. japonicus LjNPF1B* mutant plants produce a decreased number of root tips and a reduced length of the longest root (Connolly, 2018).

The first root phenotyping assay (Table 6) showed a significant increase in all variables measured for the mutant line (NF9804-1) compared to the wild type (R108) and segregating control (NF9804-4). Repetition of this initial experiment (Tables 7 and 8) reveals that the trends of the initial phenotypic assay was not reproducible, thus we are unable to make any conclusions

based upon these data sets. At present, we are unable to explain the variability between these experiments.

2.2 Conclusion

The results indicate that the transport system encoded by *NPF4.12* does not have an essential role in the development of *M. truncatula* roots. In the future, it may be of interest to perform these same assays in a growth substrate other than Turface, with one option being pouches lined with paper towels that are immersed in a liquid nutrient solution. This is how phenotypic assessments were performed by our collaborators at the University of Vermont. Turface is a gravel-like substrate that is primarily used in baseball fields. This substrate falls away easily from roots when submerged in water during harvesting, which is a very useful advantage to the collection of fragile plant roots, to ensure these remain as intact as possible. Moreover, *M. truncatula* grows readily in Turface. The plants grown in pots with Turface as the substrate have a larger and more 3-dimensional environment to grow in as opposed to pouches where they are more confined. As with the symbiosis assays, functional redundancy between multiple *NPF* genes can not be dismissed as obscuring a role of *NPF4.12* in the development of *M. truncatula* roots. Since *NPF4.12* was found to import GA_3 and ABA in the modified yeast-two hybrid tests (discussed below) it is possible that the active transport and movement of these phytohormones which play integral roles in plant development (Gultom & Silitonga., 2018; Nguyen et al., 2019) can be complemented by another *NPF* transport system with a similar or overlapping function.

3.0 NPF1B GUS/promoter fusion

The attempt to visualize *NPF1B*'s gene expression in colonized plant roots using a reporter *GUSA* fusion to the *NPF1B* promoter was unsuccessful. *M. truncatula* plants were transformed to express *NPF1Bp::GUSA* and were stained to determine whether promoter expression could be observed in root cells colonized with *R. irregularis*. Other plants were transformed with a *BCP1p::GUSA* expressing plasmid as a positive control. This promoter is known to be expressed in cells adjacent to, and in contact with, AM fungal hyphae, and we did observe expected staining as indicated in Figure 10. We also included *NPF1Bp::GUSA* plants inoculated with *Sinorhizobium medicae* strain WSM419 for use as an additional positive control, as the *NPF1B* promoter construct was previously shown to be expressed in rhizobial root nodules. Unfortunately, we did not observe any staining to indicate gene expression in nodules, thus we must conclude that the root transformation with this plasmid was unsuccessful and/or our experimental set-up or conditions did not yield gene expression of *NPF1B* as expected. In conclusion, we can not confirm whether *NPF1B* is indeed expressed in root cells with colonized by AM fungi. The same procedure should be completed for *NPF4.12* in the future to visualize its gene expression in colonized plant roots.

4.0 Modified yeast-two hybrid assays: identification of NPF substrates

4.1 Initial Assays

The modified yeast two-hybrid assays were performed to assess the phytohormone import capabilities of candidate *NPF* genes. An initial experiment was performed to demonstrate that our yeast strain (*S. cerevisiae* strain MaV203) is a suitable host to perform this modified yeast-two hybrid assay, as the original assayed developed by Chiba et al., employed a different

yeast strain that was not readily available in Canada. As described by Thermo Fisher Scientific™, the commercial strain MaV203 is an auxotroph that can not biosynthesize the amino acids histidine, leucine and tryptophan and the nucleotide base, uracil. Accordingly, this strain is unable to grow upon selective minimal media that lacks these as supplements. The plasmids that MaV203 are transformed with include complementary genes to allow the transformed yeast to synthesize its own leucine and tryptophan, so this is used to select for yeast cells that were transformed. The pDEST22 plasmid encodes a gene for tryptophan biosynthesis, the pDEST32 plasmid contains a leucine biosynthetic gene, and the pYES-DEST52 plasmid carries a gene for uracil production. The yeast strain employed by the Chiba et al., who provided us with the plasmids to carry out these modified Y2H tests was a triple auxotroph that can not biosynthesize leucine, tryptophan, and uracil. As a means of determining whether our own yeast strain is a suitable host for these assays, we confirmed that the positive control *NPF4.1* is able to import GA_3 and ABA, whereas *NPF4.6* is able to import solely ABA as expected.

4.2 NPF4.12 imports the phytohormones gibberellic acid and abscisic acid

The modified Y2H tests were performed upon media supplemented with GA_3 and ABA at different concentrations to see the array of yeast growth. At the higher phytohormone concentrations of 0.5µM and 1.0µM it was apparent that the concentration was so high that the ABA and GA_3 in the media was able to enter the yeast cells even in the absence of an uptake system. This is evident because we observed yeast growth in strains that carry the empty vector in lieu of an *NPF*, which is used as a negative control. Thus, it is most useful to look at the lower concentrations of 0.05µM and 0.1µM to assess the true capacity for phytohormone import of the candidate NPF transport systems.

NPF4.12 was the only candidate NPF gene we investigated to demonstrate an ability to import any of the chosen phytohormones (Figures 11 and 12). Yeast grew on minimal media lacking uracil, tryptophan, leucine, and histidine when supplemented with GA_3 and ABA respectively at the 0.05µM and 0.1µM concentrations. This result indicates that NPF4.12 has a capacity to import these phytohormones. This is in contrast to the empty vector negative control which did not grow on minimal media under these conditions (Figures 11 and 12). Interestingly, many NPF members that have been shown to transport GA_3 also transport other structurally unrelated phytohormones and metabolites (Wulff et al., 2019). For example, *NPF4.1* is capable of importing GA_3, ABA and Jasmonic acid-isoleucine (Chiba et al., 2015). Similar to *NPF4.12*, other NPF members that are capable of transporting both GA_3 and ABA include *NPF1.1*, *NPF2.5*, *NPF3.1*, *NPF4.2*, *NPF5.1*, *NPF5.2*, and *NPF5.7* (Corratgé-Faillie & Lacombe., 2017).

Other candidate *NPF* genes we tested were provided by our collaborators at the University of Vermont. *CaNPF1C*, *LjNPF1C*, *PbNPF1.14*, and *MtNPF1.7* were subjected to the modified Y2H tests upon media supplemented with the same phytohormones as the initial tests. All four encoded transport systems were unable to import ABA and GA_3.

It is important to note that these modified Y2H tests could determine the phytohormone import capabilities of these transporters, but not any phytohormone export capabilities. In the future, modified yeast-two hybrid tests upon media supplemented with jasmonic acid should be administered. Jasmonic acid-isoleucine was included alongside ABA and GA_3 in Chiba et al., 2015., where the original concept for this portion of the research project originated. Time constraints and the high cost of jasmonic acid-isoleucine prevented inclusion of this hormone in our modified Y2H tests. Nevertheless, there is excellent potential for one of our candidate *NPF* genes to be capable of importing jasmonic acid-isoleucine, given that six out of eight NPF

families contain transport systems that import this substrate (Corratgé-Faillie & Lacombe., 2017).

4.3 Conclusion

GA and ABA are two well described phytohormones within the kingdom *Plantae* (Schwechheimer, 2008; Hilhorst & Karsse., 1992). ABA is involved in seed dormancy, germination, cell division, and responses to stressors (Kanno et al., 2012). GA plays a role in the development of seedlings (Gultom & Silitonga., 2018), and plant growth and development (Nguyen et al., 2019). Interestingly, a majority of known (or putative) GA transport genes are within the NPF family (Binenbaum et al., 2018). The large number of these *NPF* GA transporters implies functional redundancy (Wulff et al., 2019), an issue that may have also impacted our research into the role of *NPFs* in mediating symbiosis and root development. Fewer proteins have been characterized as ABA transporters, but most of these are in the NPF family as well (Léran et al., 2020).

5.0 Overall conclusion

The relationship between plants and AMF is one of the most widespread and significant symbioses in the world. This partnership aided the plants, which were poorly suited for the new terrestrial environment, to overcome issues such as desiccation and starvation of essential nutrients (Pirozynski & Malloch, 1975). There are still many unknowns surrounding this ancient and ubiquitous symbiosis and the factors that influence it. We conducted a series of widely encompassing experiments to determine whether the transport systems that encode the genes *NPF1B* and *NPF4.12* are involved in mediating AM symbiosis. These experiments included symbiosis assays, root phenotypic assessments, GUS/promoter fusions, and modified yeast-two

hybrid tests. The results of these experiments demonstrated that *NPF1B* and *NPF4.12* are not essential for AM symbiosis. However, the modified yeast-two hybrid tests resulted in the finding that the transport system encoded by *NPF4.12* is capable of importing the phytohormones ABA and GA_3. This finding will add to the ever-expanding list of NPF transporters that are responsible for moving pivotal substrates through the plant. In the future, further studies to explore the potentially impactful role of *NPF4.12* in AM symbiosis and plant physiology should be conducted. AM symbiosis studies in plants which over-express *NPF4.12* could show a significant phenotype compared to the gene knockout studies that we have employed. Also, completing our modified Y2H tests with jasmonic acid-isoleucine and our existing candidate genes should be of prime concern. Lastly, completing the GUS/promoter fusion experiment with *NPF4.12* to visualize its gene expression during AM symbiosis would be of importance. These last two experiments should be the priority because we have the means and most of the materials to complete them.

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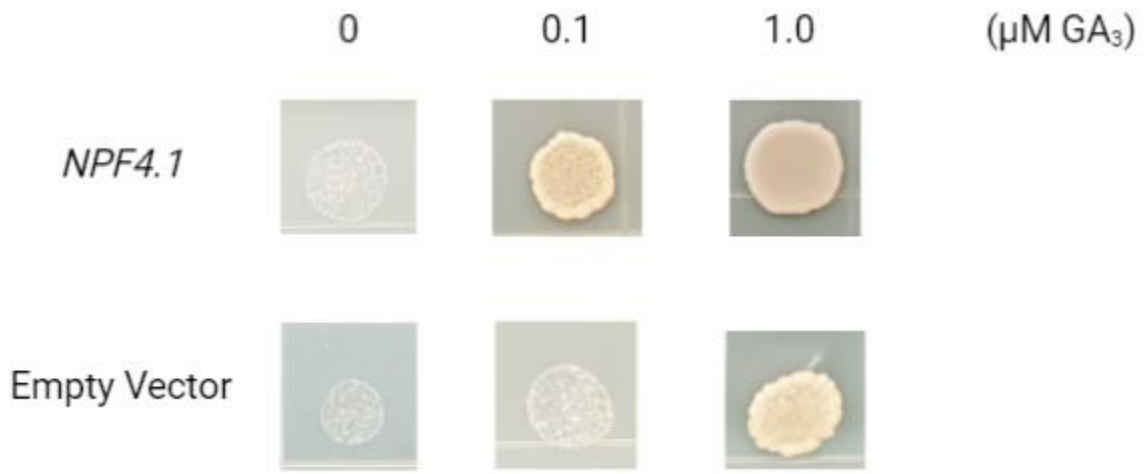
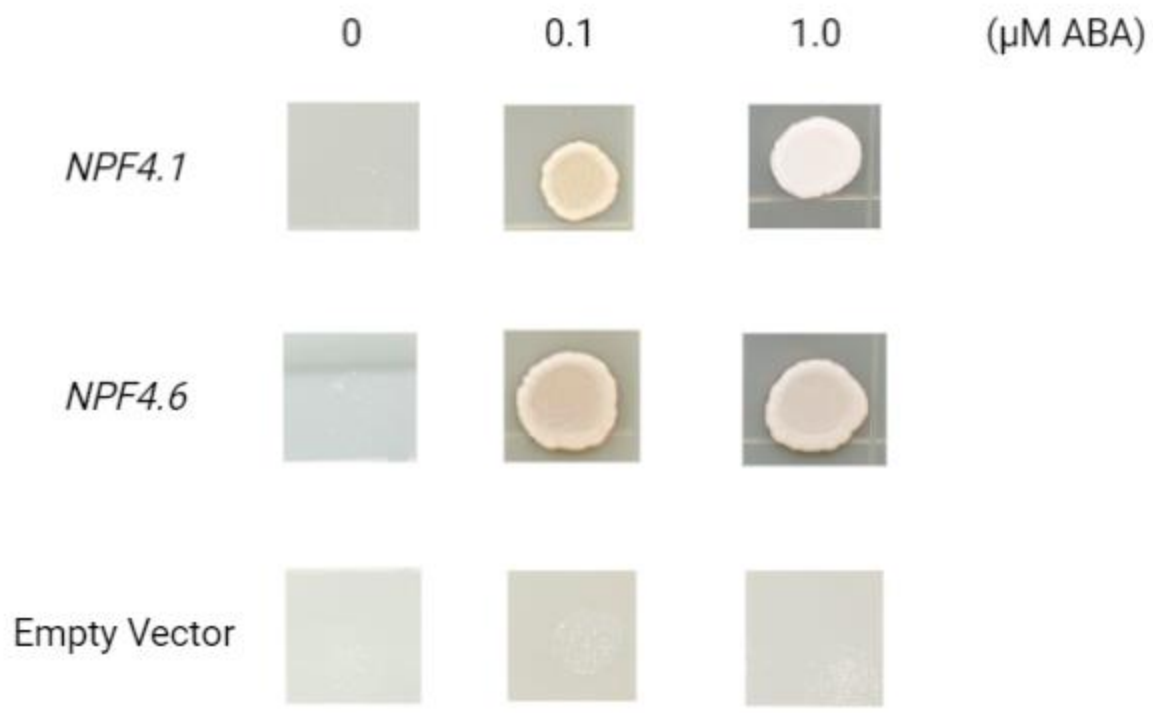
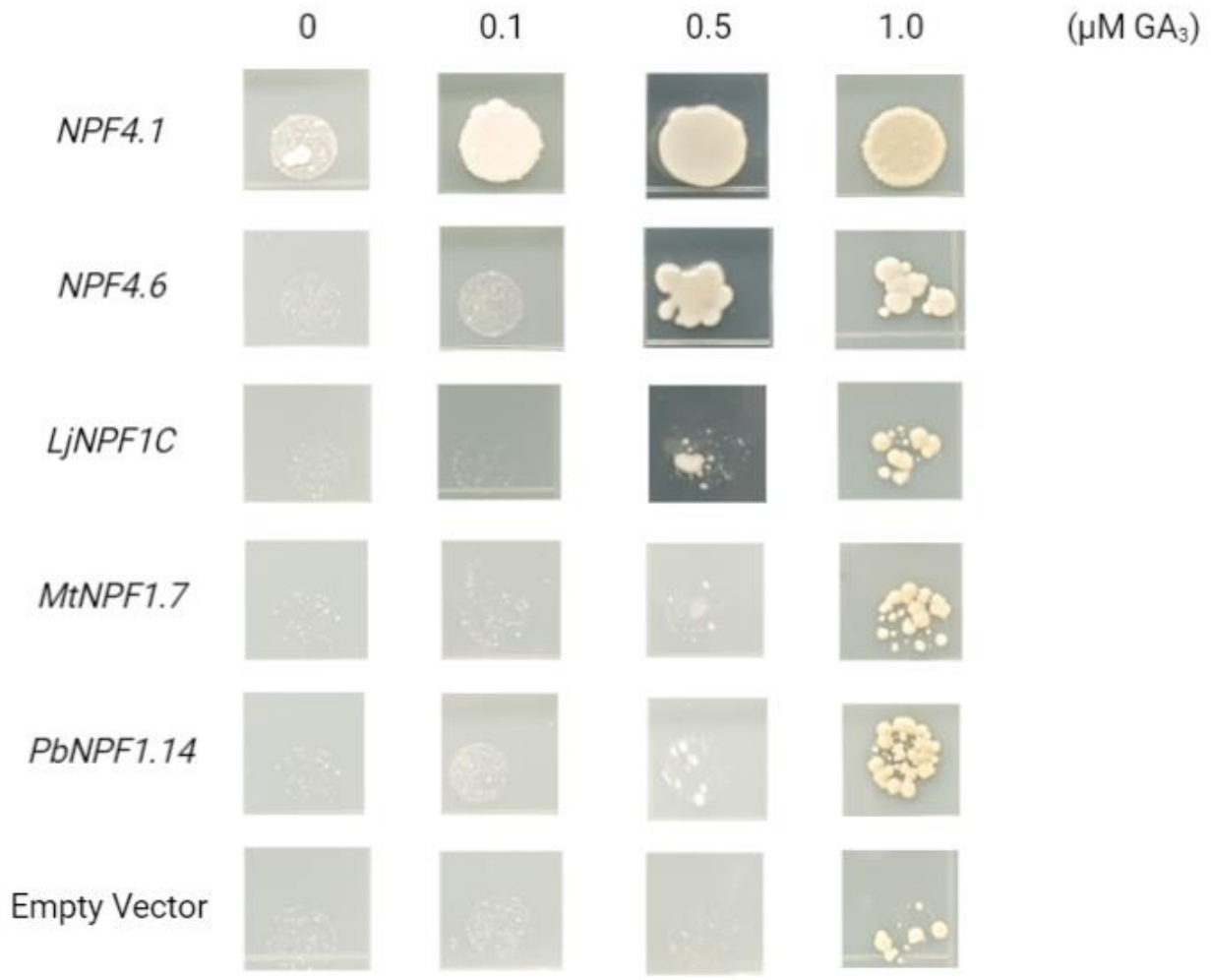
Supplementary Information



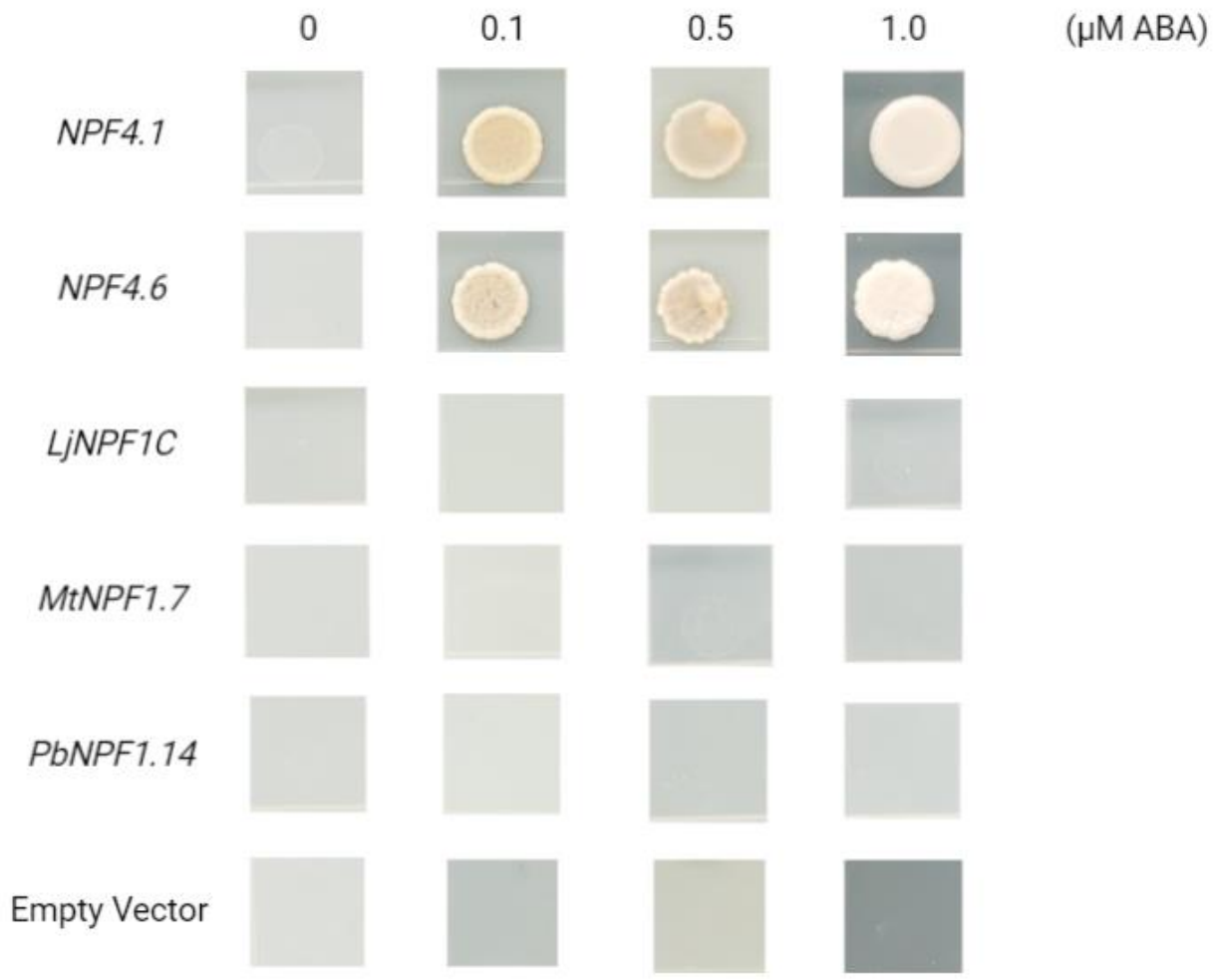
Supplementary Figure 1. Results of a modified yeast-two hybrid assay to assess ability of NPF4.1 to import GA_3. *S. cerivisiae* strain MaV203 was transformed with pDEST22/GAI and pDEST32/GID1a as its co-receptors for the presence of GA_3, along with the candidate *NPF* transporter gene *NPF4.1* to test for capacity to import GA_3. The defined minimal media upon which the yeast was plated lacked leucine, tryptophan, uracil, and histidine. Concentration of GA_3 which is supplemented in the media is denoted in the top row.



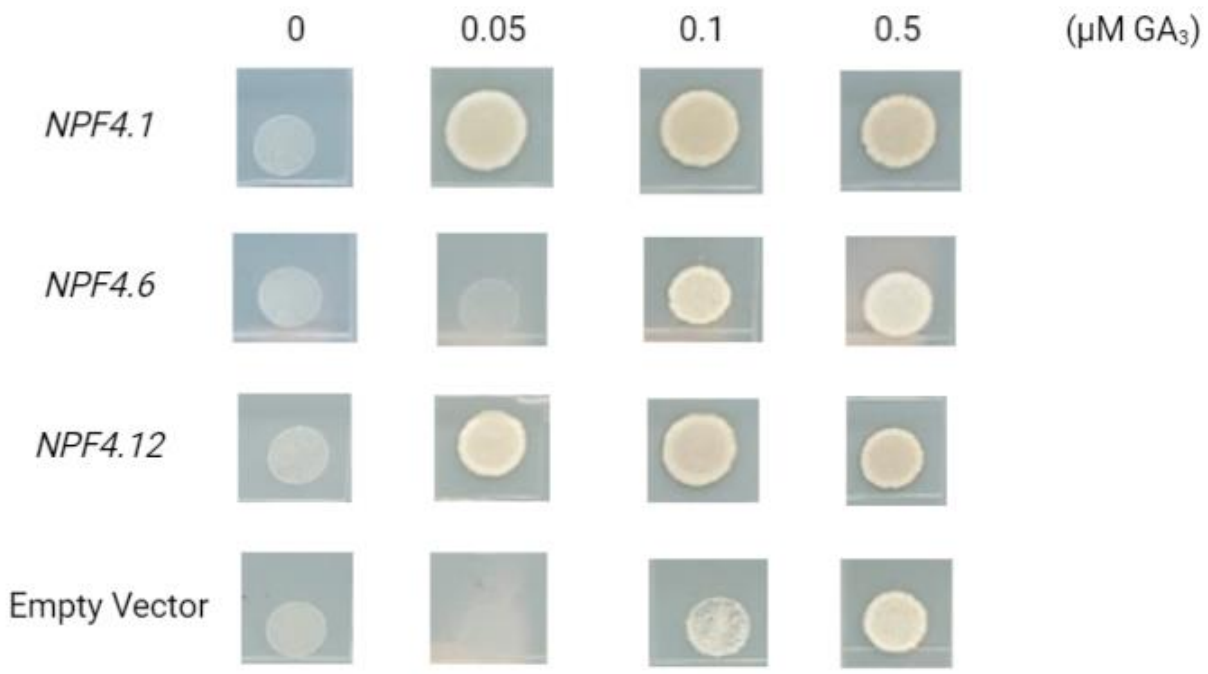
Supplementary Figure 2. Results of a modified yeast-two hybrid assay to assess ability of NPF4.1 and NPF4.6 to import ABA. *S. cerivisiae* strain MaV203 was transformed with pDEST32/PYR1 and pDEST22/ABI1 as its co-receptors for the presence of ABA, along with the candidate *NPF* transporter genes *NPF4.1* and *NPF4.6* to test for capacity to import ABA. The defined minimal media upon which the yeast was plated lacked leucine, tryptophan, uracil, and histidine. Concentration of ABA which is supplemented in the media is denoted in the top row.



Supplementary Figure 3. Results of a modified yeast-two hybrid assay to assess ability of LjNPF1C, MtNPF1.7, and PbNPF1.14 to import GA_3. *S. cerivisiae* of the strain MaV203 was transformed with pDEST22/GAI and pDEST32/GID1a as its co-receptors for the presence of GA_3, along with the candidate *NPF* transporter genes *LjNPF1C*, *MtNPF1.7*, and *PbNPF1.14* to test for capacity to import GA_3. The defined minimal media upon which the yeast was plated lacked leucine, tryptophan, uracil, and histidine. Concentration of GA_3 which is supplemented in the media is denoted in the top row.



Supplementary Figure 4. Results of a modified yeast-two hybrid assay to assess ability of LjNPF1C, MtNPF1.7, and PbNPF1.14 to import ABA. *S. cerivisiae* strain MaV203 was transformed with pDEST32/PYR1 and pDEST22/ABI1 as its co-receptors for the presence of ABA, along with the candidate *NPF* transporter genes *LjNPF1C*, *MtNPF1.7*, and *PbNPF1.14* to test for capacity to import ABA. The defined minimal media upon which the yeast was plated lacked leucine, tryptophan, uracil, and histidine. Concentration of ABA which is supplemented in the media is denoted in the top row.



Supplementary Figure 5. Results of a modified yeast-two hybrid assay to assess ability of NPF4.12 to import GA_3. *S. cerivisiae* strain MaV203 was transformed with pDEST22/GAI and pDEST32/GID1a as its co-receptors for the presence of GA_3, along with the candidate *NPF* transporter gene *NPF4.12* to test for capacity to import GA_3. The defined minimal media upon which the yeast was plated lacked leucine, tryptophan, uracil, and histidine. Concentration of GA_3 which is supplemented in the media is denoted in the top row.