Functional genetic studies of symbiotic genes in *Medicago truncatula* indicate a role for a CCAAT-box transcription factor in rhizobial infection

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ABSTRACT

Legumes form mutualistic associations with nitrogen-fixing bacteria and arbuscular mycorrhizal (AM) fungi which increases nutrient availability to the plant. Nodulation is generally restricted to legumes and has co-opted genes required for the AM symbiosis. As a result, both associations share common genes and have analogous infection strategies, including the early lipochitooligosaccharide (LCO) signals that plants perceive from the symbionts.

In order to discover novel genes required for both these symbioses, I used both forward and reverse genetic approaches. A forward genetic screen in *Medicago truncatula* revealed an H^+-ATPase mutant *Mtha1* with a defective arbuscule phenotype. The reverse screen investigated several candidates previously identified as having enhanced expression in the *nodule inception* mutant during nodulation that were also expressed in AM-colonized roots. Four of these genes were chosen for further study. The first, a novel AM-specific gene, *BiFunctional Protein* (*BFP*) has a predicted a role in lipid modification. Phylogenetic analysis revealed that *BFP* was restricted to AM host plants including a member of the liverworts, *Marchantia paleacea*. *BFP* expression during AM interactions in *M. truncatula* was found to be dependent on the common symbiotic gene *DOES NOT MAKE INFECTIONS 3*. AM colonization assays using mutants for *BFP* in *M. truncatula* and *Oryza sativa* were inconclusive.

Three CCAAT-Binding Factor transcription factors were also studied (*CBF1*, *CBF2* and *CBF3*). Promoter-reporter studies revealed that these genes are also expressed during rhizobial infection of wild type plants. While the *M. truncatula cbf1* mutant did not exhibit a consistent nodulation phenotype, mutant analysis for *CBF3*, showed it is required for normal infection during root hair colonisation of rhizobia in *M. truncatula*. Based on these results and a further analysis of expression data and published work, I propose a model in which CBF3 forms a complex with NF-YA1 and NF-YC2 during early responses to LCOs during nodulation.

Contents

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For Ciaran, Loren, Daniel and Jude X

ABBREVIATIONS

ABA	Abscisic acid
ACP	Acyl Carrier Protein
AM	Arbuscular Mycorrhiza
AMP	Adenosine monophosphate
AOC	Allene Oxide Cyclase
AOS	Allene Oxide Synthase
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
CSP	Common Symbiosis Pathway
DWA	Distilled Water Agar
dpi	days post inoculation
EM	Ecto Mycorrhiza
ER	Endoploasmic Reticulum
FAAL	Fatty acid AMP ligase
FACL	Fatty acid CoA ligase
GA	Giberellic acid
GlcNac	N-Acetylglucosamine
hpi	hours post inoculation
IT	Infection thread
JA	Jasmonic acid
LCM	Laser capture microdissection
LCOs	Lipochitooligosaccharides
LOX	Lipoxygenase
MeJA	Methyl jasmonate
MtGEA	Medicago truncatula gene expression atlas
Myc	Mycorrhiza
OPDA	12-oxo phytodienoic acid
PAM	Peri-arbuscular membrane
PCR	Polymerase chain reaction
PIT	Pre-infection thread
PPA	Pre-penetration apparatus
PUFA	Polyunsaturated fatty acid
RNAi	RNA interference
UTR	Untranslated region
wpi	weeks post inoculation
WT	wild type
qRTPCR	quantitative real time PCR

Chapter One

Introduction

1.1 Symbiosis –A general overview

Mutualisms are symbiotic associations in which both parties benefit. The two most agronomically important plant-microbe mutualisms are those involving mycorrhizal fungi and nitrogen-fixing bacteria. Most land plants can form associations with mycorrhizal fungi, which indicates that this ancient partnership performs a vitally important function. The *Fabaceae* family (legumes) almost exclusively form a symbiosis with a family of nitrogen-fixing bacteria known collectively as rhizobia.

1.1.1 Mycorrhizal Symbiosis-general overview

After nitrogen, phosphorous availability is the second major limiting factor to plant growth. As a consequence, phosphate is applied to agricultural land in high concentrations that results in widespread eutrophication of lakes, rivers and estuaries. Moreover, agricultural phosphate comes from rapidly-depleting non-renewable sources (Lambers et al, 2006). In natural populations, most plants participate in a mutualistic symbiosis with mycorrhizal fungi. AM fungi produce long hyphal networks that can extend far beyond the host's root system. Therefore, association with AM fungi effectively extends the area of soil that the plant has available beyond the natural depletion zone around the root (Rhodes and Gerdemann, 1975). This increased access to soil phosphorous that would normally be beyond the reach of the plant roots is crucial since phosphate is typically immobilised by adsorption to soil particles (Lambers et al, 2006).

As well as phosphorous, the fungi provide the plant host with a wealth of other advantages. These include: uptake of other important nutrients, including nitrogen; improved water uptake; and protection from pathogens (Killham, 2001) . In return, the plant host provides carbohydrates to the fungus. There are two main types of mycorrhization that exist; ectomycorrhizal and endomycorrhizal. Arbuscular mycorrhiza (AM) are an example of endomycorrhizal fungi where the hyphae penetrate plant cells, forming an intimate alliance (Killham 2001).

The use of AM fungi with crop plants could help towards the challenges being faced by modern agriculture. With an increasing global population, optimum crop yields are paramount, along with the need to reduce the harmful environmental impacts of chemical fertilisers. A recent study showed that inoculation of potato crops with AM spores, lead to a significant increase in yields, which equates to a 9.5% marketable yield (Hijiri, 2016).

AM fungi belong to a monophyletic phylum, the *Glomeromycota. Glomeromycota* are obligate biotrophs, requiring a photosynthetic host to complete their lifecycle. Phylogenetic evidence suggests that these are one of the earliest 'true fungi' and have remained morphologically unchanged for at least 400 million years. They are also one of the most primitive true fungi, that have simple spores and asexual reproduction (Brundrett 2002).

Over 80% of land plants are capable of forming AM associations, suggesting that the association stems from an early common ancestor (Parniske, 2008). Fossils from the Devonian period (approximately 400mya) show obvious plant-AM associations that look morphologically very similar to the ones seen today (Figure 1.1). This indicates that formation of AM associations emerged either before or during plants'colonisation of land (Remy et al, 1994; Humphreys et al, 2010). Mycorrhizal genes can be found in ancient plant species such as liverworts and hornworts. These genes can complement angiosperm plant mutants unable to mycorrhize (Wang, Yeun et al. 2010).

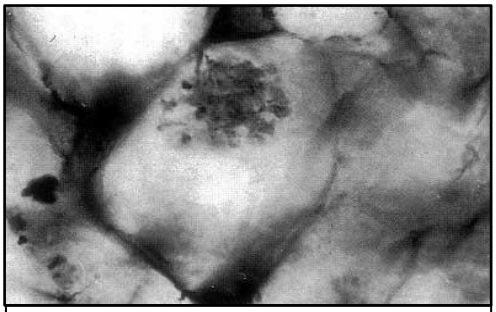


Figure 1.1 **Fossil of a plant-mycorrhizal interaction**

Mycorrhizal symbiotic fungus growing on cells of Aglaophyton from the Early Devonian Rhynie Chert X600.

(http://www.palaeos.com/Fungi/Ascomycota/C00040Pezizomycotina.html)

1.1.2 Rhizobial Symbiosis-general overview

Legumes produce nodules on their roots that house phylogenetically diverse gram-negative nitrogen-fixing bacteria known as rhizobia, which provide nitrogen to the plant host (Held, Hossain et al. 2010). Legumes are important in agricultural systems that

employ crop rotation practises; thereby leaving nitrogen in the soil for a subsequent crop (Gough and Cullimore 2011).

1.1.3 Mycorrhization and nodulation share common genes

The discovery of the first common symbiotic mutant (in pea), prompted the realisation that AM and rhizobial symbioses may share common genes (Duc et al, 1989). Several genes have since been identified to be required for both symbioses, which suggests that legumes enlisted the older mycorrhizal pathway to form an association with nitrogen-fixing bacteria (Kistner and Parniske, 2002). Molecular evidence points to rhizobial-legume associations evolving approximately 60mya; there was a genome-wide duplication of legumes around that time (Cannon *et al*, 2006). It seems likely that this genome duplication allowed for the acquisition of novel nodule-related functions for otherwise redundant gene copies, offering a potential opportunity to recruit mycorrhizal genes to nodulation (Young et al, 2011).

1.2 Early establishment of symbioses

AM and rhizobial symbioses share common genes, as well as comparable infection and cellular accommodation strategies. The legumes *Medicago truncatula* and *Lotus japonicus,* are used as models to study the similarities and differences of both associations, as they can partake in both. Non-legumes, notably rice and tomato, are also used as models for the AM symbiosis. Members of the *Brassicacae* have lost the ability to accommodate AM fungi; this includes the model plant, *Arabidopsis thaliana.*

1.3.1 Arbuscular Mycorrhiza

1.3.1.1 Early Infection

The communication between AM and a plant host in the soil, stimulates both hyphal and lateral root branching which may serve to increase the chances of the hyphae making contact with the root surface. When the hyphae and roots meet, the hyphae can adhere to the epidermal surface of a new lateral root. It does this by the formation of a special kind of appressorium, known as a hyphopodium. In contrast to fungal pathogens that use appressoria to forcefully penetrate the root, the host plant

undergoes dramatic cellular changes to accommodate its potential symbiotic partner (Mendgen et al, 1996).

The first physical response to hyphopodium contact is the migration of the plant nucleus towards the plant-fungal contact site within the epidermal cell. An accumulation of endoplasmic reticulum (ER) and actin bundles form between the nucleus and hyphopodium contact site. ER, actin and microtubule follows the nucleus as it slowly moves from the cytoplasmic side of the plasma membrane, where fungal entry is anticipated, towards the opposite end. This aggregation of cellular and cytoskeletal components forms a cytoplasmic bridge across the cell vacuole called the pre-penetration apparatus (PPA). During this pre-penetration phase, novel intracellular machinery is being assembled for the intense membrane trafficking that needs to occur for newly assembled plasma membrane to form, termed 'cytoplasmic aggregation' (Genre et al, 2005; 2008). It takes approximately 4-5 hours for the nucleus to traverse the whole cell, after which it disassociates from the cytoplasmic bridge. The nuclear migration effectively pre-determines the path of the PPA, and thus the path of fungal entry, which occurs once the PPA has completely formed across the epidermal cell. In the same way, the underlying outer cortical cells form PPAs to allow the smooth passage of the AM fungus into the root (Genre et al, 2005). It takes approximately three hours for the AM hyphae to cross the cell. By then, much of the ER and cytoskeletal elements have dissipated, suggesting that the PPA is a transient structure (Figure 1.2; Genre et al, 2005). Once the AM hyphae reach the inner cortex, they change from intracellular to intercellular movement and eventually form structures called arbuscules in the innermost layer of cortical cells (Murray et al, 2011). The word arbuscule literally means 'little tree' or 'bush'. This describes the intricately branched hyphal structure that provides a high surface area where nutrient exchange can occur. The arbuscule has an intimate association with the cortical cell but is physically separated from the cell cytoplasm by both the plant-derived peri-arbuscular membrane (PAM) that is continuous with the plant plasma membrane, and the fungal plasma membrane (Parniske, 2008).

PPAs are also required for arbuscule formation. Inner cortical cells which are in direct contact with hyphae display the cell migration and cytoplasmic aggregation seen in epidermal cells at early infection (Blancaflor et al, 2001). PPAs are also formed in cells that are adjacent to arbuscules. In cortical cells that have PPAs, the nuclei are enlarged with decondensed chromatin, indicating enhanced transcriptional activity (Genre et al, 2008). There is a great overlap of gene expression between arbuscule-containing cortical cells and adjacent cells that contain PPAs, but do not have arbuscules (Gaude et al, 2012). These genes are likely to be involved in the cytoplasmic aggregation and nuclear changes that are required for PPA formation. The genes that are exclusively expressed in arbuscule-containing cells are likely to be involved in nutrient exchange mechanisms and the formation of the peri-arbuscular membrane (PAM).

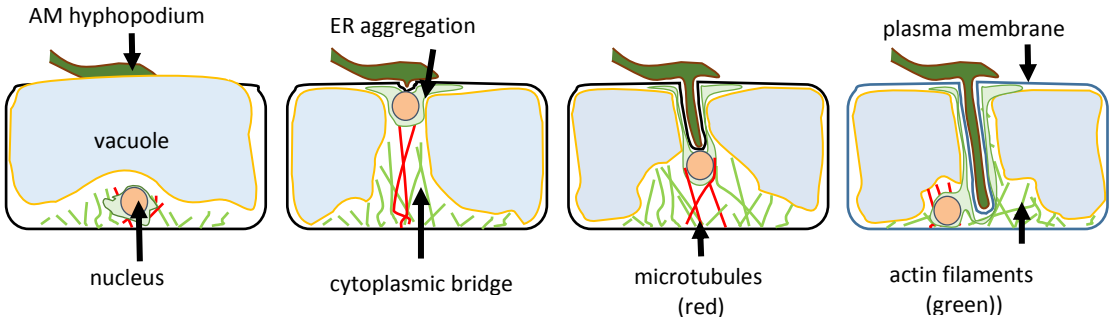


Figure 1.2 **The stages of early infection by arbuscular mycorrhiza in root epidermal cells**

The nucleus migrates to the site of hyphopodium attachment. This is associated with cytoplasmic aggregation and the formation of a cytoplasmic bridge as the nucleus traverses the epidermal cell. This forms the pre-penetration apparatus through which the AM hyphae can infect, whilst remaining in the apoplastic compartment.

1.3.1.2 Arbuscule formation

Two distinct domains have been identified in the arbuscule structure: an 'arbuscule trunk domain' and an 'arbuscule branch domain'. The branch domain is surrounded by the peri-arbuscular membrane (PAM), which is specific to arbuscules. It surrounds the highly branched arbuscule and is continuous with the plasma membrane, but has a distinct protein composition containing specific nutrient transporters, for example, the phosphate transporter *PT4*. The membrane surrounding the trunk domain appears to be more like a typical plasma membrane (Pumplin et al, 2009). The PAM and the fungal

membrane that surrounds it provide the site of nutrient exchange. The space between the membranes is called the peri-arbuscular space and is composed of fungal and plant cell walls. It is here that nutrients are released prior to being taken up by each symbiont, namely phosphate and other nutrients by the plant and sugars by the fungus (Parniske, 2008). Experiments involving promoter swap constructs, suggest that the subcellular location of proteins to the peri-arbuscular membrane is controlled by precise timing of gene expression; where all *de-novo* synthesized proteins are directed to the peri-arbuscular membrane during arbuscule branching and growth (Pumplin and Harrison, 2009).

The formation of an arbuscule in a plant inner cortical cell can be genetically divided into 5 stages: 1) PPA formation 2) fungal entry/arbuscule trunk formation; 3) birds foot stage (hyphal branching resembling a bird's foot); 4) hyphal branching which develops into a mature arbuscule; and 5) arbuscule collapse/senescence. These stages of development are linked to the transcription of particular genes (Gutjahr and Parniske, 2013-Figure 1.3).

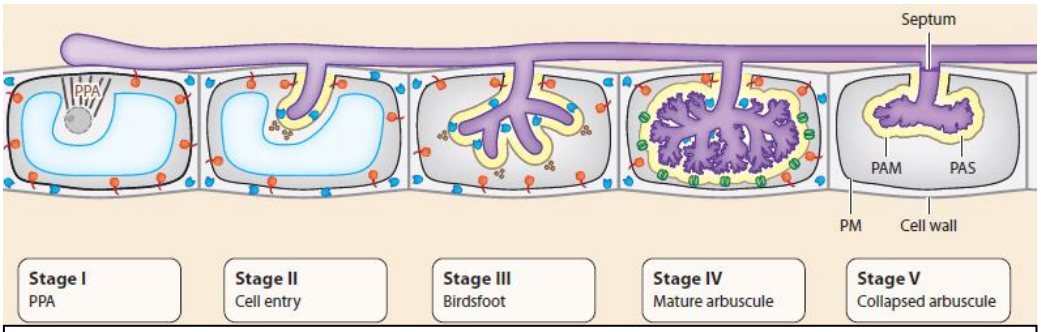


Figure 1.3 **Stages of arbuscule development**

Figure adapted from Gutjahr and Parniske (2013)

1.2.1 Signal exchange between symbionts

The soil dwelling rhizobia and arbuscular mycorrhizal (AM) fungi are able to perceive a potential plant host by detecting certain low-molecular weight products that are secreted into the rhizosphere by the plant root. Root exudates from carrot hairy roots can induce hyphal branching of *Gigaspora* AM species, and induce mitosis in *Gigaspora gigantea,* which is essential for hyphal branching (Buee et al, 2000). It was found that strigolactones extracted from *L. japonicus* roots can induce hyphal branching in the

germinating spores of *Gigaspora margarita,* as well as strigolactone synthetic analogue (Akiyama et al, 2005). This effect was also seen in monocotyledonous plant, Sorghum, where strigolactones were shown to induce metabolic changes in the AM species *R. irregularis* and *Glomus claroideum* (Besserer et al, 2006). Strigolactones do not remain in the rhizosphere for long due to a labile ether bond that spontaneously hydrolyses in contact with water. This property would make strigolactones in the soil a good indicator of nearby potential hosts for the AM fungus (Parniske, 2005). The AM exist as spores in the soil and strigolactone perception triggers spore germination and the release of low molecular-weight factors that can be perceived by the plant (Genre et al, 2013). The presence of these has been identified from experiments separating AM fungi from the roots of *M. truncatula* with a diffusible membrane and observing mycorrhizal-induced gene expression (Kosuta et al, 2003). Plant perception of these AM-derived signals also stimulates an increase in lateral root formation (Olah et al. 2005).

Similarly, rhizobia perceive flavonoids in the rhizosphere which serve as chemo-attractants and induce the production of diffusible substances called Nod factors. Nod factors are lipo-chitooligosaccharides (LCOs) that show structural similarities to chitin-based microbe associated molecular patterns that trigger early defence responses (Gough and Cullimore, 2011). LCOs are signalling molecules that induce plant developmental responses characteristic of rhizobial infection, even when no infection is present (Fournier et al, 2008). Nod factors show a high degree of plant-host specificity. Decorations on the chitin backbone are essential for this and often include an acyl chain at the non-reducing end, sometimes, a sulphate group at the reducing end (Limpens and Bisseling, 2003).

Since the discovery of Nod factors, some of the AM diffusible signals (Myc factors) have been identified that are composed of a mixture of different N-acetylglucosamine oligosaccharides. This includes a mixture of sulphated and non-sulphated LCOs that are structurally very similar to Nod factors (Maillet et al, 2011), as well as short-chain oligomers, particularly CO4 and CO5 (Genre et al, 2013).

1.2.2 Perception of LCOs

The structural similarities of the rhizobial and AM-derived diffusible signals that are perceived by the plant host is another example of nodulation and mycorrhization having analogous characteristics. The *M. truncatula* receptor NOD FACTOR PERCEPTION (NFP)

and its *L. japonicus* orthologue, NOD FACTOR RECEPTOR 5 (NFR5) are required for Nod factor perception. Rhizobial infection also requires the entry receptor LysM DOMAIN RECEPTOR KINASE 3 (MtLYK3)/ NOD FACTOR RECEPTOR 1 (LjNFR1) (Limpens et al, 2003; Radutoiu et al, 2003; Smit et al, 2007). These receptors bind Nod factors at high-affinity binding sites, and plant mutants block rhizobial entry and nodule formation (Broghammer et al, 2012).

AM colonisation is not dependent on either NFP or LYK3, suggesting that there are different Myc factor receptors, although none of these have been identified. *Parasponia andersonii* is the only non-legume that can form a symbiotic association with rhizobia. This symbiosis has evolved independently from legume nodulation but requires Nod factors for effective rhizobial symbiosis. An RNAi knockdown of a *NFP*-like putative LysM Nod factor receptor in *P. andersonii* showed that it is essential for both nodulation and AM associations (Op den Camp et al, 2011). NFP has a paralogue, *LYR1,* which can be traced back to the genome-wide duplication, approximately 58 million years ago (Young et al, 2011). Recently, the orthologue of *PaNFP* in tomato, *SlLYK10,* was reported as being required for AM infection, confirming that *NFP* has neo-functionalised in legumes (Buendia et al, 2016).

1.3 Plant physiological responses to symbionts

1.3.2 Plant physiological responses to rhizobial infection

1.3.2.1 Root hair infection

The initiation of the rhizobial-legume symbiosis begins with the rhizobia attaching to the flank of a growing root hair. The host plant perceives Nod factors which initiates a switch from root hair polar growth to that towards the bacteria. This forms a tight curl around the rhizobia, which entraps it in an 'infection pocket'. The nucleus in the root hair doubles in size and moves to the centre of the epidermal cell. Between the nucleus and the basal end of the cell a cytoplasmic bridge is formed. The nucleus slowly moves down the root hair and across the cell, following the path created by the cytoplasmic bridge. Behind the nucleus, an invagination of the root hair is formed where growing cell membrane and cell wall is laid down. This forms a tube, the infection thread, which is the conduit for rhizobia infection (Fournier et al, 2008). The plant host is likely to be mechanistically in charge of IT progression as it forms ahead of bacterial colonisation. The progression of the IT grows in a discontinuous fashion, with rapid growth being

associated with a broad cytoplasmic bridge and a nearby nucleus (Figure 1.4). The cytoplasmic bridge is thought to be required for supplying the growing IT tip with exocytotic and endocytotic vesicles. Infection threads aligned with the epidermal infection threads are also formed in the underlying cells, creating a continuous passage into the root cortex, until reaching the developing nodule primordium (Timmers et al, 1999; van Spronsen et al, 2001).

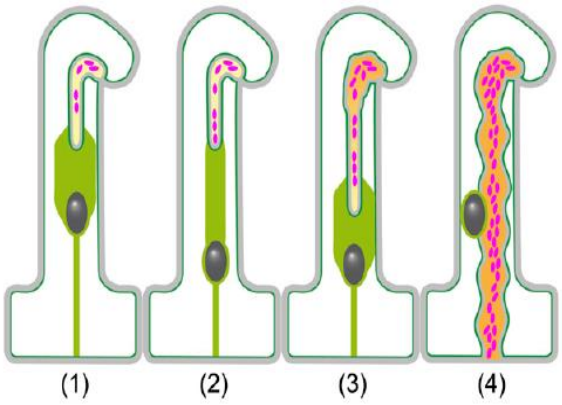


Figure 1.4 **Diagrammatic representation of infection thread (IT) progression in the root hair**

The IT has discontinuous growth, with a nearby nucleus and broad pre-infection thread (PIT) associated with faster growth. At all times there is a cytoplasmic strand linking the nucleus and the basal end of the root hair cell (Fournier et al, 2008).

1.3.2.2 Nodule development

During the very early stages of infection, cortical cells have already begun dividing in readiness for nodule organogenesis directly below the site of infection (Xiao et al, 2014). Although the two processes are coordinated, they are genetically separable (Murray et al, 2007; Tirichine et al, 2007; Oldroyd and Downie, 2008). There are two types of nodules; indeterminate and determinate. *M. truncatula* has indeterminate nodules that have a persistent meristem and an adjacent infection zone containing ITs where the nodule is continuously infected. Determinate nodules, seen in *L. japonicus,* have a fixed lifespan, lack a meristem and are not continuously infected.

Once rhizobia reach the nodule primordia, they are endocytosed and are thereby enclosed by a plant-derived membrane called the peri-bacteroid membrane where they differentiate into bacteroids. These organelle-like structures, called symbiosomes, are where nitrogen fixation takes place (Popp and Ott 2011). Plant derived leghaemoglobin binds to oxygen to support respiration of the bacteroids and protect the nitrogenase, allowing the bacteroids to fix nitrogen and giving the nodule a pink colour (Starker et al, 2006). The symbiosome is therefore the site of nutrient exchange between the plant

and rhizobia. The peri-bacteroid membrane, although derived from the plant plasma membrane, incorporates novel proteins required for its specialised functions.

Mature indeterminate nodules consist of distinct zones that progress from the nodule tip to the base (Vasse et al, 1990). Zone I, in the apical part of the nodule, is the meristematic zone that is responsible for nodule growth. Zone II is the infection zone which consists of infection threads that release rhizobia into the nodules as symbiosomes. Zone III, the largest zone, is the fixation zone which is packed with nitrogen-fixing bacteroids. Zone IV is the senescent zone where the bacteria are degraded.

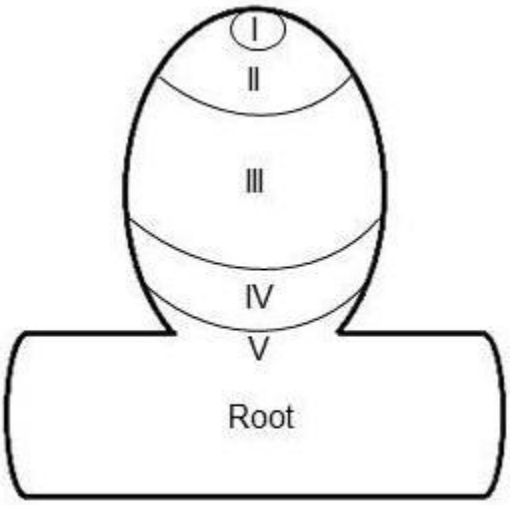


Figure 1.5 **The zones of an indeterminate nodule**
A diagram to demonstrate the zones observed during indeterminate nodule development. Zone I is the nodule meristem. Zone II is the infection zone. Zone III is the nitrogen fixation zone. Zone IV is the senescence zone.

1.4 Plant genes required for symbiosis

1.4.1 The Common Symbiosis Pathway

The perception of Nod factors and Myc factors initiates a calcium spiking response in and around the cell nucleus (Wais et al, 2000; Miwa et al, 2006; Saito et al, 2007; Charpentier et al, 2008). This is induced and perceived by a core set of genes that comprise the Common Symbiosis Pathway (CSP). Five of these genes have been identified in both *M. truncatula* and *L. japonicus*: *Does Not Make Infections 2 (MtDMI2/LjSYMRK),* a leucine-rich repeat receptor-like kinase; *MtDMI1/LjPOLLUX,,* a

nuclear envelope-localised cation channel; *MtDMI3/LjCCaMK,* a calcium and calmodulin dependent kinase; and *Interacting protein of DMI3 (MtIPD3/LjCYCLOPS)* (Endre et al, 2002; Stracke et al, 2002; Ane et al, 2004; Levy et al, 2004; Mitre et al, 2004; Imaizumi-Anraku et al, 2005; Messinese et al, 2007; Charpentier et al, 2008; Capoen et al, 2011; Horvath et al, 2011). In *L. japonicus,* nucleoporins *NUP85*, *NUP133* and *NENA,* and cation channel *CASTOR* have been shown to be part of the CSP, however orthologues in *M. truncatula* have not been identified (Kanamori et al, 2006; Saito et al, 2007; Groth et al, 2010).

The genes in the CSP are placed along the pathway according to whether they are dependent on, or are required for, for the initiation of nuclear calcium oscillations. *MtDMI3/LjCCaMK* lies immediately downstream of the calcium oscillations and is thought to decode the calcium spiking (Miller et al, 2013). Mutations that constitutively activate *DMI3* are sufficient to initiate spontaneous nodule formation in the absence of rhizobia (Gleason et al, 2006). Also, in *L. japonicus,* CCaMK activation produces PPA-like structures (Takeda et al, 2012). *MtIPD3/LjCYCLOPS* lies downstream of calcium oscillations, and is phosphorylated by *DMI3* (Yano et al, 2008; Horvath et al, 2011; Singh et al, 2014). Downstream of *MtDMI3/LjCCaMK* and *MtIPD3/LjCYCLOPS* is a suite of transcription factors that regulate gene responses required for either nodulation or AM colonisation (Figure 1.6).

Historically, the CSP refers to the genes that are responsible for the required calcium oscillations prior to successful infection of both rhizobia and mycorrhizal fungi. However, this may not be the full extent of the genetic overlap between the two symbioses. Transcriptomics from rhizobial-infected root hairs have highlight enhanced expression in genes also up regulated in AM colonised roots (Breakspear et al, 2014). This, potentially highlights a common genetic recruitment for early epidermal infection, supported by the common physiological strategies employed in response to both symbionts. Two genes downstream of the CSP have already been shown to be required for both nodulation and AM colonisation (Pumplin et al, 2010; Murray et al, 2011). It seems likely, that more 'common symbiosis' genes will be discovered in the future.

1.4.2 Genes downstream of the common signalling pathway

Research on nodulation has been more progressive than that of AM, mainly because it is easier to culture rhizobia and purify Nod factors, it has a faster infection rate (days, compared to weeks for mycorrhiza), and easier identification of nodulation mutant phenotypes. Hence, knowledge of genes downstream of the common signalling pathway, is heavily weighted towards nodulation. These genes acting downstream of the CSP are the genes responsible for specific plant physiological responses to either symbiont (i.e. development of nodules or arbuscules).

A common theme throughout the study of AM associations and the legume-rhizobial symbiosis is that there are many similar features, from the early chitin-based signalling responses of the microbes, the common signalling pathway, to the subcellular features of the plant-derived infection structures. This is probably the result of a genetic co-opting of the AM pathway during legume evolution. Although it is clear that there must be divergent gene expression for the accommodation of each symbiont, it is also apparent that the intense cellular reconstruction that occurs during the early stages of infection could also require the same genes. Genetic studies are not only easier for nodulation in forward screens, but identifying gene expression in individual cell types is also simpler. Recent studies have examined gene expression in root hairs and laser capture microscopy (LCM) of the individual nodule zones during infection (Breakspear et al, 2014; Roux et al, 2014). Similar approaches have been performed in AM colonised roots, using LCM to identify gene expression in infected cortical and epidermal cells. Because more is understood about genes required for nodulation, I will describe these first.

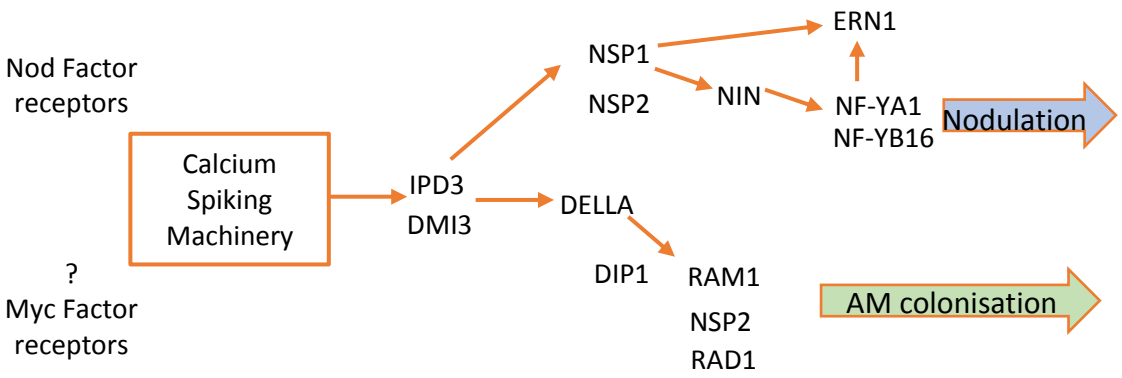


Figure 1.6 **Transcription factors downstream of the common symbiosis pathway (CSP)**

A putative transcription factor signalling pathway, downstream of the calcium spiking machinary, based on current studies. All proteins are written as *M. truncatula* proteins to make the figure simpler. The regulation of the NF-Ys by *NIN* was found in *L. japonicas* (*LjNF-YA1* and *LjNF-YB1*) (Soyano et al, 2013). *RAD1* was discovered in *L. japonicas* and found to interact with the *RAM1* and *NSP2* orthologues (Xue et al, 2015). The role of DELLA in AM symbiosis has been confirmed in *M. truncatula* (Floss et al, 2013), rice (Yu et al, 2014) and *L. japonicas* (Pimprikar et al, 2016). *DIP1* interacts with the DELLA gene, *SLR1,* in rice and the *RAM1* orthologue (Yu et al, 2014).

1.4.2.1 Genes required for nodulation

NODULE INCEPTION (NIN) is a transcription factor required for both normal infection and nodule development. *NIN* mutants have excessive root hair curling in response to rhizobia, but fail to form infection threads or develop nodules (Schauser et al, 1999). *NIN* expression is dependent on the presence of *NSP1* and *NSP2* and is also essential for the spontaneous nodule formation observed with autoactivation of *DMI3/CCaMK* (Marsh et al, 2007). NIN also plays a role in restricting the expression domain of the infection marker, *ENOD11*, indicating that it has a role in the negative regulation of infection (Marsh et al, 2007; Vernie et al, 2015). A novel *nin* mutant in *L. japonicus* (*daphne*) has no cortical *NIN* expression but retains its expression in epidermal cells. This mutant does not develop nodules, and exhibits an increased infection thread phenotype. When *NIN* is overexpressed in the *daphne* mutant, the hyper-infection phenotype is suppressed. This, along with the negative regulation seen in *ENOD11,*

suggests negative feedback regulation is mediated by *NIN* where the cortical expression of *NIN* during nodule organogenesis restricts the epidermal susceptibility to rhizobial infection (Yoro et al, 2014) (Figure 1.7).

Several genes are required for the normal bacterial progression of infection threads. Some of these are also required for nodule development (as is the case for *NIN*). *Nodulation Signalling Pathway 1* (*NSP1*) and *NSP2* in *M. truncatula*, are GRAS transcription factors immediately downstream of the CSP that are required for normal nodulation (Smit et al, 2005; Kalo et al, 2005). This is evidenced by their being required for the spontaneous nodulation phenotype seen in gain-of-function *CCaMK* mutants (Gleason et al, 2006). *NSP1* and *NSP2* are required for early infection and nodule development (Catoira et al, 2000; Oldroyd and Long, 2003). They interact to directly regulate the early infection marker, *Early Nodulin 11 (ENOD11)* which encodes a proline-rich cell-wall associated protein (Journet et al, 2001; Boisson-Dernier et al., 2005). *ERF Required for Nodulation 1 (ERN1)* also positively regulates *ENOD11,* but on a separate promoter region than NSP1/2; responding specifically to Nod factors (Cerri et al, 2012). This is also the case for *ERN2,* whose function may be partly redundant with *ERN1* (Andriankaja et al, 2007).

Other genes are more specialized for functions in infection, several of them being dependent on NIN for the up regulation during nodulation. NIN is needed for the expression of two flotillins, *FLOT2* and *FLOT4 that* are essential for both infection thread

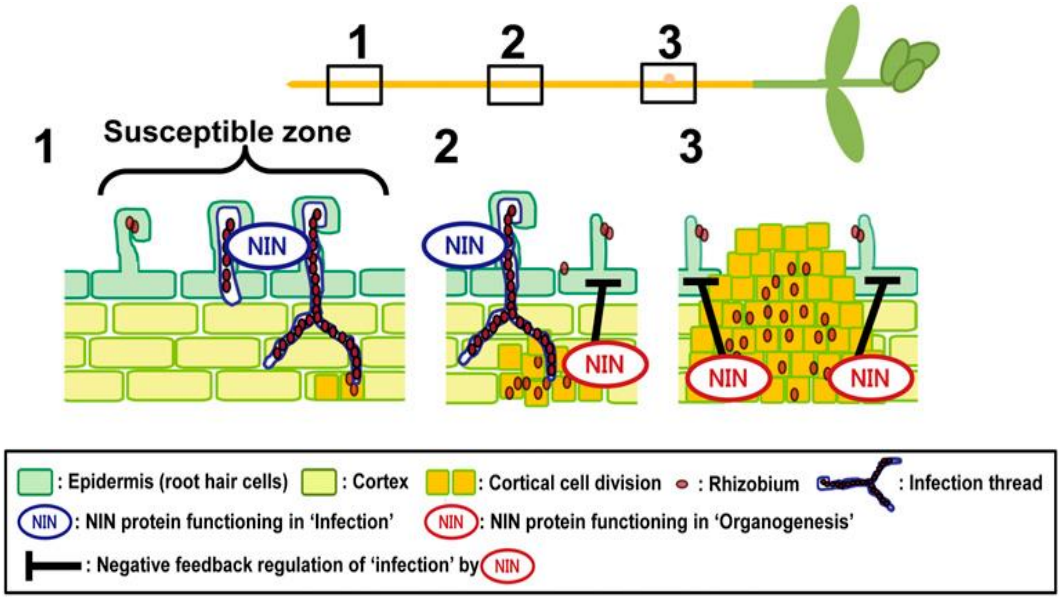


Figure 1.7 **A model of rhizobial infection processes mediated by *NIN*.**

NIN functions in both rhizobial infection (blue) in the epidermis and organogenesis (red) in the cortex. In the earlier stage **[1]** *NIN* (blue) is predominant. In the later stage **[3]** the proportion of *NIN* (red) has increased with nodule development. This model assumes that a potential negative correlation between nodule organogenesis and infection pathways (black bars) regulates the amount of infection and restricts the region of rhizobial susceptibility. Figure adapted from Yoro et al (2014)

and nodule formation. They are lipid raft markers that are strongly induced in response to Nod factors, and localise to the IT membrane (Haney and Long, 2010). Another NIN dependent gene, *Cystathionine Beta Synthase-like 1 (CBS1)* in *M. truncatula* encodes a protein with unknown function that is required for infection and nodulation, localising to the infection thread and nodule symbiosomes (Sinharoy et al, 2016). *NIN* also regulates the *L. japonicus* gene *Nodulation Pectate Lyase* (*NPL*), which is required for normal infection thread progression. It is thought to be instrumental in plant cell wall degradation during rhizobial infection (Xie et al, 2012). The *L. japonicus* CCAAT-box transcription factors, *NF-YA1* and *NF-YB1,* (orthologues of *NF-YA1* and *NF-YB16* in *M. truncatula*) are also directly regulated by *NIN* (Soyano et al, 2013). *MtNF-YA1* is required for normal infection thread and nodule development (Combier et al, 2006; Laporte et al, 2013). A double knockdown of both *MtNF-YA1* and its closest homologue, *MtNF-YA2* result in down regulation of *ERN1* and *ENOD11,* but not *NSP1. MtNF-YA1* can bind to the *ERN1* promoter and positively regulate its expression, but not to the *ENOD11* promoter; this shows that *MtNF-YA1* regulates *ENOD11* transcription via *ERN1*

activation (Laloum et al, 2014). NF-Ys are currently gaining a lot of attention for their potential roles in nodulation. These transcription factors will be examined in greater depth later in this introduction, as they form a significant part of this study.

Another gene required for normal infections is *Lumpy Infections (LIN)*, which encodes an E3 ubiquitin ligase; mutants display a four-fold reduction in the number of infections. (Kuppusamy et al, 2004). *LIN* is required for the expression of two other infection genes: *RHIZOBIUM DIRECTED POLAR GROWTH (RPG)* and *VAPYRIN (VPY).* The putative *L. japonicas* orthologue of *LIN* is *CERBERUS,* which is required for normal infection thread formation (Yano et al, 2009).It is also induced in AM roots, and aids in the hyphal elongation between cortical cells during colonisation (Takeda et al, 2013). *RPG* is expressed strongly during rhizobial infection. Mutants exhibit delayed and abnormal infection threads, however the few nodules that do develop are normal, indicating that it is an infection-specific gene (Arrighi et al, 2008). *VPY* encodes a protein that features a Major Sperm Protein domain which is associated with those involved in protein trafficking and membrane biogenesis. Mutants have abnormal infection threads and fewer nodules. *VPY* is Nod factor-responsive and dependent on the CSP, but not on *NIN* (Murray et al, 2011). It is also required for normal AM infection which, along with *CERBERUS,* provides evidence of the assumed co-opting of common genes for cell restructuring during early rhizobial and AM infection (Pumplin et al, 2010; Murray et al, 2011).

Another receptor has been identified in *L. japonicus* that perceives bacterial exopolysaccharides. *Exopolysaccharide Receptor 3* (*Epr3)* encodes a receptor-like kinase that is induced after Nod factor perception in root hairs and epidermal cells, and required for normal bacterial entry. This gene could be up stream of the CSP, but that has not yet been ascertained (Kawaharada et al, 2015).

In *M. truncatula* a remorin gene, *SYMREM1,* is essential for normal rhizobial infection in root hairs and in the nodule infection zone. It is induced within 24 hours of both Nod factor application and rhizobial infection. *SYMREM1* RNAi knock down lines have aborted infection threads and a few small, white uninfected nodules. The protein localises to the plasma membrane of root hair infection threads, and infection threads in the nodule (Lefebvre et al, 2010).

To understand the transcriptional responses in root hairs to rhizobia and Nod factors, an infectome study using isolated root hairs was carried out (Breakspear et al, 2014). This identified the induction of genes required for flavonoid synthesis and also cell

cycle-related genes. Genes involved in the synthesis of gibberellic acid, strigolactone and brassinosteroids were identified as up-regulated, and jasmonic acid (JA)-related genes were repressed. Auxin-responsive genes were also induced, and *AUXIN RESPONSE FACTOR 16a (ARF16a)* was shown to be required for the normal number of infection events, compared to WT plants.

1.4.2.2 Genes involved in AM infection

The GRAS transcription factors *NSP1* and *NSP2* that act immediately downstream of the CSP were initially considered to be nodulation-specific genes. More recently they have been associated with a possible role in AM symbiosis. NSP2 is required for Myc LCO signalling, and *nsp2* mutants show a slower onset of colonisation (Maillet et al, 2011; Lauressergues et al, 2012). *LjNSP1* was shown to be induced in AM-colonised roots during fungal contact and particularly in the arbuscule-containing cells. The *Ljnsp1* and *Mtnsp1* mutants appear to prevent some fungal entry into the root, although the arbuscules look like those in WT roots (Takeda et al, 2013; Delaux et al, 2013). Both *NSP* genes are also implicated in strigolactone biosynthesis (Liu et al, 2011). Furthermore, *NSP2* can interact with the AM-specific GRAS transcription factor *REDUCED ARBUSCULAR MYCORRHIZA 1 (RAM1).*

RAM1 is CSP-dependent and is directly activated by *CYCLOPS* in *L. japonicus* (Pimprikar et al, 2016). Mutants of *RAM1* were initially shown to have an early AM phenotype, often unable to form hyphopodia on the root surface. However, this was later refuted (Park et al, 2015). *RAM1* is thought to regulate the gene *REDUCED ARBUSCULAR MYCORRHIZA 2 (RAM2)* which encodes a glycerol-3-phosphate acyl transferase (GPAT). It is responsible for the synthesis of cutin monomers, without which AM fungi cannot form hyphopodia/appressoria. The few arbuscules that do form are abnormal (Wang et al, 2012). The arbuscules in the *ram1* mutant are underdeveloped, and in the stronger *ram1-3* allele, only arbuscule trunks are formed (Park et al, 2015). However, this allele did not exhibit any problems with initial penetration; this phenotype was confirmed in *ram1* mutants of *L. japonicus* (Pimprikar et al, 2016). Over-expression of *RAM1* can induce arbuscule-related genes, suggesting that it has an important role in arbuscule development (Park et al, 2015).

DELLA genes are also required for normal AM symbiosis. DELLA proteins are repressors of the hormone GA. In *L. japonicus,* AM-colonised roots exhibit GA accumulation and the

induction of GA biosynthesis genes (Takeda et al, 2015). However, the GA status of roots has opposing effects. With GA treatment, AM colonisation is reduced in a dosage-dependent manner. This coincides with a suppression in the induction of the *L. japonicus* orthologues of *RAM1* and *RAM2.* Conversely, low GA conditions reduce AM hyphal branching in the roots, and suppression of the AM marker gene *SUBTILISIN-LIKE SERINE PROTEASE 1* (*SbtM1*). The differential effects of GA on AM status are thought to promote AM infection in non-colonised areas of the root, whilst negatively controlling colonised areas (Takeda et al, 2014).

DELLA double mutants in *M. truncatula* have severely impaired arbuscule formation (Floss et al, 2013). In rice, the DELLA mutant *slr1* does not form arbuscules. *SLR1* interacts with a GRAS transcription factor called *DELLA Interacting Protein 1 (DIP1).* Also, *DIP1* can interact with *RAM1,* but *RAM1* cannot interact with *SLR1* (Yu et al, 2014). A dominant DELLA protein can restore arbuscule formation in the *cyclops* mutant, and also increases *RAM1* expression (Floss et al, 2013; Park et al, 2015; Pimprikar et al, 2016). This indicates that DELLAs' role in AM reflects its regulation of *RAM1,* acting upstream of *RAM1.*

REQUIRED FOR ARBUSCULE DEVELOPMENT 1 (RAD1) is a transcription factor described in *L. japonicus.* It is expressed in arbuscule-containing cells and *rad1* mutants in *Medicago* have lower colonisation rates at later time points (5 and 7 wpi), suggesting that it makes colonisation rates slower, and arbuscules collapse at earlier time points compared to WT plants (Xue et al, 2015; Park et al, 2015). It can interact with *L. japonicus RAM1* and *NSP2* orthologues (Xue et al, 2015).

A recent study shows an interesting role of a karrikin receptor in rice. *DWARF14LIKE (D14L)* encodes an alpha/beta-fold hydrolase that acts with growth promoting genes in response to karrikins; compounds found in smoke that induce seed germination. The mutant is unable to establish a symbiosis with *Rhizophagus irregularis* or *Gigaspora rosea,* with no hyphopodia formation or internal colonisation (Gutjahr et al, 2015).

1.4.3 Some AM-induced genes are up regulated in *nin* root hairs in response to rhizobia

To complement the root hair infectome data, the same methodology was used in the *nin* mutant (D. Guan thesis, 2014). This revealed an interesting phenomenon where some genes induced during AM root colonisation were also induced in *nin* root hairs 5 days after inoculation with rhizobia. These were not previously considered to be involved in rhizobial infection as they had not been induced in whole root transciptome data that (available on the MtGEA database- http://mtgea.noble.org/v3/).

There are two potential explanations for why a mycorrhizal-specific gene would be induced in response to rhizobia in the absence of *NIN*. The first is that the presence of *NIN* could directly or indirectly down regulate certain AM-specific genes that are not required for, or may interfere with, nodulation in individual cells. Legume roots can be colonised by both rhizobia and mycorrhiza concurrently. It is possible that AM responses may be the default pathway and that the co opting of the common signalling pathway (CSP) for nodulation requires *NIN* both to induce nodulation genes and repress some AM genes within individual cells.

The second explanation is that *NIN* may directly or indirectly be responsible for the negative regulation of the common symbiotic pathway during nodulation. In the *nin* mutant this repression would not occur, and result in increasing gene expression of common symbiosis pathway genes.

1.4.4 Identifying novel AM genes

Most plant genes that are known to be required for AM associations have been identified by screening mutants that are defective in the legume-rhizobial symbiosis, or by forward genetic screens. Recently a novel phylogenomics approach to identify candidate AM genes in plant hosts has recently been employed (Delaux et al, 2015; Bravo et al, 2016). This looks for the evolutionary conservation of plant proteins throughout the history of plant-AM interactions, essentially identifying mycorrhizal genes by virtue of their absence from non-host genomes. This produces candidate

genes that have a selective pressure to be retained for the association. The model plant *A. thaliana,* along with other *Brassicaceae,* has lost the ability to form AM associations and many genes specific to mycorrhization have been lost in *A. thaliana* as a result (Wang et al, 2010). The legume *Lupinus angustifolius* is a good resource for this type of analysis; it is a legume that has lost the ability to from mycorrhizal associations, but can still nodulate. Any genes that have been conserved in plants that can form AM associations, but not in *A. thaliana* and *L. angustifolius,* are promising candidate AM symbiosis genes.

Liverworts are rapidly becoming popular as a model to study AM interactions. The majority of known plant AM genes are found in plants as early as the non-vascular Bryophytes (Delaux et al, 2015). Most liverworts of the *Marchantia* genus can form associations with many AM species. There are a few exceptions, including *M. polymorpha* (Russell and Bulman, 2005). They can be grown quickly and easily on agar using vegetative reproduction, and they have a dominant haploid gametophyte phase which can be exploited for forward and reverse screens, as only one copy of each gene is present. *Marchantia paleacea* is an example of a liverwort that can form symbiotic associations with AM fungal species (Humphreys et al, 2010).

1.5 NF-Y (CCAAT-box transcription factors) and their role in symbiosis

1.5.1 Introduction

NF-Ys (Nuclear factor Y's) are transcription factors ubiquitous in eukaryotes (Bucher, 1990). NF-Y was originally characterised as the Nuclear Factor binding to the Y box of the promoters of MHC Class II genes (Dorn et al, 1987). They are also known as HAPs (Heme Activated Proteins) and CBFs (CCAAT-Box Factors). The latter name describes the pentanucleotide sequence on a gene promoter that NF-Ys bind to either activate or repress transcription.

Three different NF-Y subunits are required to form a heterotrimeric complex to enable DNA binding to occur. These are known as NF-YA (or HAP2/CBF-B), NF-YB (HAP3/CBF-A) and NF-YC (HAP5/CBF-C). Apart from plants, eukaryotes possess only one of each subunit. In yeast, another subunit (HAP4) is present. HAP4 is not required for sequence-specific DNA-binding (McNabb et al, 1995) but it is required for transcriptional activation (Forsburg and Guarente, 1989).

The vast majority of research on NF-Ys has been within animal systems, which have a single copy for each sub unit. They have shown to be fundamental during the cell cycle, amongst other functions. In plants, there has been a huge expansion of all three NF-Y subunits which makes studying these genes more complex, as there is scope for functional redundancy within each the subunits. Despite these potential hindrances, functions of new plant NF-Ys are now being reported and research into plant NF-Ys is a rapidly expanding area.

Amongst these NF-Ys are those that are implicated in either rhizobial or AM symbiosis, downstream of the common symbiosis pathway (CSP). Of these *NF-YA1* (previously HAP2.1) in *Medicago truncatula* (orthologue of *LjNF-YA1 in Lotus japonicus)* is the most studied.

In this introduction I will discuss the observations found in animal systems, particularly concerning the formation of the NF-Y heterodimer, the binding of DNA, the regulation of NF-Ys and potential interactions with other transcriptional regulators. The expansion of plant NF-Ys and the implications of this will be examined, as will similarities and differences between plants and other eukaryotes. I will present a general overview of what roles these transcription factors play in plants, and whether any generalised functions can be identified. Finally, I will review the current understanding of NF-Ys that are required for the development of normal symbiotic associations.

1.5.2 Roles in mammals and yeast

In mammalian systems CCAAT-box motifs are found in the promoters of genes implicated in the cell cycle, metabolism, growth, apoptosis and cell proliferation. Within the human genome, CCAAT motifs are abundant in genes associated with cancer (Fleming et al, 2013). Their role in development has been revealed by experiments in both *Drosophila melanogaster* and mouse. Overexpression and knockdown of *Drosophila* NF-YA results in lethality at several developmental stages (Yoshioka et al, 2007). Also, NF-YA knockouts in mouse result in death at very early stages of embryogenesis, highlighting the fundamental role of NF-YA (Bhattacharya et al, 2003). HAP (Heme Activated Protein) reflects the role that NF-Ys play in iron homeostasis, mostly studied in yeast, but found to be conserved in humans (Hortschansky et al, 2007). Both iron deficiency and excess is deleterious to cell functions, so tight control of iron levels is necessary. Ferritin is a protein that stores iron in a non-toxic form. In humans, CCAAT boxes are located in ferritin promoter (Wang et al, 2010).

1.5.3 NF-Y subunits form a heterocomplex

All three NF-Y subunits are ubiquitous and highly conserved amongst eukaryotes. The conserved regions relate to DNA binding and interaction sites between the subunits. The DNA-binding motifs of NF-YA are not homologous to any other proteins. It comprises two distinct halves; the N-terminal for binding to the NF-YB and NF-YC subunits, and the C-terminal for DNA binding (Mantovani et al, 1994; Xing et al, 1994; Romier et al, 2003). Human NF-YA was found to be expressed as both a short and long splice variant (Xiao-Yan et al, 1992), and the NF-YA family is generally known to be regulated by alternative splicing (Laloum et al, 2012). Only one form of each NF-YA variant is present in any given cell type. The NF-YC subunit has four isoforms, also generated by alternative splicing (Chen et al, 2002). This occurs mainly in the Q-rich activation domain. This post transcriptional modification could explain how one gene for each subunit can accommodate all NF-Y functions (Ceribelli et al, 2009). No different isoforms of NF-YB have been observed in mammals (Dolfini et al, 2012).

Whilst NF-YA shows no homology to any other DNA-binding proteins the NF-YB and NF-YC subunits have a remarkable homology to the core histones H2B and H2A, respectively (Bexevanis et al, 1995). They are also highly homologous to the histone-like

proteins αNC2 and βNC2 (Sinha et al, 1996), which are the subunits that recognise and bind to the preformed TATA/TBP complex (Kamada et al, 200).

Formation of the NF-Y heterotrimeric complex begins with the formation of a tight NF-YB/NF-YC heterodimer, interacting through the histone fold domains (Xing et al, 1993; Sinha et al1996; Kim et al, 1996) in a head to tail fashion (Guerra et al, 2007). This dimer can then associate with NF-YA to form an unstable heterotrimer. Nuclear localisation of the B/C dimer and A subunit occur independently in mammals, competing for interaction with importins. This suggests that the trimeric complex assembles in the nucleus (Frontini et al, 2004; Kahle et al, 2005).

Neither NF-YB nor NF-YC can associate with NF-YA as an individual subunit (Kim et al, 1996). Only once the NF-Y complex has bound to DNA is the trimer stable (Bi et al, 1997; Liang and Maity, 1998). Both NF-YA and NF-YC contain glutamine (Q)-rich activation domains which are highly conserved in distant phyla (Serra et al, 1998). In the yeast species *S. cerevisiae* and *S. pombe* the three subunits (HAP2/3/5) homologous to NF-YA/B/C are missing any Q-rich domains. However, yeast has another subunit (HAP 4) which is not involved in DNA binding, but is required for gene activation (Forsburg and Guarente, 1989).

1.5.4 The NF-Y complex directly associates with DNA

The association of the NF-YB/NF-YC dimer to NF-YA is very weak. It is only stabilised once the complex interacts with DNA. Although all three subunits directly interact with the DNA, it is the A subunit that binds to the CCAAT-box motif in a sequence-specific manner, which can be found either in the forward or reverse orientation (Liang and Maity, 1998). This motif is highly conserved, resulting in loss of or reduced NF-Y/DNA association with the addition of mutations (Dorn et al, 1997; Liang and Maity, 1998). It is thought that NF-YB and NF-YC subunits interact with DNA via the HFDs to the phosphate backbone, like the H2A and H2B core histones, which they are analogous to (Romier et al, 2003).

Nucleosome formation requires the binding of H3/H4 tetramers to DNA, followed by the association of H2A/H2B (Motta et al, 1999). Studies suggest that the NF-YB/NF-YC subunits can utilise their homology to H2A/H2B and be recruited by H3/H4 core histones; thereby recruiting the NF-Y complex on active promoters on otherwise inaccessible areas of the nucleosome (Dolfini et al, 2012).

It appears that the presence of the NF-YA complex can not only compete with H2A/H2B, preventing nucleosome formation (Caretti et al, 1999; Motta et al, 1999), but are also essential for histone modifications associated with activation and repression of gene expression (Donati et al, 2006; Gurtner et al, 2008). This is achieved by recruitment of the relevant enzymes (Dolfini et al, 2012). Core histone H2B undergoes mono ubiquitination at the Lys120 residue, located in the HFD αC, in order to create a chromatin environment conducive to transcription. The analogous Lys138 in NF-YB is also mono ubiquitinated which, if changed to an arginine residue, does not affect DNA binding, but eliminates transcriptional activation (Nardini et al, 2012).

1.5.5 Interactions with other transcription factors

On DNA binding, a α-helix of NF-YA inserts into the minor groove of DNA. This produces a bend that allows binding of other transcription factors in the major groove (Nardini et al, 2013). The NF-Y complex is involved in interactions with other transcription factors and co factors (Dolfini et al, 2012 and refs therein). The CCAAT box is usually flanked by at least one other important promoter element, and NF-Y has been shown to increase the affinity of the other element for DNA binding. The distance between these elements is functionally important, as small changes have dramatic negative effects on transcription (Dolfini et al, 2012). Many such interactions have been described for NF-Ys controlled stress responses, including DNA damage (Romano et al, 2006). For example in Arabidopsis, bZIP and NF-Y transcription factors assemble into a complex in response to ER stress (Lui and Howell, 2010). NF-Y also interacts with TFIID as part of the RNA polymerase II preinitiation complex (Bellorini et al, 1997; Coustry et al, 1998). The q-rich regions of NF-YA and NF-YC have shown to bind to TATA-box binding protein associated factors ($TAF_{II}s$) (Coustry et al, 1998). This could suggest that the NF-Y complex has an important role in helping the binding of TAFIIs to DNA, but also stabilising them within the protein complexes required for transcription (Liberati et al, 1999).

1.5.5 Plant NF-Ys

Whereas most eukaryotes have one example of each type of NF-Y subunit, plants have expanded to around 10 genes encoding each subunit. This potentially allows the formation of different heterotrimeric combinations to allow subtle changes in transcription in response to different environmental conditions. This makes studying plant NF-Ys challenging, as there is a great deal of scope for functional redundancy.

Furthermore, yeast interaction studies have shown that most plant NF-Y subunits can bind promiscuously with each other (Calvenzani et al, 2012; Hackenberg et al, 2012), as well as the yeast counterparts. Plant NF-Ys can also interact with mammalian ones (Calvenzani et al, 2012).

Like other eukaryotes, plant NF-Ys have roles in cell cycle and development, including roots and the nodules of legumes (Combier et al, 2006; Zanetti et al, 2010; Soyano et al, 2013; Sorin et al, 2014). The expansion of NF-Ys in plants has allowed for other functions. The most well-studied is the *LEAFY COTYLEDON 1 1(LEC1)* and *Lec1-like (L1L)* NF-YB genes. These are expressed specifically in seed and play essential roles in regulating embryogenesis. *LEC1* and *L1L* interact with abscisic acid (ABA) responsive element binding factors, implicating *A. thaliana* NF-YBs in ABA signalling. (Laloum et al, 2012 and refs therein).

Many plant NF-Ys have been implicated to abiotic stress responses often, but not always, through ABA signalling (Kumimoto et al, 2013; Li et al, 2013; Xu et al, 2015). Many NF-Y subunits implicated in drought tolerance and responses to salinity have been studied in *Arabidopsis,* maize, soy bean, poplar and rice (Nelson et al, 2007; Li et al, 2008; Zhiyong et al, 2013; Han et al, 2013; Lee et al, 2015).

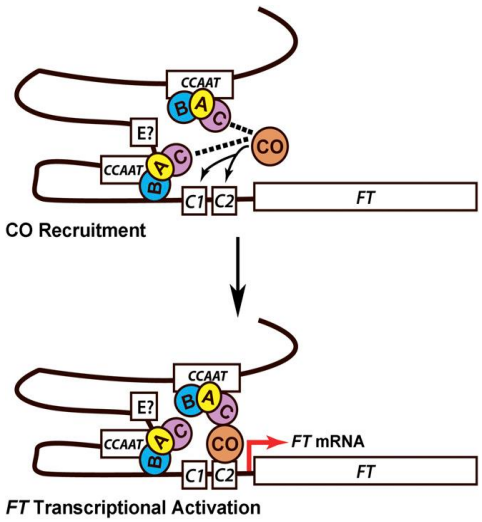


Figure 1.8 **Proposed action of an NF-Y complex in the interaction of *CONSTANS (CO)* in the regulation of flowering time.**

Two NF-Y heterotrimeric complexes bind to distal CCAAT-box sites on the *FLOWERING TIME (FT)* promoter. Chromatin loops to recruit *CO* by interacting with NF-Y. *CO* can then bind to the *CORE* sites close to the start codon of *FT* and allow transcription.

(Figure from Cao et al, 2014)

Another well-studied role for NF-Ys is in interactions with the flowering time regulator *CONSTANS (CO),* to regulate photoperiod flowering. *CO* is a transcriptional activator of *FLOWERING TIME (FT),* which accumulates during the day and is rapidly degraded at night. Some NF-Y mutants have phenotypes similar to *co* mutants; low *FT* expression and delayed flowering. *FT* has two CO-responsive elements (CORE 1 and 2) in its promoter (-220bp and -161bp from the transcriptional start site) (Tiwari et al, 2012); however its functional promoter length is 5.3 kilo bases (Adrian et al, 2010). A recent study has proposed that, in *Arabidopsis,* NF-Y complexes bind to CCAAT motifs on distant enhancer sites on the *FT* promoter. This has the effect of looping the chromatin to bring the NF-Y complexes close to the proposed *CO* binding sites. The NF-Y recruits *CO,* and enables it to bind to the promoter, allowing for *FT* transcription (Figure 1.8; Cao et al, 2014). Many NF-Y subunits have been implicated in flowering time regulation, particularly in relation to photoperiod responses (Cai et al, 2007; Chen et al, 2007; Kumimoto et al, 2008; Kumimoto et al, 2010; Li et al, 2011; Kim et al, 2016).

NF-Ys also have a role in epigenetic regulation of *Suppressor of Overexpression of CONSTANS 1 (SOC1),* a major floral pathway integrator. Under certain environmental conditions, an NF-Y complex can bind to the *SOC1* promoter, regulating demethylation by recruitment of a demethylase; thus allowing *SOC1* expression.

SOC1 is a major floral pathway integrator, regulated by presence/absence of DELLAs, GA, CO, and NF-Ys. DELLAs, which are degraded in the presence of GA, inhibit NF-Y binding on the *SOC1* promoter. The NF-Y complex then interacts with the promoter, which results in demethylation at *SOC1,* allowing transcription. Under long day

conditions, *CO* interacts with NF-Y regardless of the presence of DELLAs, allowing demethylation and subsequent transcription (Hou et al, 2014).

In addition to these plant roles, NF-Ys have also been implicated in shoot apical and root nodule meristem maintenance (Combier et al, 2006; Kneusting et al, 2015); photosynthesis regulation (Kusnetsov et al, 1999; Stephenson et al, 2010; Stephenson et al, 2011); fruit ripening (Li et al, 2016); chloroplast biogenesis (Miyoshi et al, 2003); rhizobial infection of legumes (Laporte et al, 2013); and pollen tube orientation (Yu et al, 2011).

Micro RNAs (miRNAs) are around 21 nucleotides long and are implicated in post transcriptional regulation in plant development (Jones-Rhoades and Bartel, 2004). Several plant NF-YAs have shown to be regulated by miR169 (Laloum et al, 2012), and are implicated in nitrogen responses (Zhao et al, 2011), nodulation (Combier et al, 2006), root architecture (Sorin et al, 2014), as well as tolerance to drought and salinity (Zhao et al, 2009; Sun et al, 2015; Yang et al, 2016).

1.5.6 NF-Ys involved in symbiosis

Several NF-Ys have been associated with the legume-rhizobia symbiosis downstream of the common symbiotic pathway. The most well studied of these is *MtNF-YA1* (previously called *HAP2.1*) in *M. truncatula* and *LjNF-YA1* in *L. japonicus*. *MtNF-YA1/LjNF-YA1* is involved in early infection events, early nodule development and in nodule meristem maintenance (Combier et al, 2006; Laporte et al, 2013). It is regulated by the transcription factor, NIN, which has been shown to bind to the *LjNF-YA1* promoter (Soyano et al, 2013; Breakspear et al, 2014). The *Mtnf-ya1* mutant has aberrant infection threads which appear to abort in the root hairs and rarely reach the cortex. This seems to produce a feedback mechanism which results in the initiation of a greater number of infection events compared to WT. However, few infections result in nodule formation (Laporte et al, 2013). The nodules that do form in *Mtnf-ya1* are round, rather than the elongated WT phenotype. Closer inspection shows that the *nf-ya1* nodules have no nodule meristem, which is required for nodule elongation (Combier et al, 2006). *MtNF-YA1* expression in nodules is restricted to the meristematic and infection zones (Roux et al, 2014). The expression in the infection zone is post-transcriptionally regulated by miR169, reducing mRNA transcripts in this part of the nodule (Combier et al, 2006). *MtNF-YA1* is also subject to alternative splicing during root nodule development, which leads to a reduction in the *MtNF-YA1* transcript levels

(Combier et al, 2008). It is proposed that the effects of miR169 and alternative splicing work to regulate the spatial-temporal concentrations of *MtNF-YA1* transcripts in mature nodules.

MtNF-YA1 promoter:GUS analysis has shown that it is accumulates transcripts in root hairs at the micro colony stage and expression continues to be tightly associated with colonisation and the cells surrounding the infection threads. There is also accumulation in the dividing cortical cells of nodule primordial. In mature, differentiated nodules, it is restricted to the meristem and infection zones (Laporte et al, 2013).

MtNF-YA2 is the closest homologue to MtNF-YA1. Unlike NF-YA1, it is not regulated by NIN (Breakspear et al, 2014), but has been shown to act redundantly in the *Mtnf-ya1* mutant. This was shown by knocking down both genes with a single RNAi construct which led to a much stronger infection phenotype than was previously described. Knocking down both genes resulted in most infections aborting at the micro colony stage, and only 50% of plants having any nodules present (Laloum et al, 2014).

MtNF-B16 (LjNF-YB1) can form a complex with MtNF-YA1 (Soyano et al, 2013; Baudin et al, 2015). And as mentioned above, it is also regulated by *NIN*, which can bind to its promoter (Soyano et al, 2013). Unlike *Mtnf-ya1/Ljnf-ya1,* a knock down in *Mtnf-yb16* or *Ljnf-yb1* does not show a nodulation phenotype (Soyano et al, 2013; Baudin et al, 2015).

MtNF-YC2 is an orthologue of the Phaseolus vulgaris NF-YC1, which was initially discovered to be important for rhizobial infection and normal nodule development. It was shown to be involved in the regulation of cell cycle genes (Zanetti et al, 2010). *MtNF-YC1,* a close homologue of *MtNF-YC2,* does not show a nodulation phenotype when knocked down by RNAi. However, silencing both *Mtnf-yc1/Mtnf-yc2* together gave a stronger nodulation phenotype than *Mtnf-yc2* RNAi alone. The redundancy between *MtNF-YC1/C2* and that of *MtNF-YA1/2* are comparable. *MtNF-YA1* and *MtNF-YC2* are both dependent on *NIN* (Breakspear et al, 2014) and have a nodulation phenotype when knocked down (Zanetti et al, 2010; Laporte et al, 2013). *MtNF-YA2* and *MtNF-YC1* are not regulated by *NIN*, do not exhibit a phenotype in the knockdown, but contribute to the phenotype when knocked down with their homologous counterparts (Laloum et al, 2014; Baudin et al, 2015).

Phenotypic and expression data of NF-Ys in nodulation strongly implicate MtNF-YA1/NF-YB16/NF-YC2 as acting in the same complex. Recently, it has been shown that yeast 3

hybrid interactions are successful using any combinations of MtNF-YA1/NF-YA2/NF-YB16/NF-YC1/NF-YC2. Confirmation of the MtNF-YA1/NF-YB16/NF-YC2 heterotrimer was confirmed using CoIP experiments in *Nicotiana benthamiana* (Baudin et al. 2015).

The majority of research in the role of NF-Ys in the establishment of symbiosis has been studied in nodulation. Very little is known about the roles that NF-Ys play during the AM symbiosis. Since the start of my PhD, two NF-YC subunits (*MtNF*-YC*6* and *MtNF-YC11* have been identified as being expressed in roots colonised by AM (Hogekamp et al, 2011). This research will be discussed in greater depth in Chapter 5, as this is part of a research collaboration with the Kuster lab. More recently, two soy bean orthologues of *MfNF-YA2* (*GmNF-YA1a/b)*, have a positive role during arbuscular mycorrhizal (AM) associations (Schaarschmidt et al, 2013).

The main aim of this project was to discover novel AM genes, as few had yet been identified. The strategy for this was to undertake both a forward and reverse screen. Forward screens can identify clear phenotypes, which can then be further studied and the gene of interest subsequently identified. The challenge to this approach is that large numbers of mutant plants need to be screened in order to increase the chance of finding a mutant of interest.

The reverse screen approach focuses on potential candidate genes chosen by expression analyses. The strength of this is that the gene of interest is already known. It can also identify those which would not be picked up in a forward screens; in the case of functional redundancy, for example. However, this method may not yield a phenotype. My aim was to find genes that are required for the early stages of infection, notably the formation of the pre-penetration apparatus (PPA). The comparable physiological strategies employed in PPA and infection thread formation suggests that there may be common symbiotic genes other than those downstream of the known CSP. My aim was to explore this hypothesis by identifying common infection genes.

This project focuses on the preliminary forward and reverse to find AM genes and, possibly, common infection genes. I describe four genes: a novel AM-specific gene involved in lipid modification, and three NF-Y genes. One of these, a NF-YB, is induced early both symbioses, and is required for early infection events during nodulation.

Chapter Two

Materials and Methods

2.1 Plant Methods

2.1.1 *Medicago truncatula* lines and growth conditions

Medicago truncatula ecotypes Jemalong A17 (Barker et al, 1990) and R108 seedlings (Hoffmann et al, 1997) were used in this study. All mutants and transgenic plants described in this study were derivatives of either ecotype. Plants were grown in a 1:1 mixture of Terra green and sharp sand (TG::SS) or in John Innes Cereal Mix (loam based) or Barley mix (N100 P200 K200). Plants were watered regularly as needed and kept in controlled environment chambers with a 16 hour photoperiod at 20°C and 80% humidity.

On plates, seedlings were either grown on distilled water agar (DWA) or Fahraeus plant medium (FP) using a filter paper sandwich method. Briefly, Whatman paper (Grade 0858 Cellulose Qualitative Filter Paper) was cut to size to fit square tissue culture dishes (Fischer scientific) and sterilized. Seedlings were grown vertically on 1.5% agarose slants between two filter paper squares.

2.1.2 Growth conditions of Barley and Wheat plants

During the establishment of the AM inoculum protocol, Barley (*Hordeum vulgare* 'Golden Promise') and wheat (*Triticum aestivum* 'Paragon') were grown in the same conditions as *Medicago* plants, except some were watered with M media (Table) instead of water.

2.1.3 Growth conditions of rice plants

Rice plants were grown, inoculated and bulked in the lab of Uta Paszkowski, at The University of Cambridge, UK.

2.1.4 *M. truncatula* seed sterilization, scarification and vernalisation

Seed pods were collected from mature dried *M. truncatula* plants. These were dried for 3-7 days in 37^{0}C incubator. Seeds were extracted by crushing pods with wooden blocks covered in corrugated rubber. To germinate, seeds were lightly scarified with sand paper (or treated with sulphuric acid (98%) for 10 mins, followed by ten rounds of rinsing with water for 2 minutes each). Seeds were then treated with 10% Sodium hypochlorite (Sigma Aldrich) for 2 minutes, and then rinsed in sterile water 5 times for 2 minutes each, until all traces of bleach had gone. Seeds were immersed in sterile water to imbibe for at least one hour until they had started to swell. They were then plated on Distilled Water Agar (DWA) plates and inverted to allow for downward root growth. Seeds were put in the dark at 4^{0}C for stratification, or for 14 days for vernalisation and early flowering (https://www.noble.org/medicago-handbook/).

2.1.5 Germination of wheat and barley seeds

Seeds were placed in a petri dish on water-soaked filter paper and left at room temperature to germinate.

2.1.6 Plant materials

All *Tnt1 Medicago* insertion lines and rice *Tos17* insertion lines were identified during this study, except the *nin-2* mutant.

Line	Background	Description	Allele	Source
***M. truncatula* (barrel medic)**				
A17		ecotype	WT	
R108		ecotype	WT	
NF16919	R108	*Tnt1* insertion at position 238	*cbf1*	Samuel Roberts Noble Foundation, USA
NF11498	R108	*Tnt1* insertion at position 109	*cbf3-1*	Samuel Roberts Noble Foundation, USA
NF1242	R108	*Tnt1* insertion at position 332	*cbf3-2*	Samuel Roberts Noble Foundation, USA
NF11571	R108	*Tnt1* insertion at position 404	*cbf3-3*	Samuel Roberts Noble Foundation, USA
NF12356	R108	Tnt1 insertion at position 560	*cyp450-1*	Samuel Roberts Noble Foundation, USA
NF12182	R108	Tnt1 insertion at position 638	*lecrk, not recovered*	Samuel Roberts Noble Foundation, USA
NF13226	R108	Tnt1 insertion at position 6677	*bfp-1*	Samuel Roberts Noble Foundation, USA

NF10789	R108	Tnt1 insertion at position 6980	*bfp-2*	Samuel Roberts Noble Foundation, USA
NF12826	R108	Tnt1 insertion at position 2281	*bfp-3*	Samuel Roberts Noble Foundation, USA
NF18637	R108	Tnt1 insertion at position 3534	*bfp-4*	Samuel Roberts Noble Foundation, USA
F9542H	A17	Tnt1 insertion at position 331	(SYMAQ) not recovered	Christine Lesignor at L'Unité de Recherche en Génomique Végétale (URGV), Dijon, France
NF18323	R108		*max4-1*	Samuel Roberts Noble Foundation, USA
	A17	71 kb deletion	*ram1-1*	John Innes Centre
nin-1	A17	11bp deletion starting at position 1850	EMS	
nin-2	R108	*Tnt1* insertion 20 bp upstream of ATG	*Tnt1*	
***O. sativa* (rice)**				
NE7031	Nipponbare	Tos17 insertion at position 3765	*Osbfp-1*	National Institute of Agrobiological Sciences, Japan
NE0660	Nipponbare	Tos17 insertion at position 1707	*Osbfp-2*	National Institute of Agrobiological Sciences, Japan
Nipponbare		*Oryza sativa*	WT	National Institute of Agrobiological Sciences, Japan
***H. vulgare* (barley)**				
'Golden Promise'		variety		
***T. aestivum* (wheat)**				
'Paragon'		variety		
***Allium schoenoprasm* (chives)**				
Chives		garden variety		Mr. Fothergill's Seeds http://www.mr-fothergills.co.uk/

Table 2.1 Plant lines used in this study

2.1.7 Hairy root transformation of *M. truncatula* using *Agrobacterium rhizogenes*

Germinated *M. truncatula* seeds were removed from the vernalisation conditions and incubated at room temperature overnight. Under sterile conditions, the meristem of the root tip was removed using a scalpel; the cut end was then dipped in the transformed *A. rhizogenes* culture. Twelve seedlings were put on modified (ModFP) plates and kept upright in controlled environment rooms for 7 days. At this stage, any root growth is from untransformed roots. These were removed, and the seedlings

placed onto new ModFP plates. Plants were grown for 3 weeks, until hairy roots had developed. If a dsRED selection marker was used, plants that showed positive fluorescence were transferred to the soil substrate for nodulation assays.

2.2 Manufacture of mycorrhizal inoculum using chive plants

2.2.1 Initial manufacture axenic spores

This technique requires adherence to sterile technique procedures to produce an inoculum free from contaminating microbes, particularly rhizobia. All surfaces, trays, instruments etc are washed down with 70% ethanol and gloves worn. Terragreen, sharp sand and Levington's F1 low nutrient compost at a ratio of 2:2:1 was autoclaved. Seed trays were filled with this soil substrate and approximately 300 chive seeds were evenly distributed on the surface and then lightly covered with soil. This was watered well and a plastic transparent lid was applied to prevent cross-contamination of rhizobia in the growth rooms. One week after sowing, each germinated plant was inoculated at the base of the stem with 200 sterile *R. irregularis* spores. Plants were grown for eight weeks in the same conditions as *M. truncatula* plants, watering to prevent the soil substrate from drying out.

After eight weeks, chive plants were trimmed to the base of the stem, and removed. The soil substrate/chive root mixture left was then mixed by hand, and chive roots ripped into smaller sections to achieve as much homogeneity as possible. The inoculum was then transferred into zip-locked plastic bags in amounts convenient for experimental use. This was to reduce the risk of rhizobial contamination when needed for an experiment. The inoculum was stored in the dark at 4^0C.

2.2.2 Bulking of chive inoculum from initial production

Previously made chive inoculum was evenly spread on the bottom of a seed tray, ensuring an even distribution of chive roots, using 20% volume of the tray. The soil substrate previously described was added on top and chive seeds sown with growth conditions as before. The inoculum was then harvested eight weeks after planting as described above.

2.3 Forward Screen Protocol

655 inbred *Tnt1 M. truncatula* lines were used for the forward screen. These were comprised of Tnt1 mutant lines provided by the Noble Foundation that were advanced

four generations by self-pollination (single seed descent). Four seeds from each line were put in 1.5µl Eppendorf tubes with dH_2O and put at 4°C overnight to imbibe. P15 trays had 20% chive inoculum (500ml per tray) added to the bottom of each well, ensuring an even distribution of chive roots, and then filled with 1:1 terragreen:sharp sand . Each *Tnt1* line (4 seeds each) were sown in the same well, spaced evenly apart. For each P15 seed tray, 2 wells were sown with WT R108 seeds as a control. Two extra trays of WT R108 seeds were also planted for controls, with some *ram1-1* and seeds or comparison. Trays were incubated at 4^0C for two weeks to vernalise, and then were moved into normal *M. truncatula* growth conditions. At 30dpi, enough root was removed from each plant for AM fungal staining, leaving enough root for the plants to be transferred into new pots. Two plants of each line were re-potted in one litre pots in Barley mix for bulking. The other two plants of each line were re-potted in the P15 trays for possible re-phenotyping.

Stained roots were scored in a qualitative way: obvious reduction of AM structures, hyper infection, branching external hyphae trying to enter roots. The short listed candidates from this initial screen were scored quantitatively. Seeds of shortlisted candidates were germinated and grown in the normal way described in this chapter using P60 trays, using the chive inoculum as before. Roots were stained for phenotyping at two time points: 2wpi and 4wpi. Roots were scored using both quantitatively and qualitatively.

2.4 Microbiological methods

2.4.1 Bacterial strains and growth conditions

Escherichia coli cultures were grown at 37°C for 16 hours overnight at 250 RPM in 10 ml cultures. *A. rhizogenes* strain AR1193 (Stougaard et al, 1987) was used for hairy root transformations of *M. truncatula.* Single colonies were used for inoculating 5 ml TY medium and grown on shaking cultures at 28°C as required. *Sinorhizobium meliloti* strains were grown overnight at 28°C under shaking conditions. Long term storage of bacteria was in 20% glycerol at -80°C.

Strain	Resistance	Species	Description
DH5α	-	*Escherichia coli*	*E. coli* strain for plasmid amplification
AR1193	Rif & Carb	*Agrobacterium rhizogenes*	For transformation of *M. truncatula* roots
Sm2011	Tet	*Sinorhizobium meliloti*	Rhizobial symbiont of *M. truncatula*
Sm1021	Tet	*Sinorhizobium meliloti*	Rhizobial symbiont of *M. truncatula*

Table 2.2 **Bacterial strains used in this study**

2.4.2 Bacterial plasmid preparation and transformation by heat-shock or electroporation

Overnight grown cultures of *E. coli* were pelleted at 10,000 RPM for 10 minutes in 2 ml Eppendorf tubes. Plasmid was isolated by the alkaline lysis method using the Qiagen miniprep kit following manufacturer instructions. *E. coli* chemically competent cells were transformed by a 45 second heat shock at 42°C followed by an immediate cold shock on ice. SOC medium was added to each aliquot and transformed cells allowed to recover for an hour on a shaker at 37°C. The cells were pelleted by centrifugation and transformed cells selected on medium containing desired antibiotic. *Agrobacterium* cells were transformed by electroporation. In individual sterile cuvettes (Geneflow), 40 µl competent cell and approximately 100 ng plasmid were added. Current was applied at 2.5V for 10 seconds at a resistance of 200 ohms and SOC medium added immediately after. The transformed cells were allowed to recover at 28°C on a shaker for one hour and selected on TY medium containing appropriate antibiotics.

2.4.3 Blue White Screening

To screen for recombinant clones containing the gene of interest, blue white screening was performed in which white colonies identify clones with a disrupted *lacZ* gene in the plasmid backbone indicating the presence of an insert. To the growth medium, X-GAL (Formedium) a chromogenic substrate for β-galactosidase and a Lac operon inducer – IPTG (Isopropyl β-D-1-thiogalactopyranoside) were added to a final concentration of 40

µg/ml and 100 µM respectively along with the antibiotic. Plates were incubated overnight and colonies screened visually for colour development.

Medium	Recipe for 1 litre
Farhaeus Plant (FP) medium	0.1 g $CaCl_2$. $_2H_2O$, 0.12 g $MgSO_4$, 0.01g $KHPO_4$, 0.150 g $NaHPO_4.12H_2O$, 5 mg ferric citrate, 2.86 g H_3BO_3, 2.03 g $MnSO_4$, 0.22 g $ZnSO_4.7H_2O$, 0.08 g $CuSO_4.5H_2O$, 0.08 g $H_2MoO_4.4H_2O$, pH 6.3-6.7. For solid medium 0.5% (w/v) LabM No. 1 agar was added.
Modified FP medium	FP medium containing 0.5 mM NH_4NO_3
Buffered Nodulation (BNM) medium	390 mg MES, 344 mg $CaSO_4.2H_2O$, 0.125 g KH_2PO_4, 122 mg $MgSO_4.7H_2O$, 18.65 mg Na_2EDTA, 13.9 mg $FeSO_4.7H_2O$, 4.6 mg $ZnSO_4.7H_2O$, 3.1 mg H_3BO_3, 8.45 mg $MnSO_4.H_2O$, 0.25 mg $Na_2MoO4._2H_2O$, 0.016 mg $CuSO_4.5H_2O$, 0.025 mg $CoCl_2.6H_2O$, pH 6.5. For solid medium 11.5 % (w/v) LabM No. 1 agar (Formedium) was added.
Distilled water agar (DWA) medium	1.5 % (w/v) Lab M No. 1 agar (Formedium, UK, pH 5.7 (adjusted with KOH).
LB (Luria-Bertani) medium	Tryptone 10.0g Yeast Extract 5.0g NaCl 10.0g pH 7.0 10g added for solid medium
TY (Tryptone-Yeast agar) medium	Tryptone 5.0g Yeast Extract 3.0g $CaCl_2$ $6H_2O$ 1.32g

Table 2.3 **Media used in this study**

Buffers	Recipe for 1 litre
Z Buffer	100 mM Sodium phosphate buffer (100 mM Na_2HPO_4, NaH_2PO_4 each) 10 mM KCl, 1 mM $MgCl_2$ pH 7.4
GUS Buffer	50 mM Sodium phosphate buffer, 1 mM EDTA, 1% Triton-X

Table 2.4 **Buffers used in this study**

Antibiotic	Solvent	Final-concentration (µg/ml)
Carbenicillin	Water	100
Kanamycin	Water	100
Rifampicin	Ethanol	50
Spectinomycin	Water	100
Streptomycin	Water	200
Tetracyclin	Ehtanol	5

Table 2.5 Antibiotics used in this study

2.5 Molecular Biological methods

2.5.1 Agarose gel electrophoresis

DNA fragments were resolved by running the samples on a 1% agarose gel at 100 V in 1x TAE (Tris acetate EDTA). An ethidium bromide bath prepared at a concentration of 0.5 µg/ml was used for visualization of the DNA bands. Analytical gels were photographed using Geneflash Syngene Bioimaging system.

2.5.2 PCR cycling conditions

All PCR reactions were carried out using the G-Storm or PTC 225 Peltier thermal cyclers. For all cloning purposes the Phusion® High-Fidelity DNA Polymerase (New England Biolabs) was used, using manufacture recommended concentrations. For general purpose genotyping and colony PCRs the GoTaq green master mix was used.

Stage	Temperature (°C)	Time-period (Phusion)	Time-period (GoTaq)	Number of cycles
Initial Denaturation	96	5 minutes	5 minutes	x1
Denaturation	96	30 sec	30 sec	
Annealing	55-60	30 sec	30 sec	x30-35
Extension	72	30 sec per Kb	1 minute per Kb	
Final extension	72	10 minutes	10 minutes	x1

Table 2.6 Standard PCR cycling Parameters

2.5.3 DNA extraction

All DNA extraction was carried out using a Qiagen DNeasy Plant Kit by Richard Goram, Norwich. All plasmid extraction was carried out using Qiagen Mini-prep spin columns as per manufacturer's instructions.

2.5.4 Restriction digestion

Sequence specific digestion of DNA was carried out using restriction enzymes from NEB or Roche. The reaction was setup with 1 µg plasmid or PCR purified fragments. Wherever compatible a double digest was setup in the same buffer otherwise sequential digest carried out.

2.5.5 Sequencing

DNA sequencing was carried out by performing the BigDye® reaction with either purified PCR product or plasmid according to manufacturer's protocol and then completed by The Genome Analysis Centre (TGAC), Norwich or Eurofins (MWG Operon), UK. Purified plasmid of Golden Gate constructs was sent for Next Generation Sequencing by IMGM Laboratory, Germany.

2.5.6 qRT-PCR

Root material was ground in liquid nitrogen using a pestle and mortar and RNA was extracted using a QIAGEN® RNeasy mini kit. The RLT buffer was pre-warmed to 50°C and the elution volume was reduced to 30µl. RNA was then treated with Turbo DNAse (Life Technologies™) to remove the DNA. The quality of the DNA was tested on a 1% Agarose gel containing ethidium bromide and the quantity was tested on a NanoDrop (Thermo Fisher Scientific). cDNA was synthesised using SuperScript®II Reverse Transcriptase (Life Technologies™). The input RNA was normalised to the lowest sample with a lower threshold of 400ng/ total RNA.

qRT-PCR was carried put on a CFX96 Touch™ c1000 thermal cycler (Bio-Rad) using SYBR™ Green JumpStart Taq ReadyMix (without MgCl2) in a 96 well plate. Each well contained the following: 2.6µl MgCl2; 5µl SYBR Green, 2µl diluted cDNA, 1.6µl each 20µM primers to give a total of 10µl. The primer efficiencies were calculated using a serial dilution of control cDNA. The following parameters: 95°C 30s, [94°C 30s, 60°C 30s, 72°C 30s]x49. Melt curve 65°C to 95°C in 0.5°C increments in 5s.

The efficiency of all primer pairs was calculated using a dilution series and linear regression of the resulting Ct data points. *Ubiquitin, EF1α* and *TIP41*-like protein were determined to be the most stable references. Normalized relative quantities were calculated using the qBase model (Hellemans et al, 2007), which allows for multiple housekeeping genes and primer specific efficiencies. Values were based on 3 technical reps per sample. The expression was then calculated relative to the control. Standard error was calculated as relative standard error to the control. See Appendix I for primers.

2.5.7 Gateway Cloning

For cloning into the pENTR/dTOPO vector (Invitrogen) a four base pair CACC tag on the forward primer was added. The purified fragment was used for cloning according to the manufactures protocol. The reaction was allowed to proceed overnight and 2-5 µl was

used for transforming 50 µl of competent cells. Colonies were screened by restriction digestion or colony PCR and confirmed by sequencing.

For the LR reaction, the desired entry clone and the destination vector plasmid were purified and the concentrations noted using the Nanodrop 2000 UV-Vis spectrophotometer. 150 ng of each vector was added to 2 µl of LR Clonase (Life Technologies) and the volume made up to 10 µl. The reaction was allowed to proceed overnight at 25°C and terminated the next day by addition of 1 µl of Proteinase K. 2-5 µl of the reaction was used for transforming chemically competent cells and plated onto appropriate selection medium. Colonies were screened by restriction digestion and confirmed by end sequencing of the clones inserts.

2.5.8 Golden Gate assembly: Level 1 and Level 2 (Binary) vector assembly

The protocol was adapted from Engler et al, (2008). Individual components to be assembled were synthesised by GeneArt^{R} (Life Technologies). To construct 'level 1' vectors, 100 ng of the linearized vector backbone and equimolar amounts of the other assembly pieces were added to a 15 µl total reaction mixture volume. The reaction mixture contained a final concentration of 1x NEB T4 buffer, 1x BSA, and 1 µl of *BsaI* and T4 ligase (New England Biolabs) each. The tube was placed into a thermocyler and cycling parameters setup as follows. (37°C/ 3min//16°C/4min) x25 cycles (50°C/5min//80C/5°min) x1 cycle. 2 µl of the assembly reaction was transformed into 20 µl of competent *E. coli* cells. Only white colonies selected on the basis of blue white screening were screened by restriction digestion and confirmed by sequencing. Construction of level two vectors was done using the same protocol but the *Bsa*I was replaced with *Bpi*I restrictions enzyme. Selection of untransformed colonies was based on red-white selection; the untransformed colonies appeared red.

2.5.9 General sample collection and RNA isolation

Tissue was in 2 mL Eppendorf tubes or wrapped in aluminium foil and immediately snap frozen in liquid nitrogen. The samples were ground in liquid nitrogen using a pre-chilled mortar and pestle treated with RNAseZAP (Invitrogen). The ground samples were collected in 2 mL Eppendorf tubes. RNA was isolated using RNeasy plant mini kit (QIAgen) following the manufacturer's protocol. The eluted RNA was treated with DNase (Invitrogen) following the manufacturer's protocol and the quality evaluated by agarose gel electrophoresis.

2.5.10 Root hair tissue collection and RNA isolation

The root hair harvesting protocol was performed as that published (Breakspear et al, 2014). Root tips were removed and the roots plunged into liquid nitrogen contained in a Teflon-coated loaf tin (Dunelm Mill). A Daler Rowney number 2 filbert paint brush (Dunelm Mill) was used to brush root hairs and collected in the tin. Around 120-150 roots were used per RNA sample and the remaining nitrogen was poured into a 45 ml PTFE-coated conical centrifuge tube (VWR) and the nitrogen left to boil off. RNA was isolated from this purified root hair sample using RNeasy plant micro kit (QIAgen) according to the manufactures protocol and quality analysed using a Bioanalyser.

2.6 Assays used in this study

2.6.1 Promoter-GUS analysis

Promoter:*GUS* constructs for *CBF1, CBF2* (Hogekamp et al, 2011) and *CBF3* (unpublished) were obtained from H. Kuster, University of Hannover.

2.6.2 Histochemical localization of GUS

To visualize spatial patterns of gene expression, X-GlcA staining of β-Glucuronidase activity was performed. To 50 ml of GUS buffer, 196 ul of 250 mg/ml X-GlcA (Melford) in DMF (Dimethyl formamide) was added and finally mixed. Tissue samples were taken in small petri dishes and covered in the staining solution and the plates kept at 28 °C in dark. After the desired colour intensity developed, the staining solution was removed and the samples washed with fresh GUS buffer.

2.6.3 Infection thread staining and quantification

M. truncatula seeds were sterilised and germinated on DWA plates as described in section 2.6. The plates were then transferred to room temperature and incubated overnight. Seedlings were placed on top of filter paper on BNM + 0.1 µM AVG square (120x120 mm) agar plates. A culture of *S. meliloti* 2011 *lacZ* (2.2) was grown overnight in 5 ml TY broth with streptinomycin and tetracycline (2.4) at 28 °C with vigorous shaking until 0.3 < OD600 < 0.8 (exponential phase). The *S. meliloti* 2011 *lacZ* culture was diluted in sterile deionised water to OD600 = 0.001 and was used to inoculate the plants using an intranasal Mucosal Atomization (MADS) device (LMA, San Diego, USA) for even coverage (1 ml per plate) and another sterile filter paper placed on top. Plates

were incubated in a growth chamber (20 °C/15 °C, day/night cycles of 18/6 h with 32 % relative humidity, 300 µmol m-2 s-1 light intensity).

After 6 days the roots were fixed in Z buffer (100 mM sodium phosphate pH 7.0, 10 mM KCl and 1 mM MgCl2) containing 2.5% (w/v) glutaraldehyde under vacuum in a fume hood for 15-30 mins. Then fresh Z buffer with 2.5% (w/v) gluteraldyhde was added and the roots left for at least one hour at room temperature. The roots were then washed 3 times in Z buffer (no gluteraldehyde).

To stain the roots X-Gal staining solution was made up. For 1 ml: 880 µL Z buffer, 50 µl K3[Fe(CN)6] (potassium ferricyanide) 100 mM, 50 µl K4[Fe(CN)6] · 3H2O (potassium ferrocyanide) 100 mM and 20 µl 5-bromo-4-chloro-3-ondolyl-beta-D-galactopyranosid (X-Gal) 4% (w/v) in dimethylformamide. The X-gal staining solution was added to the roots and incubated in the dark at 28 °C overnight. The roots were washed in Z buffer with one quick wash followed by one for an hour. The stained roots were then placed in 5 ml fresh Z buffer with 2-3 drops of 0.5 M Na-EDTA to inhibit fungal growth. The roots were then analysed under a microscope and the number of infection threads quantified or images captured.

2.6.4 Histochemical staining procedure

Root material was submerged in 2.5% glutaraldehyde and placed under a vacuum for 15 minutes. The glutaraldehyde was removed and fresh glutaraldehyde was added before leaving at room temperature for a minimum of 1 hour. The fixed material was then washed with Z buffer with one 5 minute wash followed by a 1 hour wash. Fresh *LacZ* staining solution was made according to the following: 1mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) or 5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside (Magenta-gal); 0.1M sodium phosphate; 10mM Potassium chloride; 1mM Magnesium Sulphate; 5mM Potassium Ferricyanide; 5mM Potassium Ferrocyanide.

2.6.5 Nodulation assay

To compare nodule number between different genotypes seeds were sterilized, scarified and vernalized as described before. Overnight germinated seedlings were transferred to sterile terragreen and sharp sand mixed to a 1:1 ratio and covered by a transparent lid to maintain humidity. Specifically, P40 (2 inch diagonals) trays were used for all nodulation assays. After allowing seven days of growth, plants were inoculated with 1 ml of rhizobia at a final absorbance of 0.02 at OD600 diluted in water. The plants were allowed to grow

for three weeks under long day conditions and watered regularly. To count the number of nodules, soil was completely removed from each pot without damaging the roots and the roots gently washed in water. Pink (nitrogen fixing) and white nodules were scored separately and the numbers recorded.

2.6.6 Growth media and inoculation

Several sources of AMF inoculum were used in the course of this study. Seedlings were planted directly into one of the following inoculums.

Chive inoculum: *Rhizophagus irregularis* was grown in a 50:50 mix with chive plants. Inoculum was made by chopping up the root mass and soil containing *R. irregularis*. A 50:50 mix of terragreen and sand was made and then mixed with the chive root inoculum at a ratio of 1:20 inoculum : sand mix.

PlantWorks inoculum: A commercial inoculum was obtained from PlantWorks, UK, and mixed with the 50:50 sand mix at a ratio of 1:10 inoculum : sand mix.

Symplanta inoculum: Another commercial inoculum was obtained from Symplanta, Munich, 10,000 spores/ml of *Rhizophagus irregularis* (syn: *Glomus irregulare*) SYMPLANTA-001 research grade. For a strong inoculum, 750 spores/plant was use. For a weak inoculum, 500 spores/plant was used.

2.6.7 Mycorrhization assay and Ink staining

To compare differences in percentage colonisation by the fungus *Rhizophagus irregularis*, seedlings were germinated as described. The seedlings were allowed to grow on plates for seven days on DWA medium and gently removed from the plates with forceps without damaging the roots. They were then transferred to 1:1 terragreen:sharp sand low nutrient growth medium mixed with 20% chive inoculum containing roots of chive plants infected with spores of the AM fungus. Alternatively, seedlings were transferred to inoculum containing growth medium directly after germination. Plants were covered with a lid to maintain humidity and allowed to grow for 4-5 weeks before harvesting the root tissue. Roots were washed and approximately one inch of each sample from around two third of the total root length was collected for analysis. The fungus was visualized using an ink staining protocol (Vierhilig et al, 1998). Roots were placed in float racks containing 2 ml eppendorf tubes with holes at the bottom to allow drainage. The rack was placed in boiling 10% w/v potassium hydroxide solution for 10 minutes to clear the roots and the excess solution allowed to drain off by blotting onto a blue roll. It was then

placed into the staining solution containing 5% ink and 10% acetic acid at 96°C for 6 minutes. Finally, the samples were washed with distilled water to remove excess stain.

2.6.8 RNA interference of BFPL

Primers were designed for specificity to *BFPL*, BFPL RNAi F1 and BFPL RNAi R1 (Table 2.6), amplifying a 90bp region, which was checked by sequencing. This was cloned into the pENTR-D TOPO entry vector. This was then cloned into the destination vector pK7GW|WG2D(II)R (modified from pK7GW|WG2D(II); Karimi et al, 2002), using GATEWAY® LR reaction, Invitrogen.

The RNAi construct was transformed into *A. rhizogenes* and used for hairy root transformation. Successful transformation was determined by presence of dsRed. Plants grown on 1:1 terragreen:sharp sand using 20% commercial AM inoculum (PlantWorks, UK). After 4 weeks, roots were stained and scored for AM colonisation.

2.7 Bioinformatics Analyses

2.7.1 *In silico* gene expression analysis (MtGEA)

In silico gene expression analysis was carried out using the Medicago Gene Expression Atlas (MtGEA) Database (Benedito et al, 2008), Samuel Roberts Nobel Foundation. Probesets were identified using the BLAST function using the CDS.

2.7.2 Determining orthologues and homologues of BiFunctional Protein (BFP) and BFP-Like (BFPL)

To determine plant orthologues, a BLASTP of most plant species against the *BFP* and *BFPL* amino acid sequences were done using Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), others with their species-specific genome portals. This included the unpublished *Lupinus angustifolius* genome sequence made available courtesy of K. Singh. *Marchantia paleacea* sequence courtesy of G. Radhakrishnan. Potential orthologous sequences were checked for the presence of *BFP/BFPL* predicted protein domains in the same order sequence, using InterPro (http://www.ebi.ac.uk/interpro/).

Fungal homologues of *BFP* were determined using NCBI BLASTP server, and protein domains determined using InterProScan.

2.7.3 Sequence alignments

Sequences were aligned using pairwise alignment in Geneious ®

2.8 Phylogenetic Analysis

2.8.1 BFP tree

Protein sequences from the genomes of several plant species were downloaded into Geneious® software. These were aligned with reference to *MtBFP* using ClustalW in Geneious®. Tree was built in Geneious® using the Neighbour-Joining method (100 replicates to determine bootstrap values), with *Amborella trichopoda* as a root.

2.8.2 NF-Y trees

The trees were constructed using Phylogeny.fr (http://www.phylogeny.fr/) server, using the 'advanced setting', which uses appropriate likelihood test (aLRT) to measure branch support values (Anisimova and Gascuel, 2006).

2.9 Light Microscopy

Light microscopy was performed on either a Nikon Eclipse E800 with a Pixera Pro 600ES camera or a Zeiss Axiophot with a Retiga-2000R Fast 1394 Color camera, QImaging. Fluorescence microscopy was performed on a Zeiss Axiophot with a Retiga-2000R Fast 1394 Color camera, QImaging, or a Leica MZFLIII Fluorescence stereoscope.

Primer name	5' to 3' sequence	Description
Tnt1 F	TCCTTGTTGGATTGGTAGCC	*Tnt1* forward
Tnt1 R	CAGTGAACGAGCAGAACCTGTG	*Tnt1* reverse
NFYC Gt F2	CAAGCAGGTGCATATTCAGG	*cbf1* genotyping forward
NFYC Gt R1	CCATTTGCATGACAGTGGTA	*cbf* genotyping reverse
CytP450 Gt F2	TTGCTTCGGAAGAAGGAAGA	*cyp450* genotyping forward
CytP450 Gt R1	TTCCAATCAAAGTGGCACAA	*cyp450* genotyping reverse
BFP Gt F1	TTTTCATCGCATCGGTGTTA	*bfp-1+2* genotyping forward
BFP Gt R1	TGGCTGAATGAAGAGTGCTG	*bfp-1+2* genotyping reverse
BFP-3 Gt F	AACCGCCCTTCTGTTTTCAC	*bfp-3* genotyping forward
BFP-3 Gt R	ACCCCTTCAGAGATGACAGC	*bfp-3* genotyping reverse
BFP-4 Gt F	AGAGCTCCAAGGTTCAAGGA	*bfp-4* genotyping forward
BFP-4 Gt R	AAACAGGTTGTGGCACCAAA	*bfp-4* genotyping reverse
Tos17-tail6	AGGTTGCAAGTTAGTTAAGA	3' end of *Tos 17*
OsBFP F6	AAGATGCATTCCCATGAAGC	*Osbfp-1* genotyping forward
OsBFP R6	CGAGCACATACCAAAGGGAT	*Osbfp-1* genotyping reverse
OsBFP F1	GAAATGCCAGTGTGCTCTGA	*Osbfp-2* genotyping forward
OsBFP R1	TGTTGTGCTGTGGTCGGTAT	*Osbfp-2* genotyping reverse
BFPL RNAi F1	GCTGTTGTTCAAAACGGAAGTGT	*BFPL* RNAi forward
BFPL RNAi R1	CGACTTTAGCACATGCTCGC	*BFPL* RNAi reverse
BFP pPCR F2	GGTATTCCAATGCACAAGATTTTTC	*BFP* qPCR forward
BFP pPCR R2	ATCAATTCTTGCATCATCATCAGCA	*BFP* qPCR reverse
Cbf3 F whole gene	ATGTCAGGTAATAAGAGAAACCAAACAA GTCCTG	*CBF3* rtPCR forward
Cbf3 R whole gene	TCAACCCTGTAACGTAGGTTTGGTG	*CBF3* rtPCR reverse
GG3	CCCGCCAATATATCCTGTC	golden gate sequencing
GG4	GCGGACGTTTTTAATGTACTG	golden gate sequencing

Table 2.7 Primers used in this study

CHAPTER 3

Tnt1 Forward Screen for mycorrhizal-defective mutants

3.1 Introduction

Forward screens of mutagenized lines, where gene expression is disrupted or altered, is an effective way to discover novel genes that are required for specific biological functions. In order to find mutants, thousands of plants need to be screened, which is extremely time consuming. Discovering AM-specific genes in this way is difficult as phenotyping for AM colonisation requires staining and microscopy; a huge undertaking with thousands of plants. Paszkowski et al (2006) were able to bypass this by screening a mutator-mutagenized maize (*Zea mays*) population. Maize accumulates a yellow pigment in the roots when colonised by AM fungi (Klingner 1995). The initial screening simply isolated plants that displayed differences in the concentration and distribution of the pigment.

The majority of mycorrhizal-defective mutants have been discovered on the back of early nodulation screens. Phenotyping roots for the presence or absence of nodules on a large quantity of plant roots is more manageable than screening for AM phenotypes. Many mutants defective in nodulation were discovered to also be impaired in AM symbiosis. This established the concept of the common signalling pathway (CSP), a suite of genes essential for both symbioses (Parniske, 2008).

Nodulation and mycorrhization have since been shown to share common genes downstream of the CSP that are recruited for effective symbiosis, particularly during early infection (Breakspear et al, 2014). However, the distinct physiological differences in accommodation strategies for the symbionts (i.e. the formation of plant-derived nodules compared to the cortical modifications required for arbuscular formation) suggests that there could also be many AM-specific genes yet to be discovered.

As previously described, the colonisation of AM fungi in plant roots undergoes various stages of infection, from the initial molecular dialogue between plant and fungus to nutrient exchange at the arbuscular interface, and subsequent arbuscule senescence. Mutants defective in the early stages of epidermal penetration can be easier to identify, as they often show no AM colonisation. However, the potency of the inoculum and the time points used to phenotype are important to discover these defects. For example, *ram1* in *M. truncatula* has defects in hyphopodia formation on host roots (Gobbato et al, 2012). However, using a strong inoculum can mask this defect in the *ram1* mutant to a small degree and arbuscules can form similar to those in WT plants. Once hyphae can penetrate the epidermis, if the mutant is only defective in penetration, identifying the phenotype becomes more difficult. The AM hyphae can freely colonise the root once the epidermal barrier has been breached.

AM forward screens have been undertaken in various plant species, including *M. truncatula*, which led to the identification of *RAM1* and *RAM2* (Gobbato et al, 2012; Wang et al, 2012), *L. japonicus* (Groth et al, 2013; Kojima et al, 2014), petunia (*Petunia hybrida*) (Reddy et al, 2007), maize (Paszkowski et al, 2006) and tomato (*Solanum lycopersicum*) (Barker et al, 1998). The vast majority of mycorrhizal mutants discovered by these screens have been penetration mutants.

3.1.1 Noble *M. truncatula Tnt1* mutagenesis library

M. truncatula is a useful model legume due to its small genome (~5 x 10^8bp), ability to self-fertilise, and the quick turnover of large numbers of progeny (Cook, 1999; Oldroyd and Guerts, 2001). Resources for both forward and reverse genetics have been established in *M. truncatula* including ethyl methane sulfonate (EMS) lines, which tend to produce point mutations, and transposon insertions which disrupt whole genes (Young and Udvardi, 2009). The tobacco retrotransposon *Tnt1* has been used to develop a large library of mutant *M. truncatula* lines in the R108 ecotype (d'Erfurth et al, 2003; Ratet, 2006; Tadege et al, 2008). *Tnt1* is 5.3kb long and integrates stably into the genome and generates between four to forty insertions per plant, with a preference for open reading frames. The Samuel Roberts Noble Foundation is home to a large *Tnt1* insertion library in *Medicago truncatula*. Locating genes affected by *Tnt1* involves a user-friendly PCR protocol to screen for the end of the *Tnt1* sequence. The library can be used for both forward and reverse genetics.

This chapter describes the establishment of a new mycorrhizal inoculum in our lab and how this was used to develop a forward screen protocol for AM mutants of *M. truncatula.* The aim of this was to identify mutants defective in the AM symbiosis. Phenotypes could include an inability to allow fungal entry (penetration mutant), resulting in no colonisation. If the number of hyphopodia are absent or reduced, this could indicate a signalling defect, as in *RAM2* (Wang et al, 2012). Otherwise, it could indicate a defect in the correct formation of the PPA. Fungal structures within the host root are also observed for abnormal development. For example, underdeveloped arbuscules could suggest a defect in the cellular trafficking machinery during arbuscule development (Pumplin and Harrison, 2009). Mutants with defects in peri-arbuscular membrane-specific transporters also exhibit this phenotype (Harrison et al, 2002; Wang et al, 2014). Also a quantitative difference in the number of fungal structures could indicate a perturbation, or a defect in the autoregulation of the symbiosis.

3.2 Results

3.2.1 Development of a mycorrhizal inoculum

In order to undertake a large scale AM experiment, an inoculum was required to fulfil the following criteria:

1) Cost-effective and easy to produce, as a large amount of inoculum is required.

2) Consistently reliable in colonising *M. truncatul*a roots.

3) Free from pathogens.

4) Presence of only one AM fungi of choice (in this case *R. irregularis*).

To date, the inoculum used in AM phenotypic experiments in the lab consisted of a commercial inoculum which contained five different AM fungal species and, possibly, various growth promoting bacteria and pathogenic micro-organisms. Also, sterile commercial spores were sometimes used but sometimes did not colonise reliably.

I used a systematic approach to design a suitable inoculum for the forward screen. To decide the best plant host to produce the AM fungi, three different species were trialled and tested for their ability to colonise the fungus. For this *M. truncatul*a was not used in

order to avoid possible contamination of pathogens which may preferentially infect the *M. truncatul*a plants used in the screen. Along with the ability to colonise, was the suitability of the plants to grow in a relatively low nutrient substrate, as this would become part of the inoculum; substrate high in nutrients could prevent AM colonisation.

Three different species were tested for their ability to be colonised by *R.irregularis* spores; wheat (*Triticum aestivum* 'Paragon'), barley (*Hordeum vulgare* 'Golden Promise') and chives (*Allium schoenoprasm*). These were grown with sterile *R. irregularis* spores for six weeks, half were grown on minimal nutrient substrate and half with the addition of limited nutrients. The wheat and barley plants exhibited low, variable colonisation compared to the chive plants (Table 3.1).

Table 3.1 **AM colonisation after 6wpi with *R. irregularis* spores**

	Minimal nutrients	Added nutrients
Wheat	25%*	0%*
Barley	35%*	0%*
Chives	Unhealthy plants	40%*

*mean percentage of arbuscules

Chives were considered to be the better candidate and an experiment was set up to determine the optimum growth requirements for healthy chive plants with a soil substrate with minimal phosphate, as phosphate can prevent AM colonisation. It was considered desirable to grow the chives in a similar soil substrate to that of *M. truncatula* used during AM experiments (1 part terragreen: 1 part sharp sand). The chive plants were grown in four alternative ways (Figure 3.2):

1. 1 part terragreen: 1 part sharp sand
2. As above but watered with M medium
3. 2 parts terragreen: 2 parts sharp sand: 1 part low nutrient compost
4. As above but using normal potting compost

In order to create an AM inoculum that was free from rhizobia, the soil substrate mixes were autoclaved beforehand and care was taken to keep conditions as clean as possible. This involved cleaning all surfaces and trays etc. with 70% ethanol and wearing gloves. By trying to eliminate rhizobia, I could potentially avoid any interacting effects that initiating the nodulation symbiosis may have on a mycorrhizal phenotype.

After 8 weeks post inoculation with sterile spores, the chives grown on substrates without added compost (No.1 & 2) were small and yellowing. The plants in the other conditions were green and healthy looking. Condition number 3, was chosen as the best substrate, as it had the least amount of added nutrients of the remaining experimental conditions. Twenty chive plants were checked for AM colonisation, of which only 50% were colonised by AM. The inoculum produced from this first experiment was then used to set up trays of more chives, in order to increase the potency of the inoculum. After this, a sample of ten chive plants observed had a mean value of 76% arbuscules. Chive shoots were removing by cutting them very close to the base of the stem. The inoculum was kept under conditions that would keep it as free from rhizobia as possible; in sealed plastic bags at 4^0C.

To determine its effectiveness of the inoculum, *M. truncatula* plants were grown using the inoculum. After three weeks, the roots were absent of nodules, indicating that there were no rhizobia present (n=15). A further fifteen plants were checked at 4wpi for effective AM colonisation. Arbuscules were abundant (65%) and looked well-developed (Figure 3.1).

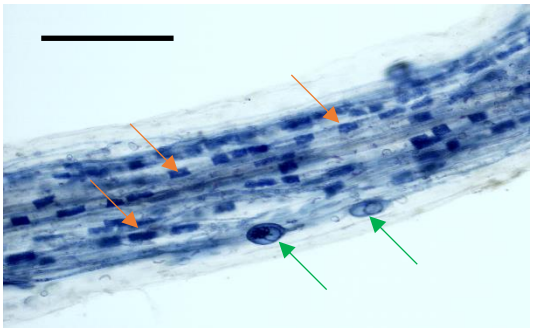


Figure 3.1 **Initial testing of chive inoculum in *M.* truncatula R108 (WT) roots**

M. truncatula seedlings were grown with *R. irregularis* (20% chive inoculum). After 4 weeks, WT R108 roots had abundant arbuscules (orange arrows) and vesicles (green arrows). Scale bar=500µm.

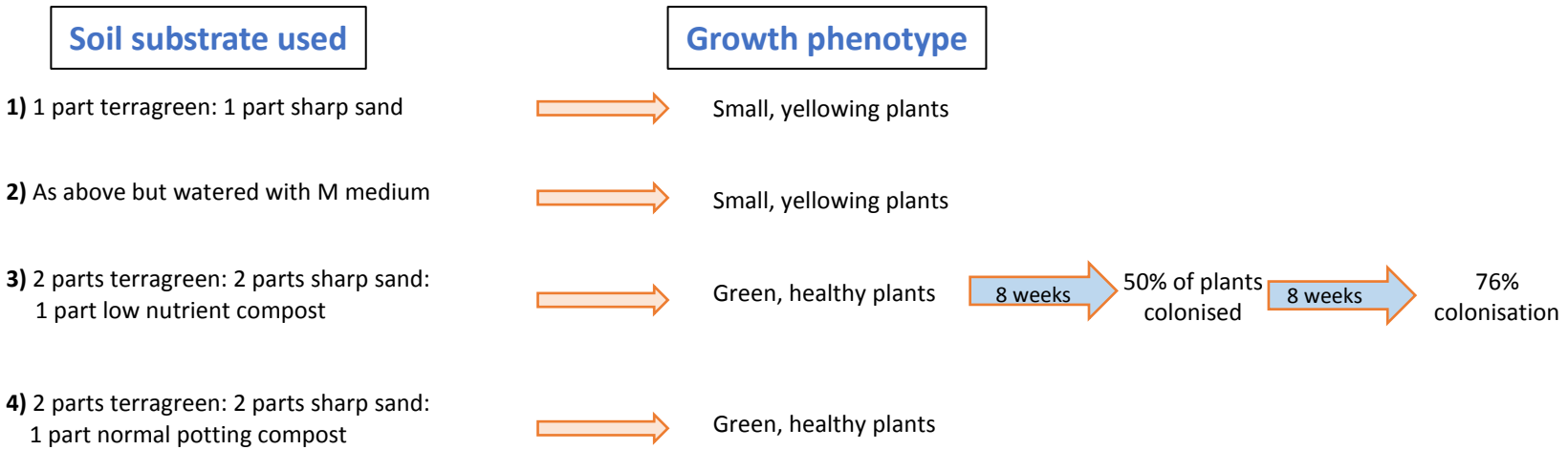


Figure 3.2 **Protocol in which the growth conditions for chive AM inoculum were chosen**

Chive plants were grown for eight weeks on terragreen and sand mix, with various nutrients added. Condition no.3 was chosen as the optimum; the smallest amount of added nutrients that yields healthy plants. After 8 weeks, half the chive plants checked were colonised. The inoculum made from this was used to inoculate more chive plants for a further 8 weeks, resulting in a mean arbuscule number of 76%.

3.3 Testing of AM chive inoculum in *mtmax4* background

I wanted to test the chive inoculum to see if it would colonise *M. truncatula* over a time course experiment. I used this opportunity to test the mycorrhizal phenotype of the novel strigolactone biosynthesis mutant *mtmax4*, which had become available in the lab. Strigolactone mutants typically have reduced arbuscule numbers compared to controls, which increase over time in relation to the increase in WT plants. This mutant, therefore, would enable to me to test whether the chive inoculum could identify quantitative phenotypes over a time course experiment, including subtle phenotypes early on.

MAX4 in arabidopsis/*DWARF10* in rice/*RMS1* in pea and *DAD1* in Petunia encodes the CCD8 (carotenoid cleavage dioxygenase 8) in the strigolactone biosynthesis pathway. As described in the main introduction, AM fungi undergo increased hyphal branching in response to strigolactones exuded from plant roots. No stable strigolactone biosynthesis mutants have been described in *M. truncatula* to date. The *mtmax4* plants exhibit the classic phenotype of increased lateral root branching observed in the Arabidopsis *max4* (Schwartz et al, 2004) as well as the other *A. thaliana max* mutants (1-3) (Stirnberg et al, 2002; Booker et al, 2004). I wanted to see if a strigolactone biosynthetic mutant would show a reduction in AM colonisation, as has been displayed in rice, tomato and pea (Gutjahr et al, 2012; Yoshida et al, 2012; Koltai, 2010; Gomez-Roldan, 2008). I scored AM colonisation in *mtmax4* at 2, 4 and 6 wpi using my *R. irregularis* chive inoculum (Figure 3.3). The R108 plants show an increase of arbuscule percentage in roots over time (from 25% -81% colonisation), showing that maximal colonisation can occur within 6 weeks of inoculation. In comparison, the commercial inoculum used in the lab takes at least eight weeks to establish an arbuscule value of 80%. The chive inoculum, therefore, can reduce the time required for AM experiments. The *max4* plants show a significant reduction in arbuscule percentage colonisation at all three time points (80% at 2 wpi; and 40% reduction at 4 wpi and 6 wpi). This is the first AM phenotype of a stable strigolactone biosynthesis mutant in *M. truncatula*, and shows that it has a significant effect on colonisation, comparable to that of other legumes.

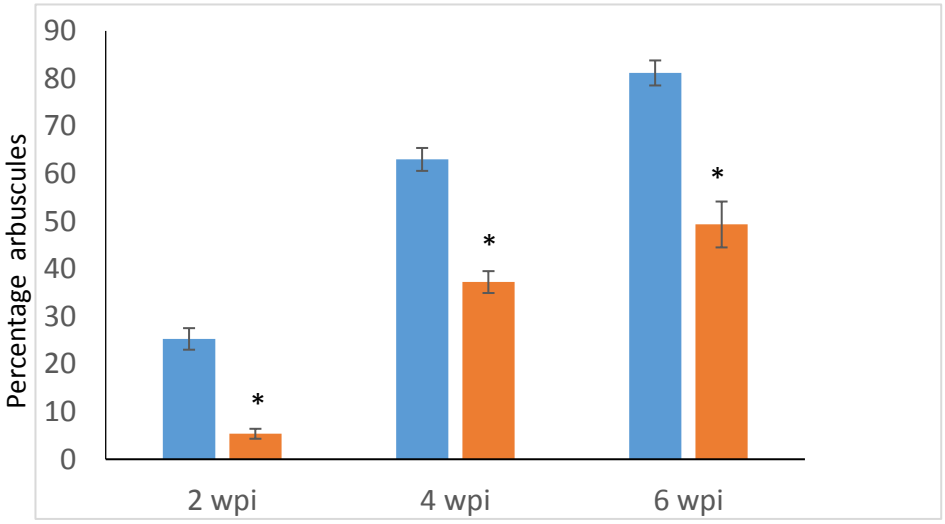


Figure 3.3 **Reduced arbuscule formation in *max4.***

A significant reduction in the percentage colonisation of arbuscules in *max4* (orange bars) roots compared to WT R108 (blue bars) in *M. truncatula*, using *R.* irregularis (20% chive inoculum) at 2wpi (n=12), 4wpi (n=13) and 6wpi (n=13). Bars indicate standard error of the mean. * p=≥0.001

3.4 Mycorrhizal forward screen

A *Tnt1 M. truncatula* population of 655 inbred lines was chosen for a mycorrhizal forward screen. *Tnt1* lines are reported to possess 40-50 transposon insertions each, approximately 50% of which are introduced into genes (Tadege et al, 2008). The plants were inbred over five generations, meaning that over 95% of the gene mutations were fixed homozygotes. This equates to approximately 8000 homozygous alleles, as half the original mutations would be fixed as WT alelles. Eighty six of these lines were from a collection in a mutant background that produces hypermorphs in both noduation and the AM symbiosis, provided by Dr. K. Mysore. These lines were included to enhance the ability to detect symbiotic mutants. Previously, C. Liu (2012-personal communication) did a preliminary assessment for nodulation phenotypes of the 655 lines, classifying mutants as either nod- or fix-; nod- plants had no nodules after 3 wpi; fix- showed only white nodules. Mature nodules are generally pink, indicating that nitrogen fixation is taking place. A fix- phenotype often indicates either a delay or block in rhizobial infection. Although these phenotypes would have to be confirmed, they give an indication on whether any AM phenotypes found could be the result of a common SYM gene.

A forward screen protocol was developed to produce optimum results in a quick and efficient manner. To help determine the parameters to be used in the screen, R108 plants were checked for colonisation at various concentrations of the chive inoculum. Concentrations of 5, 10 and 20% inoculum all yielded similar AM colonisation, although the higher concentrations showed less variability between individual plants. I decided to use 20% inoculum, as that was the minimum amount that could be used to place the inoculum on the bottom of the pots to then be filled with soil substrate before planting. Alternatively, less inoculum could have been used, but it would then have to be evenly mixed with the soil substrate, which would have been a lot more time consuming. This protocol was tested beforehand, and shown to be an efficient way to add the inoculum to the growing medium; plants show AM colonisation at 2 wpi comparable to plants grown with the inoculum mixed evenly in the soil substrate. During this initial investigation, it became apparent that the new inoculum sometimes generates chive nurse plants growing with the *M. truncatula* plants. This phenomenon is likely to occur because a small amount of the chive stems are left intact when preparing the inoculum. It is well known that chive plants can be cut and then left to grow back for later

harvesting. Alternatively, it is possible that the chive seeds that did not germinate, may have done so during the experiment.

The initial screen tested two plants of each of the 655 *Tnt1* lines for AM colonisation at 2 wpi. This was thought to be early enough to show potential penetration mutants, but enough time for some arbuscules to form and to observe their structure for abnormalities as determined from the *max4* experiment (Figure 3.3). In each tray of *Tnt1* lines, 10% were WT R108 plants. These were used as controls for both the genotype, and the different environmental conditions of each individual tray.

The *Tnt1* lines were screened for both quantitative and qualitative traits. The quantitative phenotypes were measured using the grid method (Giovannetti et al, 1980), scoring the percentage of arbuscules, vesicles and internal AM hyphae present in the roots. In addition to this, the roots were examined for the following qualitative traits:

- Arbuscule morphology
- Vesicle morphology
- Signs of difficult penetration such as hyphal branching prior to penetration, or hyphal septation.

The *Tnt1* lines were allocated different numbers during the screening process; which promoted an important element of objectivity to the AM phenotyping.

The initial screening identified 34 AM putative mutants, which were then scrutinised more carefully. From this second evaluation, 15 mutants were identified (Table 3.2). Two of these (NF4489-11 and NF6898-86) were known to have a mutation in the *VAPYRIN* gene and had very severe AM phenotypes. With these plants, the hyphae were unable to penetrate the epidermal walls, and there was obvious fungal stress. The hyphae had clearly septated and were unable to penetrate the epidermis. They had also been designated as nod- mutants, suggesting a common symbiotic role. This validated the approach, demonstrating that the protocol I designed can identify known AM mutants.

The thirteen remaining candidate lines were then further evaluated for AM phenotypes. Forty plants per line were grown; 20 of each for a 2 wpi and 4 or 5 wpi time point. These time points allowed for early phenotypes to be identified (as was already seen with the *vapyrin* mutants in the initial screen). The plants were scored for arbuscules, vesicles

and colonisation (any fungal structure, including arbuscules, vesicles and internal hyphae) (Figures 3.5 and 3.6).

This final screening reduced the number of possible mutants to five (Table 3.3).This includes NF0584, which was similar to WT controls apart from a reduction of vesicles at 2 wpi. However, very few plants of that line survived and only six were scored, whose roots were all very small. Unfortunately, there was not enough seed to score the second time point. The reduced seed production in this mutant line, could be the result of a mutation in a background gene that is required for normal seed production. Alternativley, it could be the due to pleitrophic effects of a mutated gene. There were three lines in this final screening with the mutant background in *sunn*, which has a hypernodulation phenotype (Schnabel et al, 2005). *Sunn* is an orthologue of *Har1* in *L. japonicus*, and the *har1* mutant has been shown to exhibit hypermycorrhization, with higher percentages of colonisation and arbuscules (Murray et al, 2006).

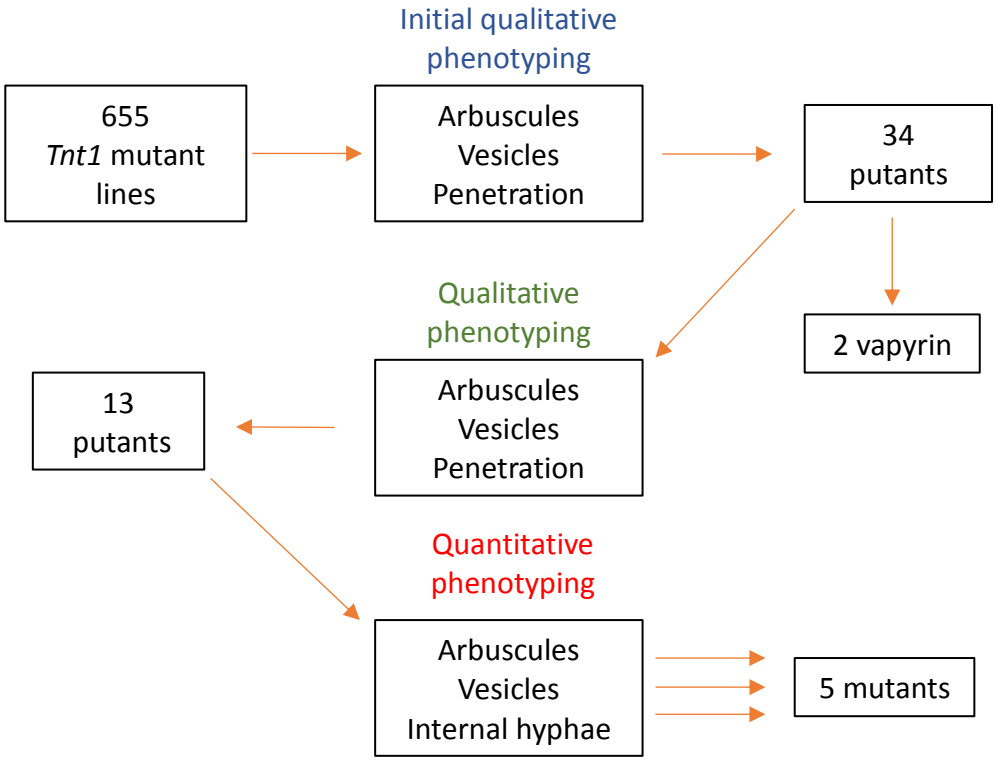


Figure 3.4 **Flow diagram of forward screen protocol**

From a starting *Tnt1* population of 655 mutants, 15 putants were identified from two rounds of phenotyping for qualitative traits (Table 3.1). This included both possible aberrant-looking fungal structures, and mutants which appeared to have fewer structures compared to the WT controls. These were further studied for both qualitative and quantitative traits (Figure 3.5 and 3.6; Table 3.3). From this, 5 mutants were identified (Figure 3.7)

Line No.	Common SYM	fix-	nod-	AM only	Reduced AM	Initial AM phenotype	Background mutation
NF0573	x	x				Low colonisation; difficult hyphal entry?	
NF0584	x	x	x		x	No colonisation (small roots)	
NF0788	x	x				Abnormal arbuscules?; hyphal septation?	
NF0807	x	x			x	Abnormal arbuscules; hyphal septation	
NF0889				x	x	No colonisation	
NF0892	x	x				Difficulty in hyphal penetration?	
NF0905	x	x			x	No colonisation; highly septate hyphae	
NF0978				x		+++internal hyphae; few arbuscules; ++vesicles	
NF1526-2_63	x		x		x	No colonisation (small roots)	*sunn*
NF1526-2_77				x		1 infection event with arbuscules; difficult hyphal entry	*sunn*
NF1526-2_84					x	+++internal hyphae; vesicles; no arbuscules	*sunn*
NF1436				x	x	No colonisation	
NF3438				x	x	No colonisation	
NF4489-11	x		x		x	Penetration mutant	*vapyrin*
NF6898-86	x		x		x	Penetration mutant	*vapyrin*

Table 3.2 **Initial AM phenotypes of putants after second screening**

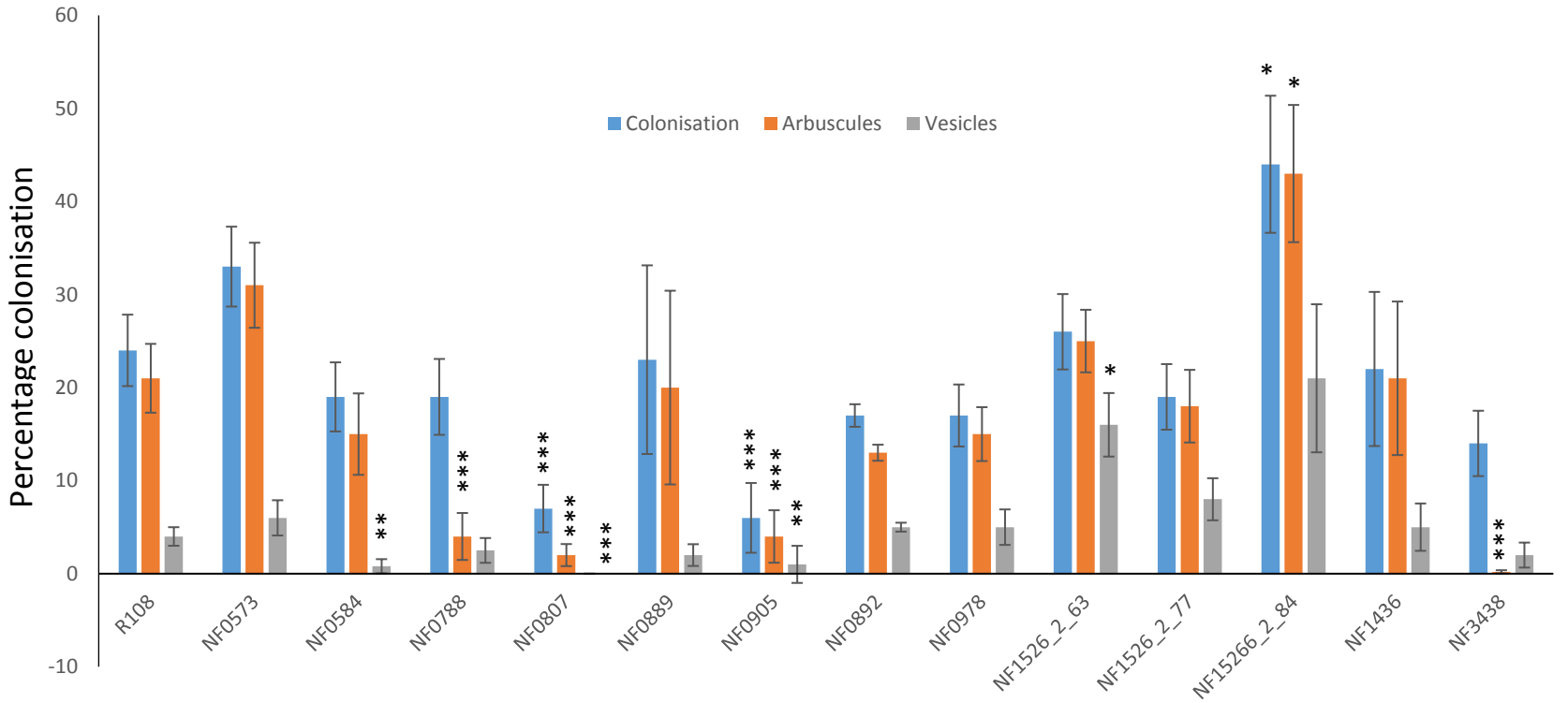


Figure 3.5 **AM phenotype of mycorrhizal putants at 2 weeks post inoculation with AM**

Thirteen putatative mutants isolated from two initial screenings were grown with *R.* irregularis (20% chive inoculum) over 2 weeks. Lines with a NF1526 prefix are in the *sunn* mutant background, 2 of these showed increased AM colonisation. *p=≥0.05; **p=≥0.01; ***p=≥0.001 Bars depict standard error of the mean.

Line No.	2wpi	2wpi	4wpi/ 5wpi	4wpi/ 5wpi
	Reduced AM	Qualitative traits	Reduced AM	Qualitative traits
NF0573		No signs of difficult entry		Normal arbuscules and vesicles
NF0584	x	Reduced number of vesicles	No seeds	No seeds
NF0788	x	Abnormal arbuscules	x	Abnormal arbuscules
NF0807	x	Abnormal arbuscules Increased internal hyphae showing septation	x	Abnormal arbuscules Increased internal hyphae showing septation
NF0889		Normal arbuscules		Many roots have fewer arbuscules per root
NF0892		No signs of difficult entry		Normal arbuscules and vesicles
NF0905	x	Few, abnormal arbuscules; septate hyphae	x	Few, abnormal arbuscules; septate hyphae
NF0978		Arbuscules look normal		Arbuscules look normal
NF1526-2_63		Increased number of vesicles (*sunn*)		Increased colonisation and vesicles (*sunn*)
NF1526-2_77		Colonisation looks normal		Colonisation looks normal
NF1526-2_84		Increased arbuscules (*sunn*)		Colonisation looks normal
NF1436		Colonisation looks normal		Colonisation looks normal
NF3438	x	Few, abnormal arbuscules; septate hyphae	x	Few, abnormal arbuscules; septate hyphae

Table 3.3 **Summary of AM phenotypes of putative mutants from forward screen.**

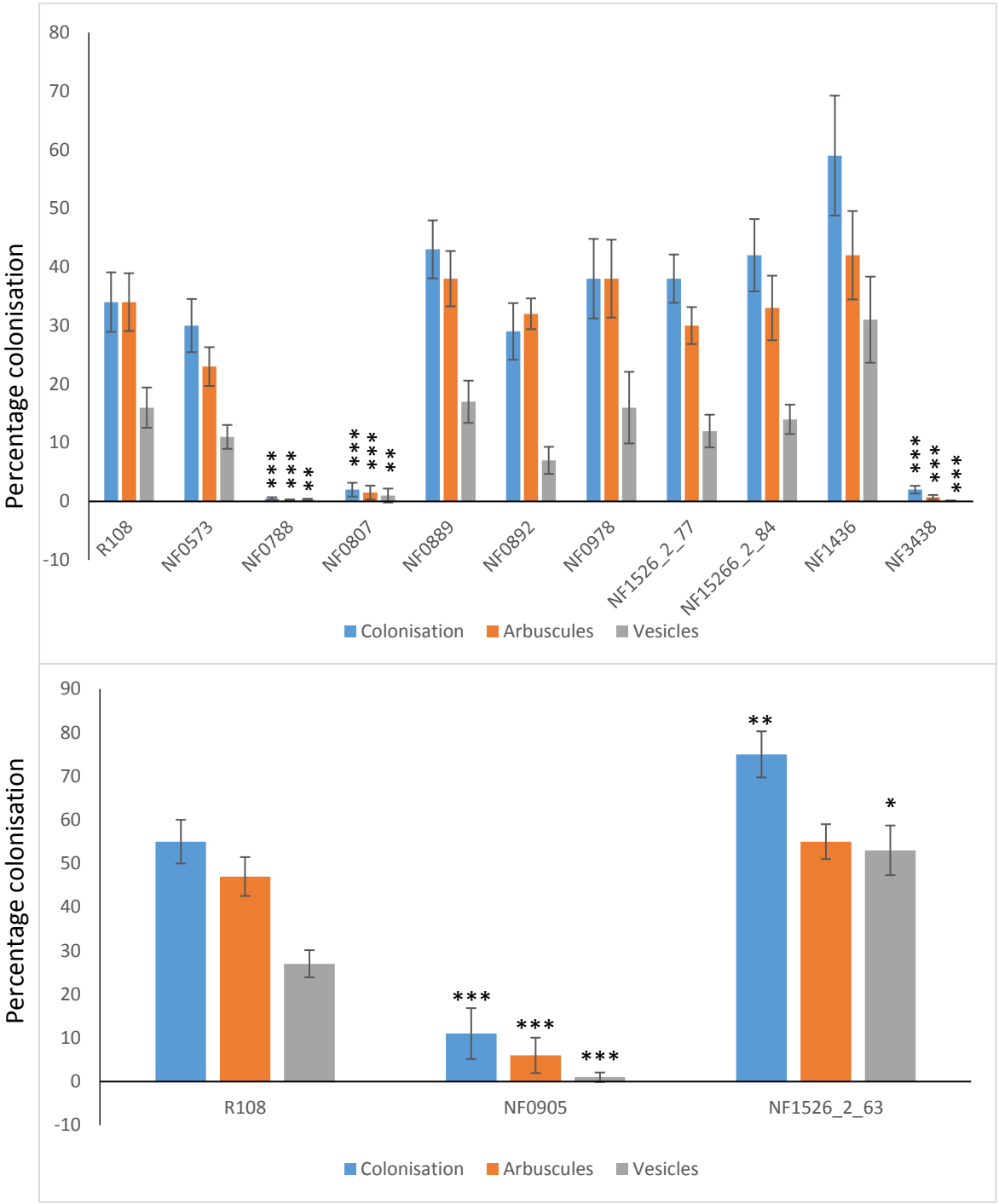


Figure 3.6 **AM phenotype of putative mutants at 4 wpi** (top) **or 5 wpi** (bottom)

Twelve putants isolated from two initial screenings were grown with *R.* irregularis (20% chive) inoculum over 4 (top) or 5 weeks (bottom). Four lines showed a reduction in colonisation. Lines starting NF1526 are in the *sunn* background, which accounts for the increase in colonisation in NF1526_2_63. *p=≥0.05; **p=≥0.01; ***p=≥0.001. Bars depict standard error of the mean.

*p=0.05-0.01; **p=0.01-0.001; ***p=<0.001

3.4.1 Final mutant phenotypes

From a forward screen of 655 mutant lines, four obvious mycorrhizal putants were identified: NF0788, NF0807, NF0507 and NF3438. Figure 3.7 shows the qualitative phenotypes of these mutant lines.

R108 colonisation increased steadily over the 2, 4 and 5 week time points (Figures 3.5 and 3.6). Arbuscule abundance was correlated with general colonisation. Arbuscules were highly branched and hyphae aseptate, indicating that the symbiosis was functioning normally (Figures 3.7a and 3.7b).

NF0788 showed a significant (81%) reduction in arbuscule formation at 2 wpi (n=11). At 4 wpi (n=14), the phenotype became more pronounced as every type of colonisation scored was significantly decreased by ≥92%. At least half the plants had no arbuscules. Those that were present were all abnormal (Figures 3.7c and 3.7d); they were very small and looked underdeveloped (Figure 3.7a). There was also increased septation of the AM internal hyphae (Figure 3.7d), a sign of hyphal degeneration (Wang et al, 2014). Vesicles looked normal (not shown). The reduction of both arbuscules and internal hyphae indicates that the mutant has difficulty with both initial penetration and arbuscule formation.

NF0807 at 2 wpi (n=13) had a significant decrease (70%) in internal colonisation, although there was a large variation (between 2% and 20%). Arbuscules were severely affected with a 90% reduction, and no plants had developed vesicles. At 4 wpi (n=12) every AM phenotype was ≥92% reduced in the mutant. Vesicles that were present looked normal (not shown). As in line no. NF0788, at least half of the plants had no colonisation at all. NF0807 showed apparent internal (Figure 3.7f) and external (not shown) hyphal septation. In the areas of root that did have arbuscules, the root was highly colonised with AM hyphae (Figure 3.7e). There were attempts at arbuscule formation, either only forming arbuscule trunks, or ones which were underdeveloped (Figure 3.7f).

NF3438 also showed a very strong AM phenotype. At 2 wpi (n=11), only arbuscules are reduced, but by 92%, with less than 1% of the roots colonised. The majority of the plants had no vesicles. There were two plants, however, with 10% and 14%, which made the mean values for vesicle abundance non-significant. At 4 wpi (n=15), however, every phenotypic AM trait measured was significantly reduced (colonisation 94%,

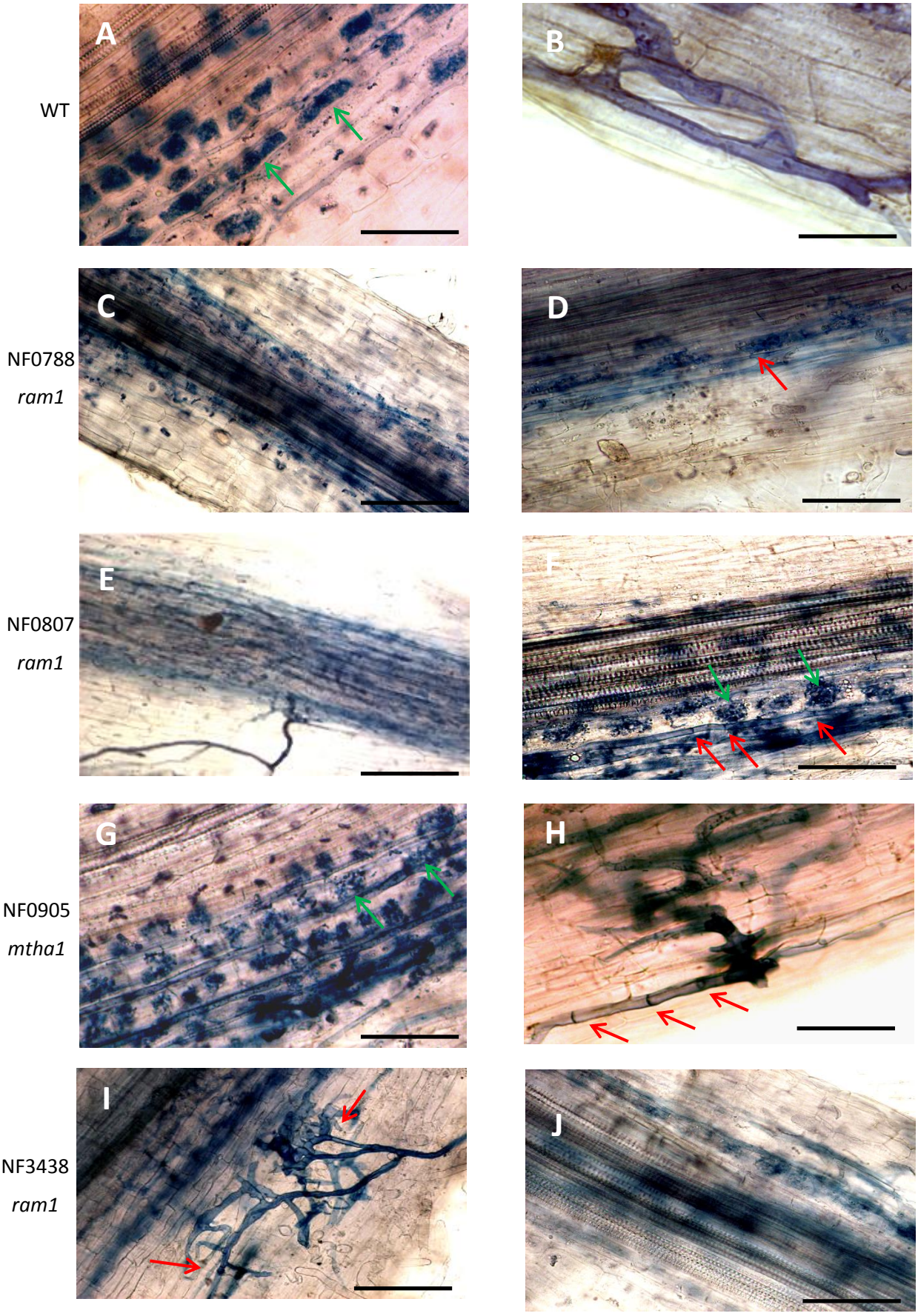

WT
A
B
NF0788
ram1
C
D
NF0807
ram1
E
F
NF0905
mtha1
G
H
NF3438
ram1
I
J

Figure 3.7 **AM phenotypes of final mutants from forward screen.**
R108 and four *Tnt1* mutant lines reduced in AM colonisation were grown in 20% chive AM inoculum for 2 and 4 weeks. WT (R108) plants displayed normal arbuscules and aseptate hyphae **[A, B]**; NF0788 had abnormal arbuscules at 2 wpi **[C]** and 4 wpi **[D]**; NF0807 had increased internal hyphae at 2 wpi **[E]**; with abnormal arbuscules and septate hyphae at 4 wpi **[F]**; NF0507 displayed abnormal arbuscules and septate hyphae at 2 wpi **[G, H]**; NF3438 also had septate external hyphae, increased internal hyphae and difficulty forming arbuscules at 4 wpi **[I]**; any arbuscules present were abnormal **[J]** (2 wpi). Red arrows indicate septate hyphae; green arrows indicate arbuscules. **A, C, E, G** bar=500µm. **B, D, F, H** bar=1000µm.

arbuscules 90%, vesicles 99%). Figure 3.7i shows a representative image of this mutant: highly branched septate hyphae at the site of the root infection followed by AM hyphae attempting to enter cortical cells, usually without any success apart from what looks like arbuscule trunks. The very few attempts at arbuscule formation do not appear to fill the cortical cells in a normal way (Figure 3.7j).

NF0905 at 2 wpi (n=13) showed a great reduction in all AM structures (≥ 70% reduction in all scored phenotypes); this was also true at 5wpi (n=12) (≥ 80% reduction). At 5 wpi there were two plants showing a high percentage of arbuscules which decreased the mean reduction in both arbuscules and colonisation. The few arbuscules that were present were smaller than WT. There were extensive septated internal and external hyphae on all plants (Figures 3.7g and 3.7h).

3.4.2 NF0788 and NF0807 are sibling lines with a mutation in *ram1*

Shortly after identifying the NF0788 and NF0807 as AM mutants, I was made aware that these two lines had also been screened as part of a PhD thesis (Bano, 2011). Bano was able to identify them as sibling lines by comparing the flanking PCR products of *Tnt1.* Sequencing the flanking DNA indicated that they both had a transposon insertion in the *RAM1* gene. It was established that they share a *ram1* allele which is a different allele to *ram1-1* (Gobbato et al, 2012), and was named *ram1-2.* This allele shows a disparity to the original phenotype described by Gobbato et al (2012), who found that *ram1-1* rarely colonised the root. When arbuscules were formed they were observed as normal compared to WT plants. Both this study (Figure 3.7) and Bano (2011), and later by Gobbato et al (2013) show that the arbuscules in *ram1-2* are underdeveloped. This study was able to identify signs of fungal stress in the form of numerous septations in the hyphae (Figure 3.7), which has not been reported for *ram1* previously.

3.4.3 NF3438 is another allele of *RAM1*

NF3438 has the most severe arbuscule phenotype of the four AM mutant lines identified in this screen. The majority of plants had no arbuscules in what is a potent mycorrhizal inoculum (Figures 3.5 and 3.6). They are able to form arbuscule trunks, but cannot develop arbuscule branches (Figure 3.7i). The gene that is responsible for this strong phenotype is obviously crucial to successful AM colonisation. Prior to taking this

further, the Schultz group declared that they are studying this line for AM colonisation. For this reason I have decided not to take it further. It has since been identified as a new *ram1* allele (Schultz, personal communication).

3.4.4 NF0905 is the H^+-ATPase *MtHA1*

The *ram1-2* mutants and NF0905 displayed very similar phenotypes: extremely low colonisation at both time points, undeveloped arbuscules and septate hyphae (Figure 3.7g and 3.7h). This line had recently been identified by another group as the H^+-ATPase transporter *MtHA1*. During this screen, I became aware that another research group had independently identified this line. I collaborated with them on the **paper 'A H^+ATPase that energizes nutrient uptake during mycorrhizal symbioses in rice and *Medicago truncatula*'** (Wang et al, 2014) (Appendix). Although this mutant had been identified as having a mycorrhizal phenotype, my images were able to further demonstrate that the AM fungus exhibits abnormal septation during attempted colonisation of the *mtha1* mutant; an indication of fungal stress. The work done by the Wang lab did not yield any images of the *M. truncatula* mutant, which I was able to provide.

3.5 Discussion

3.5.1 Efficacy of the AM inoculum for a forward screen

Initially, I sought to create a lab-made inoculum that only contained the AM species *R. irregularis*, as an alternative to the commercial inoculum that was currently being employed. I manufactured this by using chive plants to grow the AM and was able to accomplish this without contamination of rhizobia. The inoculum proved to be very potent in *M. truncatula;* reaching 80% colonisation levels by 6wpi, compared to 8wpi with the commercial inoculum. This was probably due to the way that it was harvested, by cutting chives at the base of the stems and using the soil substrate. This, essentially, kept many of the colonised roots alive and generated nurse chive plants during some of the experiments. This is something to bear in mind when using the inoculum and, perhaps, could be mitigated by cutting the chive plants at the top of the roots and, therefore, removing all the stem tissue. This would be more time consuming, but potentially worth doing to prevent the possibility that the presence of nurse plants may

rescue an AM phenotype in a mutant, as has been shown in a tomato (*Lycopersicum esculentum*) AM mutant (David-Schwartz et al, 2001). It has been demonstrated that plants can relay both allelopathic signals (Barto et al, 2011) and defence signals (Song et al, 2010; Babikova et al, 2013) from plant to plant via mycelial networks. It is possible that a plant defective in a mobile nutrient/signalling molecule could have this missing element supplied by a nurse plant via connected AM mycelia. However, keeping a part of the chive stem intact during storage, potentially preserves the potency of the inoculum over a period of time. When in chilled storage over many months, nurse plants still grow and, presumably, still retain the AM-plant symbiosis. The positive attributes of my chive inoculum is that it is cheap to make, easy to bulk in large amounts, reliable, potent, and that I have control over its content (AM fungal species and nutrients). However, the potency of the inoculum and its tendency to generate nurse plants does not make it the best choice for every AM experiment. Its use in the forward screen may have been a barrier to isolating some AM mutants that have more subtle phenotypes. I did remove any chive 'nurse' plants that started to grow in the soil during the screen, but it may still have had an effect. On the other hand, a strong inoculum is a benefit as I could rely on the inoculum to work; an essential attribute in a large scale experiment. Both the commercial inoculum and isolated spores that are used in the lab have been known to result in lower or absent colonisation.

3.5.2 Forward screen-identified AM mutant lines

It may not be a coincidence that the four short listed AM mutants in this screen had very similar phenotypes (very little colonisation and abnormal arbuscules). Whilst the experiment was validated by its ability to isolate both known (*vapyrin; ram1*) mutants as well as novel ones (*mtha1*; NF3438) they were mutants with a pronounced phenotype. I chose two time points (2 wpi and 4 wpi) in order to detect genes required for early penetration events, as well as root colonisation. However, I could have detected all of these four at 4 wpi, suggesting that my attempts at isolating mutants that have early, more subtle phenotypes may not have been achieved. One way to achieve this would be to use less inoculum and an earlier time point as this could target early penetration events and, possibly, avoid a high rate of false positives. However, initial infections occur at variable time points, many more plants per line would need to be grown in the initial screening in order to isolate a potential phenotype. An issue with using earlier time points is that the plants are very small and you may not have enough

roots to quantify AM structures in the root. However, a qualitative scoring scheme could be used. Some scientists in our lab have grown *M. truncatula* on agar plates for a week prior to planting with the AM inoculum, in order for the roots to grow first; this would be very labour intensive in a forward screen. Another way around this would be to inoculate plants already *in situ* with AM spores. This would eliminate the need to repot the plants. The potential challenges of using spores in this experiment have already been discussed. However, in order to isolate early penetration mutants, this type of strategy may need to be employed.

Another factor to consider is the potential differences in phenotype using alternative AM-species/plant host species combinations. The tomato mutant *rmc* (reduced mycorrhizal colonisation) identified by Barker et al (1998) exhibits alternate colonisation phenotypes depending on the identity of the fungal partner (Gao et al, 2001). It is worth considering, therefore, that some AM mutants could be more easily isolated using a screen with an alternate AM fungal species.

Another way to improve the experimental design of this screen would be to use more plants per line for confirmation (Table 3.3). Although I planted 20 for each time point, some of these *Tnt1* lines had less than 10 plants survive. As each *Tnt1* line can have insertions in up to forty genes, it is, perhaps, unsurprising that some of the candidate lines may have had mutations that influence plant growth and survival.

The *ram1-2* lines (NF0778 and NF0807) displayed a different phenotype to that presented by Gobbato et al (2012) in the *ram1-1* allele. The initial phenotype reported for *ram1-1* was very little formation of mycorrhiza. The few arbuscules that did form appeared like those in WT plants. The phenotype of *ram1-1* displays a lack of early penetration events, similar to the phenotype of *ram2*, which is defined as a glycerol-3-phosphate acyl transferase (GPAT) that is required for hyphopodia formation (Wang et al, 2012), and is regulated by *RAM1* (Gobbato et al, 2012). The *ram1-2* allele, however, did have a small amount of colonisation. Gobbato et al (2013) tested the *ram1-2* phenotype using nurse plants to ensure that enough arbuscules were present to examine them thoroughly. They showed, as did this study, that the *ram1-2* allele has undeveloped arbuscules (Figures 3.7c, d and f). In another study an additional *ram1* allele was characterised through a reverse screen. This mutant (*ram1-3*) has an even stronger arbuscule phenotype, where only arbuscule trunks are formed without any

smaller arbuscule branches (Park et al, 2015). The latter study did not detect any evidence to suggest that *ram1-3* has penetration difficulties and, as a result, proposed that the low colonisation in *ram1* is due to failed symbiosis. However, Park et al (2015) used a different AM species to the other studies (*Glomus versiforme* instead of *R. irregularis*) and this could be the reason behind the observed differences or apparent discrepancy. Gobbato et al (2013) showed that *RAM1* expression is detected during early AM events, even before the hyphae touches the host root surface when associated with *R.irregularis*. This indicates that *RAM1* is involved in early AM signalling events, and could indicate a role in both penetration and arbuscule development.

MtHA1 (NF0905) is a plasma membrane H+-ATPase that is proposed to facilitate nutrient uptake from the AM fungus in the host (Wang et al, 2014). Its phenotype is very similar to *ram1-2* (NF0778 and NF0807). The arbuscules have impaired development that cannot be rescued by nurse plants (Wang et al, 2014). Another allele. *Mtha1-2,* also a *Tnt1* mutant line, produces stunted arbuscules (Krajinski et al, 2014). *Mtha1-1* has a similar phenotype to mutants of the phosphate transporter mutant *mtpt4*, and both proteins are located on the peri-arbuscular membrane. It is known that plasma membrane H^+ATPases facilitate nutrient uptake (Reviewed in Sondergaard et al, 2004). The fact that *MtPT4* expression is eliminated in *ram1-3* (Park et al, 2015) could indicate a direct role for *MtHA1* in phosphate transport via *MtPT4*. Alternatively, this could be a consequence of the *mtha1* mutants being devoid of normal arbuscules, therefore, unable to express *mtpt4* (Harrison et al, 2002).

The efficacy of this forward screen was demonstrated through identification of three mutants required for effective AM symbiosis. Two are mutants of *ram1,* and one is the H^+ATPase *MtHA1.*

3.5.3 A novel strigolactone biosynthesis mutant in medicago has a reduction in AM colonisation

In this chapter, I have characterised the AM phenotype of a *M. truncatula* strigolactone biosynthetic mutant. No stable mutants in *M. truncatula* of this type have been reported to date. It is named after the *A. thaliana* orthologue *max4* (*more axillary growth 4*) and is orthologous to *dad1* (*decreased apical dominance 1*) in petunia, *rms1 (ramosus 1*) in pea and *dwarf 10* in rice. These have a mutation in CCD8, a carotenoid cleavage dioxygenase. CCD7, CCD8 and the carotenoid isomerase D27 are plastid localised enzymes that are involved in the biosynthesis of carlactone, a strigolactone precursor (Alder, 2012).

The functional role of strigolactones in AM symbiosis was initially shown in the *ccd8* mutants in pea and tomato. Both the pea and tomato *ccd8* mutants show a significant reduction in AM colonisation when inoculated with *R. irregularis* spores compared to WT controls (Gomez-Rolden et al, 2008; Koltai et al, 2010). In tomato, *ccd8* colonisation of *R. irregularis* is almost completely absent when inoculating with fungal spores. This phenotype is rescued slightly when using 'whole inoculum' (composed of spores and colonised roots). These were observed at later colonisation time points and can be compared to my *mtmax4* experiment at 4 and 6 weeks post inoculation where I saw a reduction of 40% AM colonisation with *R. irregularis* using my chive 'whole inoculum'. My results in *M. truncatula* suggest a weaker phenotype to both pea and tomato. This could be due to differences in plant species or, alternatively, with the potency of inoculums used.

I report the first early AM time point with a strigolactone mutant in this study. At 2 wpi, roots of the *mtmax4* mutant had severe (80%) reduction in colonisation. This reduction decreased to 40% at the later time points of 4 wpi and 6 wpi (Figure 3.3). This suggests that the *max4/ccd8* phenotype could be more pronounced generally at earlier time points. This is consistent with a role for strigolactones as early signalling components of the AM symbiosis.

The objective of an AM forward screen is the assumption that the discovery of novel AM-specific genes has not been exhausted. This is based on the fact that, although the physiology of rhizobial and AM infection bear similarities, they also exhibit striking differences. Forward screens for AM interactions are labour intensive, requiring staining

and microscopy. It is possible that the majority of plant genes required for AM interactions display subtle physiological phenotypes which would require more sensitive experimental protocols to detect. Perhaps the way forward would be to use a molecular based approach. Expression of a known gene required for a particular part of the symbiosis could be one way. For example, plants with reduced expression of *PT4,* the AM-specific phosphate transporter located in the peri-arbuscular membrane, would detect an aberration in either arbuscule form or function. This approach could be effective, but would also be very time consuming and costly as it requires quantitative PCR to screen the mutants. However, it would remove the subjectivity that is inherent in a conventional phenotypic screen. Another approach could be to manufacture a stable *M. truncatula* line that produces a visual pigment phenotype similar to the changes in root colour that occurs in maize roots during AM colonisation (Paszkowski et al, 2006). This stable line could then be used as the background genotype for the generation of a mutant population. For example, if the *PT4* promoter was engineered to drive the expression of a gene/genes that produces a pigment, like those that produce anthocyanins, the absence or reduction of colour could be used for the initial screen. After that, both a qualitative and/or quantitative approach to isolating AM mutants could be employed.

Chapter 4

Reverse screen of AM genes

4.1 Introduction

4.1.1 The root hair 'infectome'

In the Murray lab, much of our research is focused on the early infection events of the rhizobial and AM symbioses. As described in the introduction, both symbioses have similar mechanisms for the recognition of beneficial microbes and the cellular accommodation strategies employed by the host plant. Both pre-infection thread and pre-penetration apparatus formation are the result of extensive cellular restructuring to allow accommodation of the symbiont and, therefore, potentially utilise many of the same genes. To determine genes specifically required for early rhizobial infection and infection thread formation, Breakspear et al (2014) conducted a microarray-based transcriptome analysis of *M. truncatula* root hairs inoculated with the rhizobia *S. meliloti*, dubbed the 'infectome'. The aim was to uncouple the early infection events in root hairs from the cortical responses and subsequent nodule organogenesis that occurs in the rest of the root. It is possible that genes specific to early infection events during rhizobial colonisation would not be identified in whole roots or root segments as only a few root hair cells become infected per plant; any changes in gene expression specifically related to root hairs would be 'drowned out' by background gene expression changes in the whole root. The use of root hair isolation provides increased specificity and sensitivity to detect infection-related genes. Alongside this was an accompanying study using the same experimental protocol in the *nin* mutant (Guan, 2012; PhD thesis).

NIN is a central regulator of nodulation and is required for both early epidermal infection and nodule organogenesis. It also has a role in the autoregulation of nodulation by inhibiting infection to limit nodule number (Marsh et al, 2007; Yoro et al, 2014; Vernie et al, 2015). The root hair infectome of *nin* yielded an interesting result; several genes thought to be specific for the AM symbiosis were found to be up regulated. This suggests the possibility that *NIN* has a role in repression of AM infection processes.

4.1.2 The *Medicago truncatula* Gene Expression Atlas (MtGEA)

An indispensable resource for reverse genetics in medicago is the MtGEA database created and supported by the Samuel Roberts Noble Foundation. It is a compilation of results from a wide array of expression studies using the Affymetrix Medicago Gene Chip® from a variety of biological and chemical conditions (Benedito et al, 2008). To date, the atlas contains extensive expression data related to both nodulation and mycorrhization. This includes 'whole root' expression data at various time points of rhizobial colonisation (Carvalho et al, unpublished), the root hair infectome data (Breakspear et al, 2014), as well as laser capture microdissection of different cell types, during the AM symbiosis (Gomez et al, 2009; Gaude et al, 2012). The Oldroyd and Murray labs share a private MtGEA database which details experiments which have been undertaken in the lab but that have not yet been published. The *nin* infectome is one such experiment.

4.1.3 Reverse screening as a tool for finding novel genes

Forward screens are a powerful tool for finding genes required for particular biological processes. The screen results from Chapter 3 highlight that forward genetics is resource-intensive and tends to identify mutants with very strong phenotypes. This increases the chances of finding mutants that have already been characterised. Despite the fact that many AM screens have been carried out, relatively few novel AM genes have been found, which suggests that many interesting AM mutants may have more subtle phenotypes.

Another method of finding novel genes is the reverse genetics approach. In this type of screen, candidate genes are often identified based on their expression values in particular tissues and under certain identified biological conditions. Alternatively, phylogenetic analyses comparing gene complements across host species versus non-hosts can help identify candidates (Delaux et al, 2015; Bravo et al, 2016). An identified candidate gene can then be studied for its role in the function of interest by using an existing mutant collection or using gene knockdowns or knockouts. The two main reverse genetic resources for *M. truncatula* are the *Tnt1* population described in Chapter 3 and the TILLING resource (Le Signor et al, 2009).

For both the *M. truncatula Tnt1* population (Samuel Roberts Noble Foundation, Ardmore, USA), and the TILLING lines (John Innes Centre, RevGen UK) there are two ways to find mutants for a particular gene. A BLAST search of a gene of interest may yield a previously identified allele (http://medicago-mutant.noble.org/mutant/blast/blast.php). Alternatively, a PCR-based reverse screening service is available to screen representative DNA pools. TILLING (Targeted Induced Local Lesions IN Genomes) was developed by Colbert et al (2001). Mutations are induced by ethyl methane sulphonate (EMS) and are screened by high-throughput PCR. EMS mutations result in base pair changes, rather than complete loss of function in *Tnt1* mutations. This can result in weaker alleles which may alter the activity of the transcribed protein to produce a series of subtle phenotypes. For the reverse screen approach, *Tnt1* lines, which often provide null alleles are less expensive, are a good first step to determine if a candidate gene is important for a particular biological function. If loss of function in a candidate results in plant lethality, TILLING, which can provides mutants with differing strengths, is an attractive alternative.

The purpose of this chapter was to search for novel AM-specific genes that show an increased expression in *nin* root hairs after inoculation with *S. meliloti* and determine whether they are AM-specific or common symbiosis genes.

4.2 Results

4.2.1 Mining for AM genes negatively regulated by *NIN* during early rhizobial infection

To further investigate this phenomenon seen in *nin* root hairs during early rhizobial infection I used the following expression criteria to identify 'AM-specific' candidate genes:

1) Up regulated (≥2fold) in whole roots during AM symbiosis (Gomez et al, 2009).
2) Up regulated (≥2fold) in the *nin* infectome experiment relative to WT (Guan, 2012; PhD thesis).
3) Not up regulated in nodulated whole roots (Carvalho et al, unpublished).

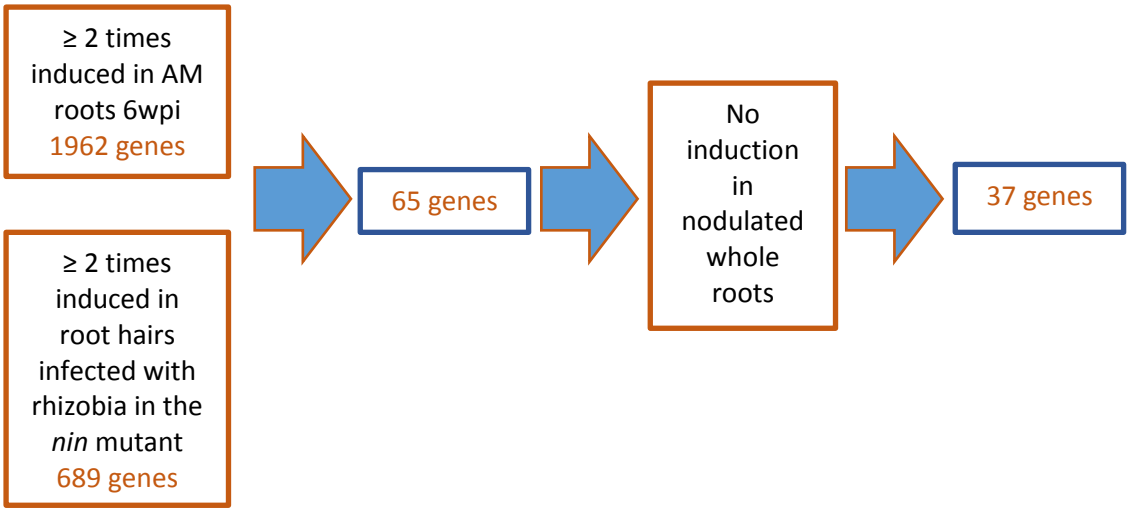


Figure 4.1 **Mining for mycorrhizal genes for a forward screen**

Thirty seven genes induced in whole roots during AM infection, but not during nodulation were discovered to also respond to rhizobia at 5pi in root hairs in the *nin* mutant. These were found by finding common genes induced in AM (Gomez et al, 2009) and the *nin* root hair infectome (Guan, D. thesis, 2014) . This produced 65 genes. Each of these were screened for induction during all of the nodulation experiments displayed in MtGEA (http://mtgea.noble.org/v3/). Only those genes showing no induction during nodulation were considered for the reverse screen.

I identified 1962 genes up regulated during mycorrhization and 689 that are induced in *nin* root hairs. Of these, there are 65 genes that are common to both sets of data. From this list of 65 genes I wanted to only look for those genes which are predominantly expressed during infection but not during later stages of nodule development. Due to the number of nodulation experiments represented, each of the 65 genes were analysed individually, by carefully looking at the relative expression of all experiments. This produced a final 37 genes (Table 4.1), of which 7 were chosen for further study:

1. A cytochrome P450. These genes are important for secondary metabolite biosynthesis, including signals for plant symbionts (Werk-Reichhart, 2003).
2. A lectin receptor kinase. Lectins are thought to mediate specificity in the legume-rhizobial symbiosis, and may have a similar role during the AM symbiosis (Hirsch et al, 1999).
3. An aquaporin. Aquaporins transport water and other solutes such as ammonia. Some have already been implicated in both rhizobial and AM symbioses (Uehlein et al, 2007).

4. Three CCAAT-box transcription factors (NF-Ys). Several are implicated in the nodule-legume symbiosis, including *NF-YA1* in *M. truncatula,* which is required for normal infection and nodule formation (Laporet et al, 2013).
5. A gene encoding a protein of unknown function that has predicted domains suggesting lipid modification (see Chapter 5).

Two of the CCAAT-box transcription factors have been previously known to be expressed in mycorrhizal roots (Hogekamp et al, 2011), named as *CBF1* (Medtr2g081600/*NF-YC6*) and *CBF2* (Medtr2g081630/*NF-YC11*). *CBF1* and *CBF2* are NF-YC subunits that are highly homologous (96% amino acid identity) and are tightly linked on chromosome 2. No functional studies have yet been reported on these genes. The third CCAAT-box transcription factor, that I have named *CBF3* (Medtr8g091720/*NF-YB7*), is a B subunit. Both *CBF1* and *CBF3* are also up regulated in WT root hairs after inoculation with rhizobia, compared to controls (Figure 4.2 and 4.3). The similarity in gene expression raises the possibility that these CBFs/NF-Ys could potentially act in the same complex together with an unidentified A subunit.

The cytochrome P450 (*CYP450*) (Medtr3g058000) also presents itself as an AM-specific gene on the public gene atlas.

The lectin receptor kinase (*LecRK*) (Medtr8g068050) shows a seven-fold increase in gene expression in WT root hairs with the addition of *S.meliloti*. This up-regulation is increased nearly six-fold again over WT levels in the *nin* mutant background. The gene of unknown function (Medtr2g098490), which I have named *BiFunctional Protein* (*BFP*) because it has two distinct domains, also shows AM-specific gene expression in roots. *BFP* only exhibits background level expression in WT root hairs this, however, it is 21-fold increased in expression in *nin* relative to WT. The features of *BFP* will be described in further detail in Chapter 5.The aquaporin (Medtr5g063930) belongs to the NIP-1 (Nodulin 26-like Intrinsic Protein) class of aquaporins (Wallace et al, 2006). The gene has a three-fold induction in rhizobia-inoculated root hairs in WT roots. I have named the gene *Symbiotic Aquaporin* (*SYMAQ*) for the purposes of this study, as it is expressed during rhizobial infection and during AM symbiosis. In *nin* root hairs, *SYMAQ* has an eight-fold induction in expression over WT in response to rhizobia.

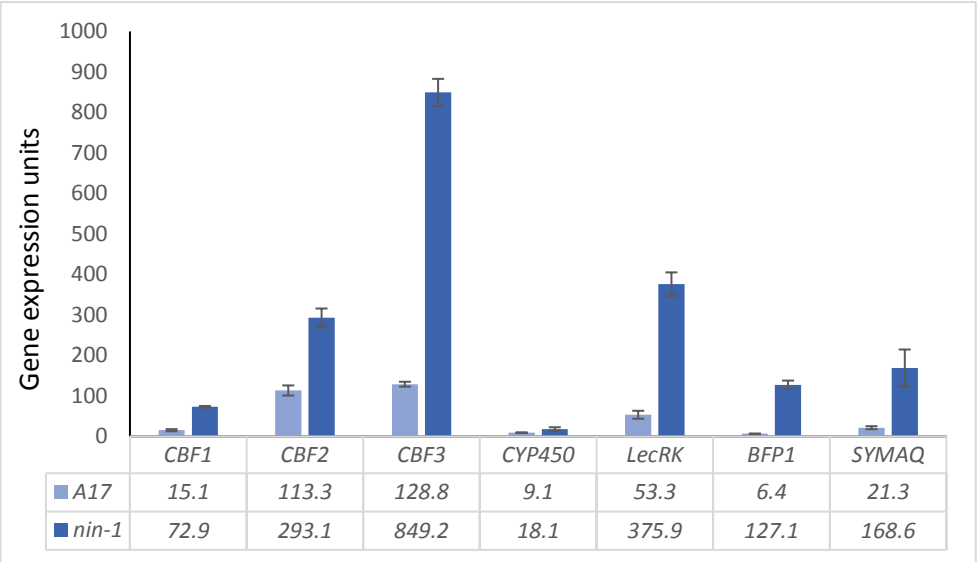
Gene annotation	Probe set no.	Gene description
Medtr8g091720.1	Mtr.4282.1.S1_at	NF-YB7 (*CBF3*)
Medtr2g081600.1	Mtr.51511.1.S1_at	NF-YC6 (*CBF1*)
Medtr2g081630.1	Mtr.16863.1.S1_at	NF-YC11 (*CBF2*)
Medtr3g086430.1	Mtr.1103.1.S1_at	ABC transporter
Medtr8g022270.1	Mtr.46524.1.S1_at	ABC transporter
Medtr1g011640.1	Mtr.32084.1.S1_at	drug resistance transporter-like ABC domain protein
Medtr8g091690.1	Mtr.12170.1.S1_at	cytochrome P450
Medtr3g057980.1	Mtr.23217.1.S1_at	cytochrome P450
Medtr3g058000.1	Mtr.47494.1.S1_at	cytochrome P450
Medtr2g030855.1	Mtr.4785.1.S1_s_at	rhicadhesin receptor
Medtr2g031270.1	Mtr.7599.1.S1_at	rhicadhesin receptor
Medtr7g086100.1	Mtr.51106.1.S1_at	blue copper protein
Medtr7g086090.1	Mtr.14511.1.S1_at	blue copper protein
Medtr8g014790.1	Mtr.20259.1.S1_at	LRR receptor-like kinase
Medtr8g087420.1	Mtr.46484.1.S1_at	LRR receptor-like kinase
Medtr8g067660.1	Mtr.45648.1.S1_at	Lectin
Medtr8g068050.1	Mtr.7279.1.S1_s_at	lectin receptor kinase
Medtr5g063930.1	Mtr.7596.1.S1_at	Aquaporin
Medtr3g408340.1	Mtr.43715.1.S1_at	derlin-2 protein
Medtr7g086160.1	Mtr.15627.1.S1_at	plastocyanin-like domain protein
Medtr4g005270.1	Mtr.31948.1.S1_at	beta-amyrin synthase
Medtr4g053630.1	Mtr.35524.1.S1_at	subtilisin-like serine protease
Medtr4g102400.1	Mtr.13963.1.S1_at	subtilisin-like serine protease
Medtr2g098490.1	Mtr.35719.1.S1_at	bifunctional protein
Medtr4g131180.1	Mtr.7993.1.S1_s_at	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase
Medtr5g005950.1	Mtr.25298.1.S1_at	late embryogenesis abundant protein
Medtr1g075550.1	Mtr.25607.1.S1_s_at	PLAC8 family protein

Medtr7g104360.1	Mtr.927.1.S1_at	purple acid phosphatase superfamily protein
Medtr4g097220.1	Mtr.47198.1.S1_s_at	DUF4228 domain protein
Medtr3g067437.1	Mtr.47546.1.S1_at	albumin I
Medtr3g079600.1	Mtr.10497.1.S1_at	serine carboxypeptidase-like protein
Medtr2g437880.1	Mtr.12632.1.S1_at	Isoflavone 3'-hydroxylase
Medtr7g102840.1	Mtr.35987.1.S1_s_at	heparan-alpha-glucosaminide N-acetyltransferase-like protein
Medtr1g115195.1	Mtr.37204.1.S1_at	glutathione S-transferase
Medtr1g100610.1	Mtr.40286.1.S1_at	unknown protein
Medtr5g018610.1	Mtr.29593.1.S1_at	unknown protein
AC141923_37.4	Mtr.35700.1.S1_at	unknown protein

Table 4.1 **Medicago AM induced genes that are upregulated in *nin* root hairs 5 dpi with *S. meliloti***

Mutant line name	Mutant line no.	Status
cbf1	NF1248	WT
cbf1	NF16919	homozygotes
cyp450	NF12356	homozygotes
cyp450	NF3185	no seeds available
lecrk	NF12182	WT
lecrk	NF4687	no seeds available
bfp	NF5628	WT
bfp-1	NF13226	homozygotes
bfp-2	NF10789	homozygotes
symaq	F9542H	WT
cbf3-1	NF11498	homozygote
cbf3-2	NF1242	homozygote
cbf3-3	NF11571	homozygote

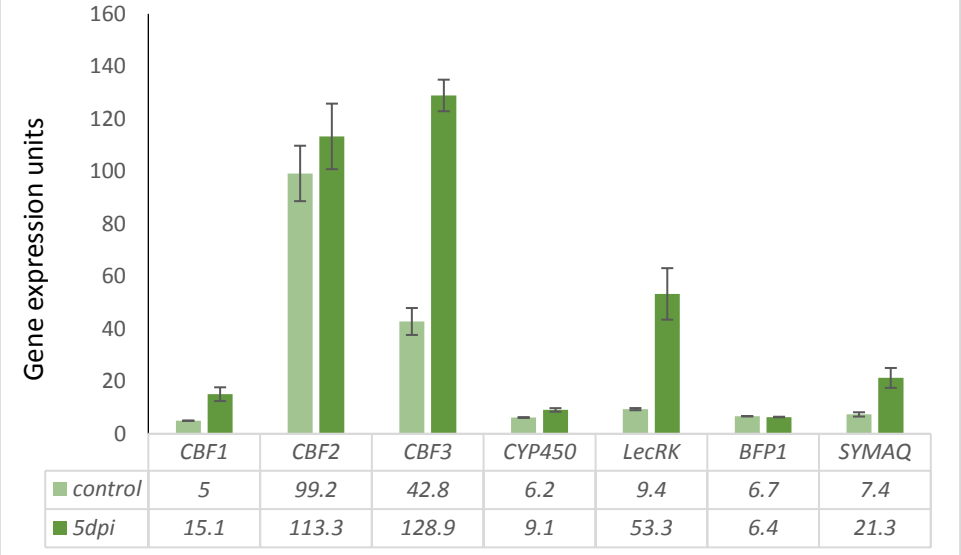
Table 4.2 **Genotypes of *Tnt1* lines**



	CBF1	CBF2	CBF3	CYP450	LecRK	BFP1	SYMAQ
A17	15.1	113.3	128.8	9.1	53.3	6.4	21.3
nin-1	72.9	293.1	849.2	18.1	375.9	127.1	168.6

Figure 4.2 **Root hair expression of candidate genes in the *nin* mutant is increased when inoculated with *S. meliloti***

Microarray root hair expression of AM genes (≥2 fold up regulated in whole roots). WT (A17) and *nin* roots were inoculated with *S. meliloti*. Root hairs were extracted at 5 dpi (Guan thesis, 2012). Expression was ≥2 fold up regulated in the *nin* background. Bars represent the standard error of the mean.



	CBF1	CBF2	CBF3	CYP450	LecRK	BFP1	SYMAQ
control	5	99.2	42.8	6.2	9.4	6.7	7.4
5dpi	15.1	113.3	128.9	9.1	53.3	6.4	21.3

Figure 4.3 **Root hair expression of candidate genes inoculated with *S. meliloti* in WT (A17) plants**

Microarray root hair expression of AM genes (≥2 fold up regulated in whole roots). WT (A17) roots were inoculated with *S. meliloti*. Root hairs were extracted at 5dpi (Breakspear et al, 2012). Bars represent the standard error of the mean.

4.2.2 Isolation of homozygous mutants

Two strategies were used to try to identify *Tnt1* insertion mutants for my genes of interest, BLAST searches of the *Tnt1* flanking sequence tag database, and the PCR screening service offered by the Noble Foundation. *CBF3* had been previously screened by C. Liu: three lines containing insertions in *CBF3* were identified and homozygous mutants were isolated. I was unable to find a mutant line for *CBF2* using either of the strategies. Seeds were requested for eight lines in total, and I was able to isolate homozygotes for four of them. This included *CBF1* and *CYP450* (one allele each), and *BFP* (two alleles) (Figure 4.5). All these mutants have a *Tnt1* transposon insertion in an exonic region (Figure 4.5).

4.2.3 AM phenotypes of isolated mutants

As the candidate genes are all up-regulated during AM symbiosis, the isolated homozygous lines were tested for AM phenotypes. After 6 wpi with *R. irregularis* (chive inoculum), the *bfp* alleles had a 19-29% (n=11-21) reduction in arbuscule colonisation (Figure 4.6). These are small, but significant phenotypes ($p \leq 0.05$).

The strongest AM phenotype found was from *cyp450*. This line showed a 93% reduction in arbuscule colonisation (n=19). This was surprising as this CYP450 only shows a 2-fold up regulation in AM whole roots at 6 wpi, and the expression is relatively low (not shown) (Gomez et al, 2009). In addition, a laser capture microscopy (LCM) study of arbuscule-containing cortical cells in *M. truncatula* indicates that this gene is not up regulated in arbuscules (Gaude et al, 2012). The arbuscules that did form were normal-looking. Neither *cbf1* (n=12) or the three *cbf3* (n=14) alleles showed a reduction in AM colonisation (Figure 4.6). These mutants also had normal looking arbuscules.

4.2.4 Nodulation phenotype of candidate mutants

All my gene candidates apart from *BFP* and CYP450 have ≥two fold higher expression in WT root hairs after *S. meliloti* application (Figure 4.3). In this sense they are not truly 'mycorrhizal specific', but they met the criteria as they were not expressed in nodules, based on the criteria described above. On this basis, these mutants were tested for a

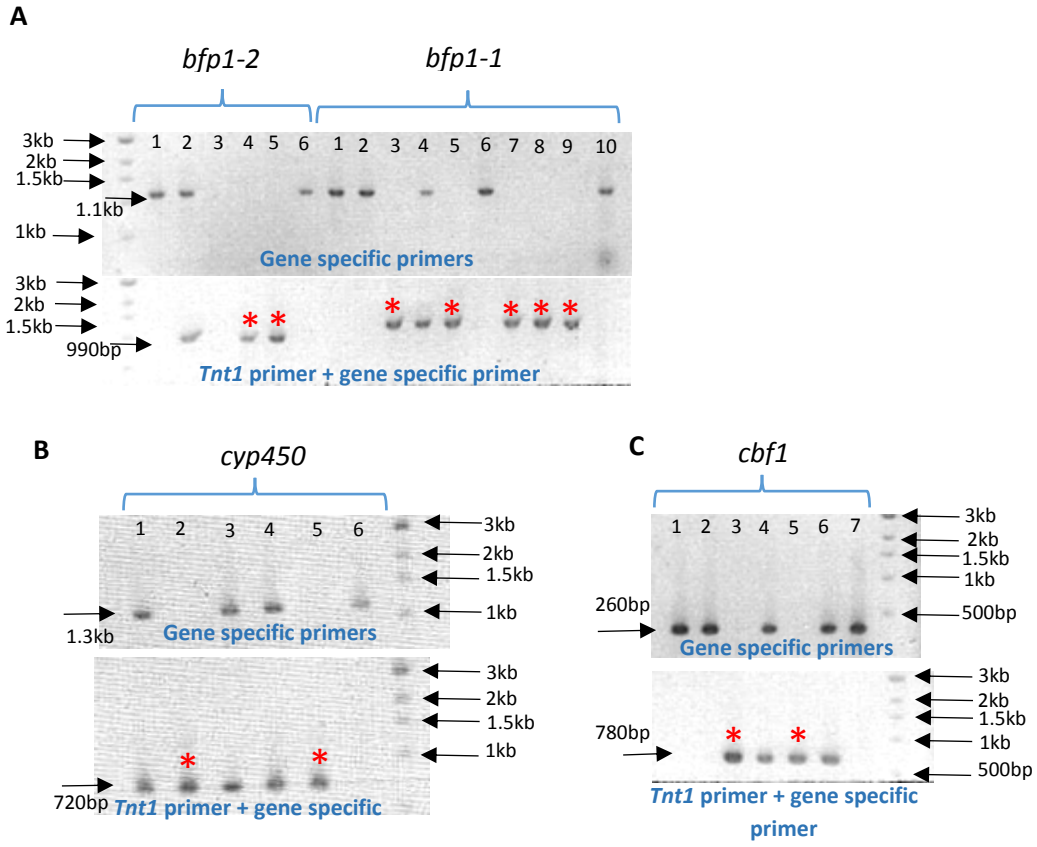


Figure 4.4 **Isolation of homozygous *Tnt1* insertion mutants**

Gels showing PCR amplification products of *bfp1* **[A]**, *cyp450* **[B]** and *cbf1* **[C]** to detect homozygous lines. The upper gels for each mutant line show amplification products for gene-specific primers that flank the *Tnt1* insertion. Lower gels show amplification products for *Tnt1* insertion junctions using a gene-specific primer and a *Tnt1*-specific primer. Red asterisk indicates a line homozygous for *Tnt1* insertion.

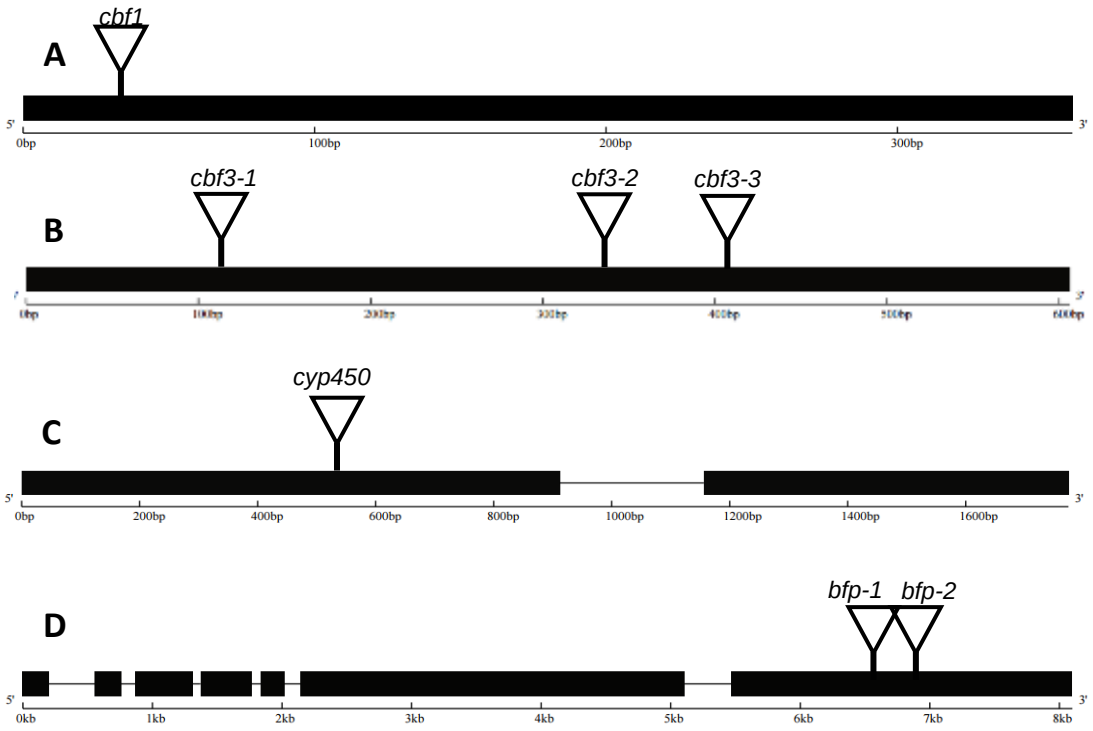


Figure 4.5 **Gene structures and *Tnt1* insertion sites of homozygous lines**

CBF1 **[A]**; *CBF3* **[B]**; CytochromeP450 **[C]**; *BFP* **[D]**.

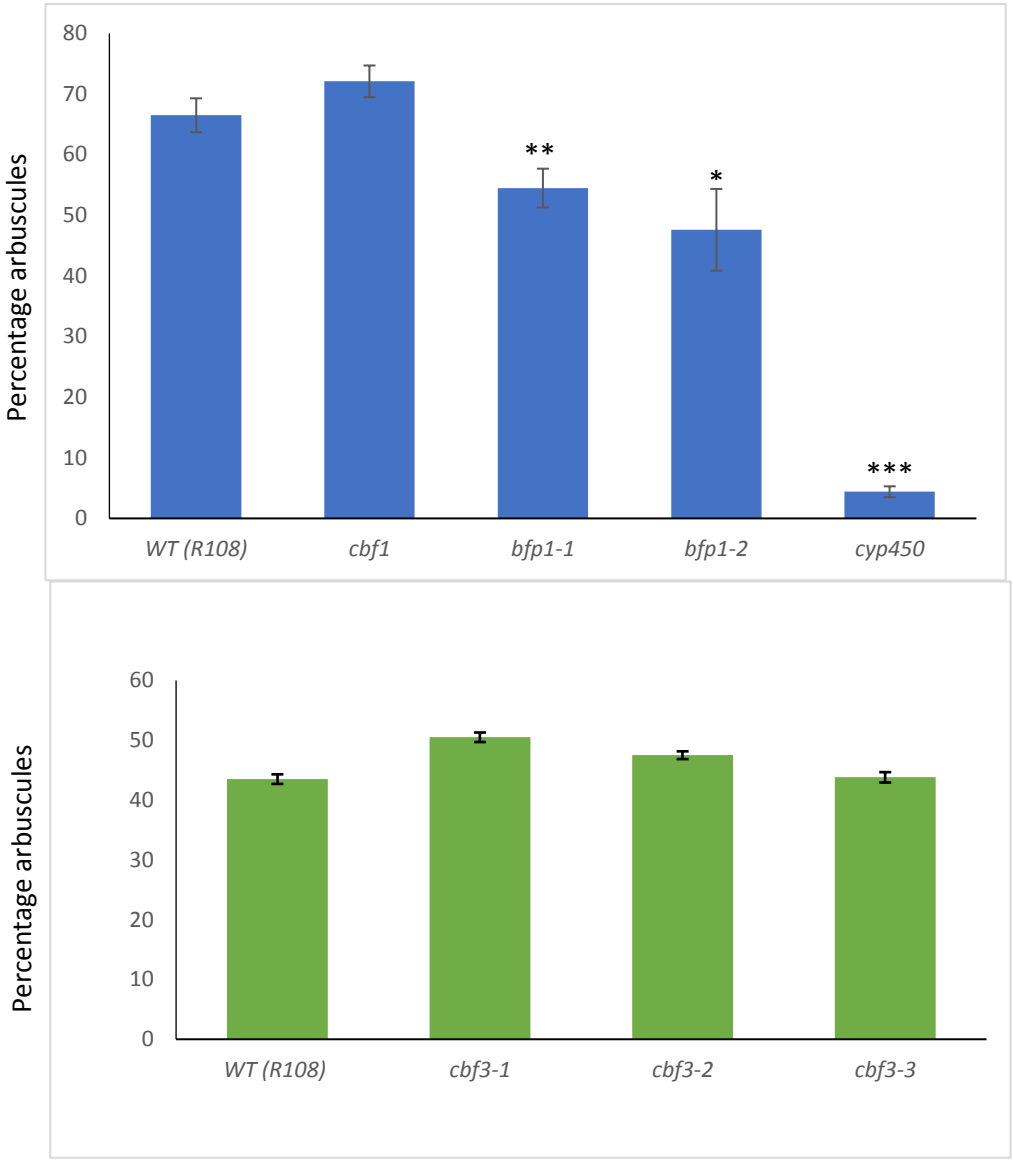


Figure 4.6 **Initial AM phenotyping of candidate mutants**

Plants grown with *R. irregularis* (20% chive inoculum) and screened after 6 wpi (top graph) or 4 wpi (*cbf3* bottom graph). Bars represent standard error of the mean. *=p≤0.05 **=p≤0.01 ***=p≤0.001

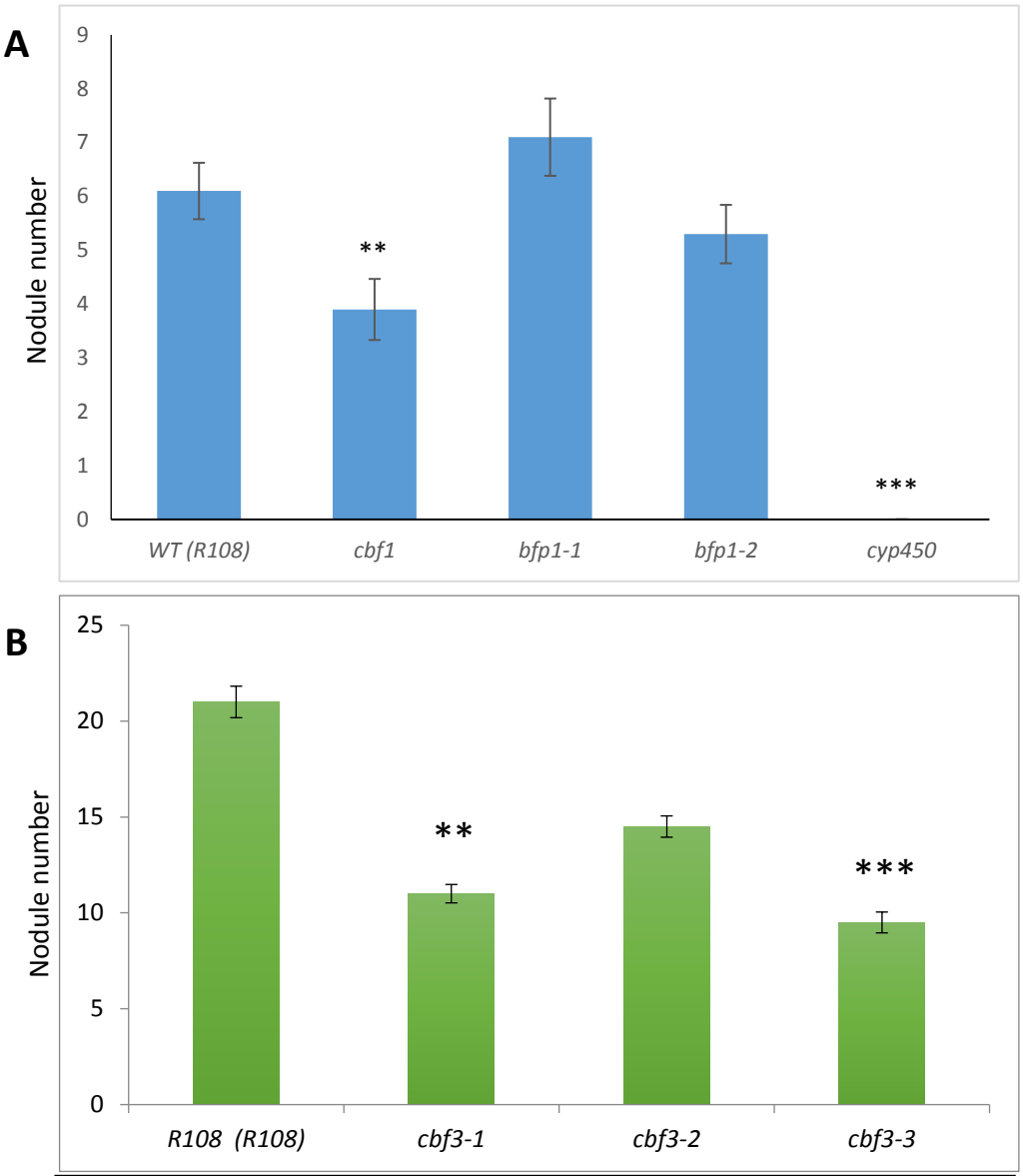


Figure 4.7 **Nodulation phenotype of candidate mutants**

Plants inoculated with *S. meliloti* 1021 and nodules counted at 3 wpi.

**=p≤0.01 ***=p≤0.001. Bars represent the standard error of the mean.

nodulation phenotype three weeks after inoculation with *S. meliloti*. The *bfp* mutants did not show a difference in nodule number (n=≥14) (Figure 4.7). Unexpectedly, there were no nodules present on any of the *cyp450* plants (n=24), despite the fact that it exhibits no expression during nodulation (apart from the increased expression in the *nin* root hair experiment). For *cbf1* there was a 36% reduction in the number of nodules (n=11) (p=0.004). All three *cbf3 alleles* (n= ≥16) showed a reduction in nodule number (31-55%), although the reduction in *cbf3-2* was not significant (p=0.06). All plants from this nodulation phenotypic assay had normal looking nodules.

4.2.5 The cytochrome P450 common symbiotic phenotype is the result of a background mutation

In initial testing, the cytochrome P450 had greatly reduced AM fungal colonisation and was unable to form nodules. This was surprising, as CYP450 is not expressed during nodulation. Also, as many forward screens looking for nodulation mutants have been done, it seemed unlikely that a mutant with such a strong phenotype would not have been previously identified. This raised the possibility that the phenotypes observed in NF12356 were due to a mutation in a gene other than *CYP450*. To study this hypothesis, the progeny of a plant heterozygous for an insertion in *CYP450* were tested for a nodulation phenotype. Every plant failed to develop nodules (n=90), indicating that the parental line carries a homozygous insertion in another nodulation gene. I also searched for known flanking sequence tags in NF12356, but none had been identified.

4.2.6 *nin* mutants have no obvious AM phenotype

I have identified several genes that are associated with AM colonisation, but not with nodulation, but are ectopically up-regulated in early rhizobial infection in the *nin* background. This raises the question to whether *NIN* has some control over the down regulation of mycorrhizal genes during nodulation. To test this an experiment was set up using two *nin alleles; nin-1* and *nin-2*. The *nin-1* allele is the result of EMS mutagenesis in the WT A17 background and has an 11bp deletion starting at position 1850. The *nin*-2 allele is a *Tnt1* transposon insertion line in the R108 WT background with the insertion lying 20bp upstream of the ATG (Marsh et al, 2007). Both *nin* alleles were scored over a

time course of 2, 3, 4 and 5 wpi with *R. irregularis* (10% chive inoculum). Half the plants were inoculated with *S. meliloti*. Ten plants were grown for each genotype/time

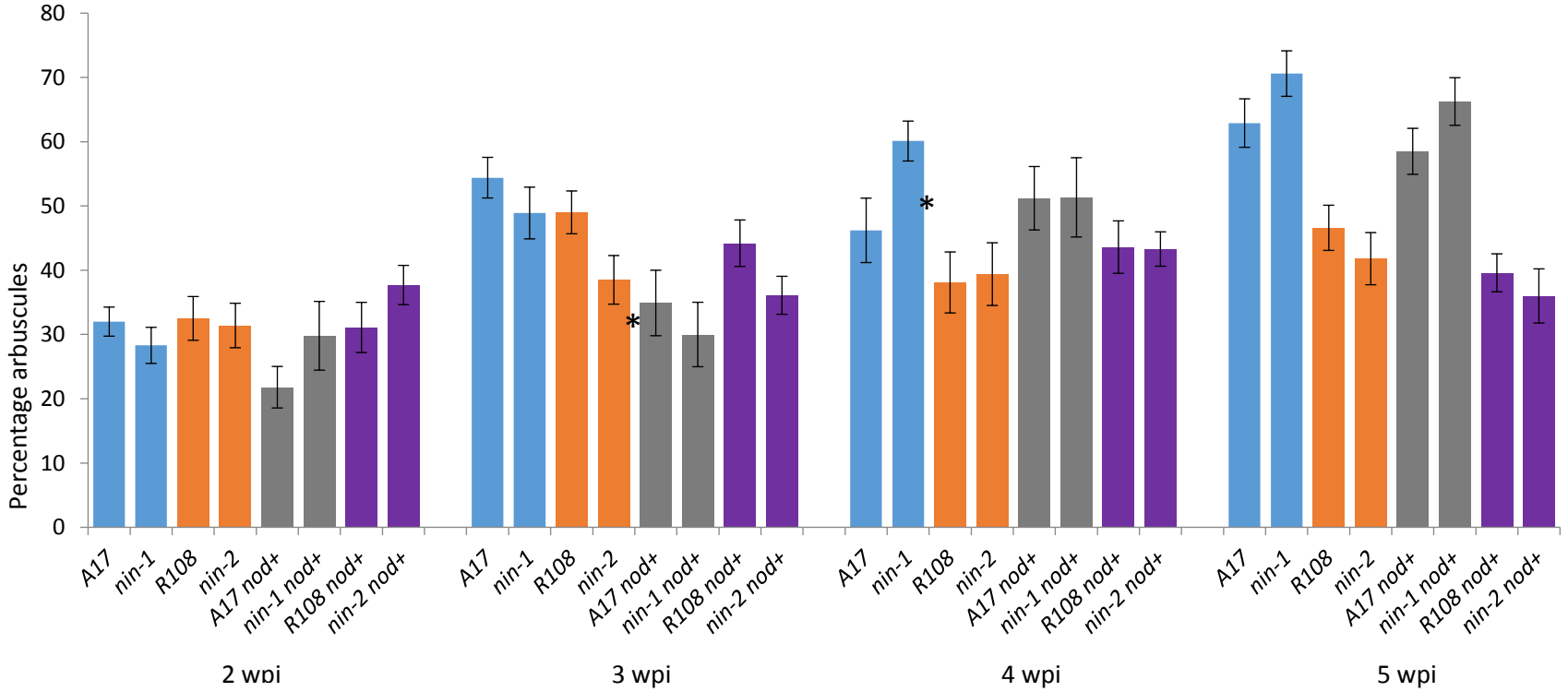


Figure 4.8 **AM phenotype of *nin* mutants with and without addition of rhizobia**

M. truncatula plants were grown with *R. irregularis* (10% chive inoculum) over a time course. Half the plants were also inoculated with *S. meliloti* 1021 .The AM phenotypes were scored at 2, 3, 4 and 5 wpi. A17 is the WT background for *nin-1,* and *nin-2* is in the R108 background. Bars represent the standard error of the mean. *=p≤0.05: nod+=nodulated

point/experimental condition by C. Liu who inoculated them and harvested the root samples. These were then stained and phenotyped by myself (Figure 4.8). Although some reductions in AM colonization were observed in certain treatments, overall the results showed no difference in AM colonisation between WT and *nin* mutants in either of the experimental conditions.

4.3 Discussion

A list of 65 genes were identified that are up regulated both during AM symbiosis and also in infected *nin* root hairs. From these genes 37 are not expressed in mature nodules, a phenomenon first identified by D. Guan (2014; PhD thesis). I selected seven of these genes for further study and was successful at isolating mutants for five of them. No mutants were identified for *SYMAQ* and *LecRK,* and the *cyp450* allele appeared to contain a mutation in a common symbiosis gene. To identify this gene would require back crossing for gene identification. For this reason, I did not pursue these genes.

4.3.1 The *CBFs* may be common symbiotic genes

Although not induced in mature nodules, a root hair transcriptome study revealed that four of these genes were also induced by rhizobia, suggesting a possible role in both rhizobial and AM infection. Mutants were isolated mutants for two of these genes, *CBF1* (*NF-YC6*) and *CBF3* (*NF-YB7*). *Tnt1*-insertion mutants for both of these genes showed a significant reduction in nodule number 3 wpi with *S. meliloti* (Figure 4.7). The up regulation in infected root hairs and nodule phenotype suggests that these are common symbiotic genes.

As NF-Ys work in a heterotrimeric complex consisting of an A, B and C subunit, it is possible that CBF1 and CBF3 work in the same complex during rhizobial infection, and that all three subunits work together in AM infection. However, mutations of *CBF1* and *CBF3* did not result in a mycorrhizal phenotype. For *CBF1* this can be accounted for by its paralogue, *CBF2,* which may functionally compensate *CBF1*. This is supported by the promoter:GUS expression analysis of both of these NF-YCs, where expression overlaps in roots during AM-colonisation (Hogekamp et al, 2011). *CBF2* does not appear to be up regulated in root hairs during rhizobial infection, which makes it seem unlikely that it

participates in nodulation (Figure 4.7). However, although *CBF2* expression is not significantly enhanced in root hairs, its baseline expression is much higher than *CBF1.* Therefore, I cannot discount *CBF2* as having as role in both symbioses. These three genes will be more closely examined in Chapter 6.

4.3.2 *BFP* is an AM-specific gene

Despite its enhanced expression during rhizobial infection in the *nin* background, *BFP* is not expressed in WT root hairs of rhizobially infected seedlings or in nodules, and otherwise appears specific to mycorrhizal colonisation. *BFP* will be studied in more detail in Chapter 5.

4.3.3 *NIN* negatively regulates some AM genes during rhizobial infection

I have identified genes involved in the AM symbiosis that are increased in their expression relative to WT in *nin* root hairs, after *S. meliloti* inoculation. We have yet to discover how a legume decides between initiating either a nodulation or AM developmental programme. It is tempting to speculate that *NIN*, as a master controller of nodulation genes, could also be responsible for down regulating the default mycorrhizal programme. However, there was no difference in AM colonisation in the *nin-1* and *nin-2* mutants either with or without the presence of rhizobia, at least in terms of arbuscule formation (Figure 4.8). However, if this action is only on individual cells that are infected, the down regulation of AM genes may not have an impact on overall AM colonization of the root.

As well as being essential for both the early epidermal infection of rhizobia and nodule organogenesis, *NIN* is also implicated with a negative regulatory role in infection to restrict nodule numbers. *NIN's* role in the autoregulation of nodulation seems to restrict the amount of root hairs that are responsive to Nod factors. This can be seen in the *nin-1* and *nin-2* documented phenotype of excessive numbers of curling root hairs compared to WT roots (Marsh et al, 2007). This is further shown by the expression of *ENOD11*, an early symbiosis marker gene, whose expression is normally restricted to infected root hairs, but has an increased expression zone in the *nin* mutant (Marsh et al, 2007). *NIN* has been shown to bind to the *ENOD11* promoter and negatively regulate its transcription (Vernie et al, 2015).

The most likely explanation for the up regulation of my candidate genes in *nin*, some of which are up regulated in infected root hairs and some not, is that this is a consequence of *NIN's* role in the autoregulation of nodulation. If *NIN* is responsible for limiting infections, it is likely to down regulate common symbiosis genes which could account for the higher expression of AM genes we see in *nin* (Table 4.1).

Chapter 5

BFP - A Novel, Conserved AM-Specific Gene

5.1 Introduction

5.1.1 *BFP* is an AM-specific gene with unknown function

In Chapter 4, I isolated homozygous mutants for a gene of unknown function that I named *BiFunctional Protein (BFP)*. Transcriptome data in the MtGEA, suggest that *BFP* expression is only induced in mycorrhizal roots. However, *BFP* is one of several *M. truncatula* mycorrhizal genes that exhibited increased expression in *nin* root hairs 5 days after inoculation with the rhizobia *S. meliloti.* The two *BFP Tnt1* insertion alleles had a small but significant reduction in arbuscule colonisation at 6 wpi with the AM fungus *R. irregularis* (Chapter 4; Figure 4.6).

Along with *M. truncatula*, rice (*Oryza sativa*) has emerged as an important model system for the study of mycorrhizal interactions. Rice is a useful model for AM symbiosis; it is a monocot like most crop species and it does not nodulate like the legume models *M. truncatula* and *L. japonicus*. Therefore, there is no concern of rhizobial contamination, which could lead to genetic cross-talk through the common symbiotic pathway. Like the tobacco *Tnt1* retrotransposon that is used to create stable mutagenesis lines in *M. truncatula*, the rice *Tos 17* retrotransposon has been used to create a rice mutagenesis library. *Tos 17* is 4114bp in length and has a relatively low (1-5) copy number in the rice genome. Nipponbare is the rice cultivar chosen for The International Rice Genome Sequencing Project (IRGSP) (Sasaki et al, 2000). It carries two copies of *Tos 17* per haploid genome which is activated during tissue culture but is inactive in regenerated plants (Hirochika, 2001). As with the *Tnt1* insertions, *Tos 17* prefers to integrate into genes. There are over 47,000 Nipponbare lines with *Tos 17* insertions, with over 42,000 mapped onto rice genomic sequences (Miyao et al, 2003).

The mycorrhizal-specific expression in plant roots of *BFP* suggests that it has a role in AM symbiosis, and makes this gene worthy of further study. In this chapter I investigate how *BFP* is highly conserved in plants that can form AM associations, but is lost in those that cannot. Predicted protein domains suggest a role in lipid modification. Based on bioinformatic analyses, some of these domains appear to be microbial in origin, suggesting an interesting evolutionary history.

5.2 Results

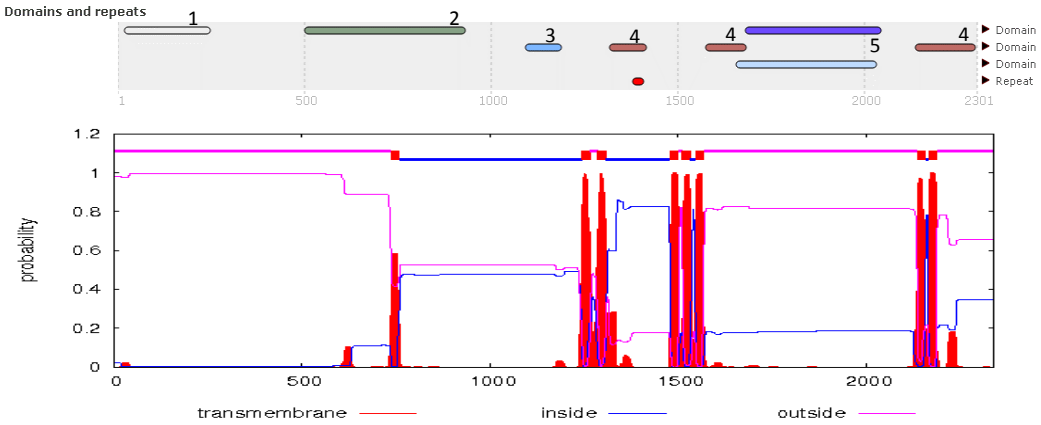
5.2.1 *BFP* encodes a protein with predicted transmembrane proteins domains and a potential role in lipid biosynthesis, and has a legume-specific homologue

M. truncatula BFP (Medtr2g09849) is a large gene (8340 bp) which encodes a protein of 2301 amino acids (99 percentile rank in size in the *M. truncatula* proteome). The amino acid sequence was analysed in protein prediction websites (InterPro, Pfam and NCBI); that all predict two main functional domains. The first is an AMP-binding synthase/ligase domain (IPR000873), and the second is a catalase-like or allene oxide synthase (AOS) domain (IPR020835) (Figure 5.1).

The AMP-binding domain belongs to a family of enzymes that act by ATP-dependent covalent binding of AMP to their substrate. Members include the long chain fatty acid CoA ligases (FACLs). The output of the *BFP* amino acid sequence in the NCBI BLASTP search identified this domain as a fatty acid AMP ligase (FAAL; cd05931), which is homologous to FACLs. For a fatty acid to participate in any metabolic process, it first has to be 'activated', a process that is generally carried out by fatty acyl-CoA synthetases/ligases. Both names are used interchangeably in the literature. Once activated, fatty acids can participate in the synthesis of more complex lipids (Watkins, 1997).

The catalase-like or allene oxide synthase (AOS) (IPR020835) domain may also be involved in lipid biosynthesis. As mentioned earlier, AOS is a CYP74 cytochrome P450 protein that has been well-studied as a fundamental enzyme in the JA biosynthetic pathway (Figure 5.1). It has a sequence and structure similar to catalase (Oldham et al, 2004). There are three known CYP74 enzymes characterised in plants, all of them acting to oxidise long chain fatty acids: AOS produces unstable allene oxides; hydroperoxide lyase is involved in the production of aldehyde and ω-oxo fatty acids; and divinylether synthase, which produces divinyl ethers (Stumpe and Feussner, 2006).

BFP Medtr2g098490.1



BFPL Medtr4g066130.1

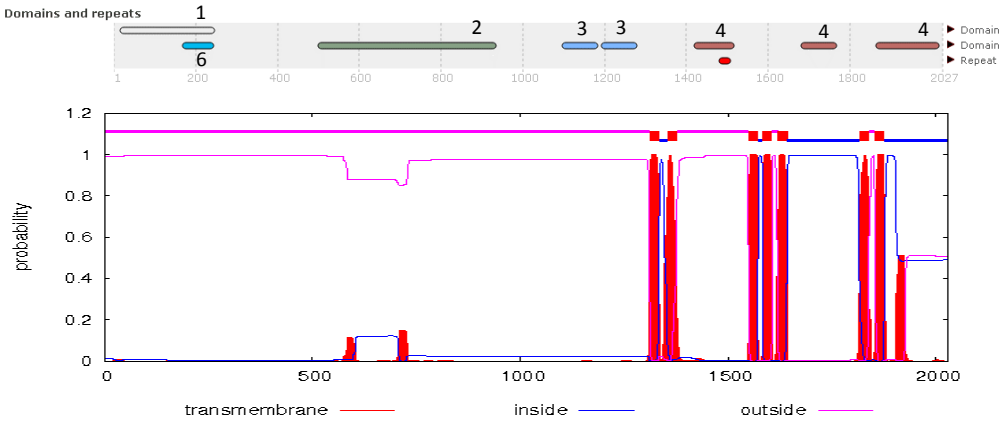


Figure 5.1 **Predicted protein and transmembrane domains of *BFP* and *BFPL***

1. FAD/NAD(P) binding
2. AMP-dependent synthetase/ligase
3. Acyl carrier-like protein with phosphopantetheine binding site
4. Trimeric LpxA-like protein
5. Catalase-like/AOS
6. Amine oxidase

Protein domain prediction was done using InterPro; transmembrane prediction was done using THMMM version 2.0.

The other predicted domains in *BFP* also suggest a role in fatty acid biosynthesis/modification. The acyl-carrier protein (ACP) domain (IPR009081) is a highly conserved carrier of acyl intermediates during fatty acid synthesis (Byers and Gong, 2007). It contains an attachment site for 4'-phosphopantetheine which is an essential prosthetic group. This acts as a 'swinging arm' for the attachment of activated fatty acids, whilst remaining covalently tethered to the ACP enzyme (Mootz et al, 2001).

In addition to these domains it also features an FAD/NAD(P) binding domain serves to bind cofactors (such as FAD, NAD, and NADP) to help catalyse enzyme reactions.

The protein also contains three Lpx domains (IPR011004) which are found in bacterial transferases such UDP N-acetylglucosamine (GlcNac) acyltransferase, and galactoside acetyltransferase

5.2.2 *BFP* is highly conserved in plants that can sustain AM symbiosis

BFP expression is only detected in AM colonised roots, which indicates that it may have an important role in the AM symbiosis. To ascertain how conserved *BFP* is in the plant lineage, I undertook a phylogenetic analysis. A BLASTP of several plant proteomes was carried out and each potential hit was further examined using InterPro and Pfam database searches to analyse domain architectures. Only genes that contained all the predicted domains in the same sequence order as *M. truncatula BFP* were assumed to be orthologues (seen in Figure 5.1).

The oldest species investigated in this analysis was *Amborella trichopoda*, which is near the base of the flowering plant lineage and is thought to have diverged from other extant angiosperms about 130 million years ago (Amborella Genome Project, 2013). Orthologues of *BFP* were present in many angiosperms, except for the AM non-hosts *A. thaliana* and *L. angustifolius*. This strengthens the argument that *BFP* function is limited to AM interactions.

Although the sequence of the *M. paleacea* genome is not yet publicly available, I was provided access to a draft genome courtesy of G. Radhakrishnan in G. Oldroyd's group. A single hit to *BFP* was found using a BLASTN analysis. The sequence was predicted to encode a 1511 amino acid protein. A BLAST alignment with *MtBFP* shows that the draft genome only has 66% coverage of the *MtBFP* protein, corresponding to the C-terminal end, with a 33% identity (Figure 5.2). The predicted protein lacks the N-terminal

methionine that would act as a start codon, which indicates that it is a partial sequence. Pfam and Interpro sequence analyses predict an acyl-carrier protein domain, LpxA domains, and a catalase-like domain (Figure 5.2). From this analysis, it seems likely that *M. paleacea* does encode an intact *BFP* protein, suggesting that this gene is conserved in ancient mycorrhizal plant lineages.

Intriguingly, the BLASTP searches of *BFP* revealed another protein with a strong homology to *BFP* that is restricted to the legume clade (Figure 5.3). This gene (which will now be referred to as *BFP-Like* (*BFPL*) (Medtr4g066130) encodes a protein similar in length to *MtBFP* (2027 residues as opposed to 2031). The gene structure indicates that exon size and number are mostly conserved (Figure 5.4). The encoded protein sequences have 85% similarity and 79% identity (Figure 5.5). Predicted domain analyses revealed that *BFPL* is missing the catalase/AOS-like domain (Figure 5.1). This appears to have resulted from a large deletion in the proteins in the *BFP* C-terminus (corresponding to exon 7). In addition, *M. truncatula BFPL* has 2 predicted acyl-carrier protein domains compared to one in *BFP*. However, this may simply reflect the sensitivity of the search as some orthologues of *BFP* are also predicted to have two of these domains. The predicted transmembrane domains of *BFPL* matched those in *BFP* (Figure 5.1). The phylogenetic and gene structure analysis indicates that *BFPL* appears to have arisen in the ancestor of legumes through duplication of *BFP*.

5.2.3 *BFP* is specifically expressed in AM-colonised roots, whereas *BFPL* appears to be evolving a new function in seed development

In Chapter 4, it was noted that, according to data in the *M. truncatula* Gene Expression Atlas (MtGEA), *BFP* appears to be a mycorrhizal-specific gene. It is 880-fold up regulated in whole roots 6 wpi with the AM fungus *R. irregularis* (Table 5.1) (Gomez et al, 2009). Data from a laser capture microdissection (LCM) study available on MtGEA reveals that *BFP* is highly up regulated in arbuscules and adjacent cortical cells (compared to uninfected cortical cells) 21 dpi with *R. irregularis* (Gaude et al, 2011) (Table 5.1). *BFP* is not expressed in any other tissues or treatments in the public/unpublished databases

Gene	Whole root[1]	Arbuscules[2]	Adjacent cortical cells[2]
BFP	880	286	106
BFPL	33	8	1

1.Gomez et al, 2009
2. Gaude et al, 2011

Table 5.1 **Fold expression values of *BFP* and *BFPL* in AM colonised roots relative to non-colonised whole roots[1] or cortical cells[2]** (data from MtGEA)

	10 dap[1]	20 dap[1]	24 dap[1]	36 dap[1]
BFP	7	7	7	7
BFPL	7	105	389	306

1.Days after pollination (Benedito et al, 2008; data from MtGEA)

Table 5.2 **Expression units of *BFP* and *BFPL* during seed filling**

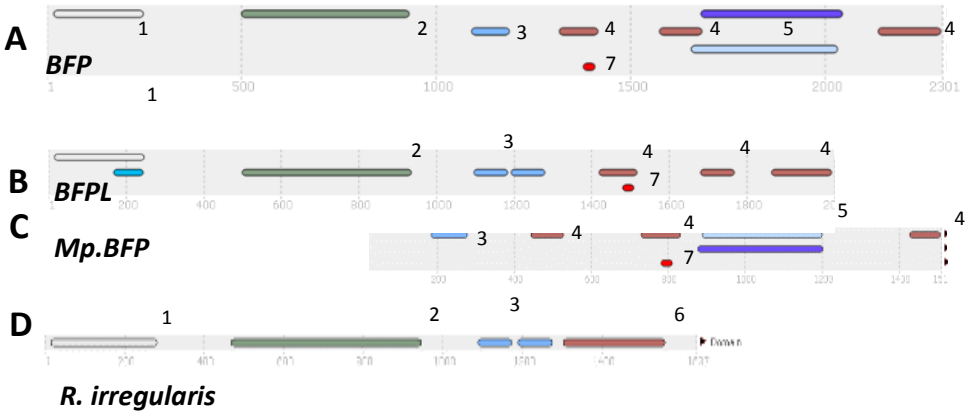


Figure 5.2 **Domains of *BFP* homologues**

Protein domains are aligned against MtBFP **[A].** MtBFPL **[B]**; The *Marchantia paleacea* homologue (prot.faa16151) **[C]**; the closest fungal homologue to BFP, *R. irregularis* (RirGEXX53363.1) **[D]**. The proteins have been aligned in the figure to represent their amino acid lengths. Analysis was done using InterPro (http://www.ebi.ac.uk/interpro/).

Domains: **[1]** FAD/NAD(P) binding **[2]** AMP-dependent synthetase/ligase **[3]** Acyl carrier-like protein with phosphopantetheine binding site **[4]** Trimeric LpxA-like protein **[5]** Catalase/AOS-like **[6]** Thioesterase **[7]** Hexapeptide repeat

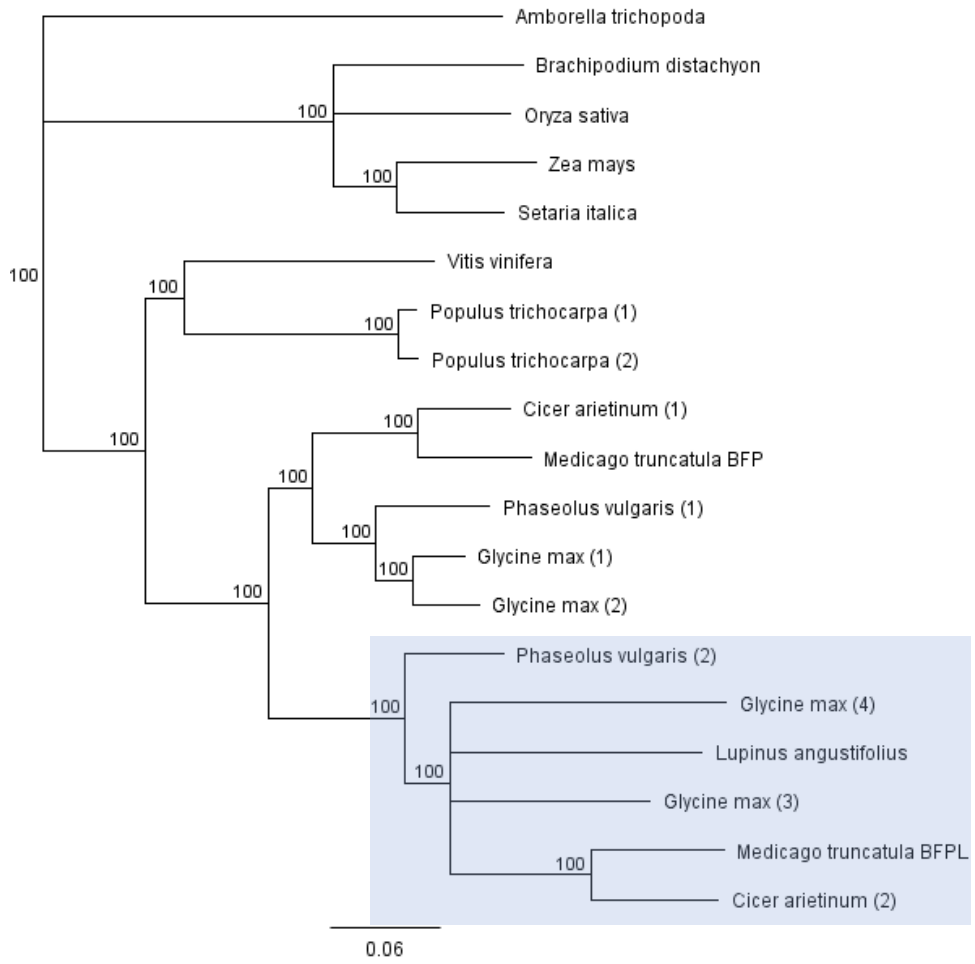


Figure 5.3 **Phylogenetic tree of *BFP* and *BFPL***

BFPL clade highlighted in blue. Rooted tree aligned using the neighbour-joining method. Percentage consensus support labels on branches. Scale bar= units of substitutions per site. Tree constructed using Geneious software.

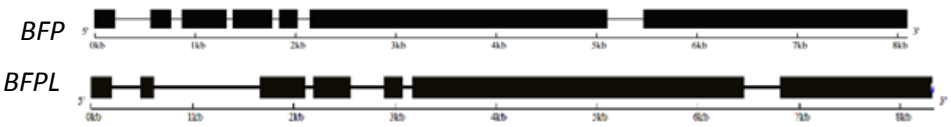


Figure 5.4 **Intronic/exonic structure of *BFP* and *BFPL***

Both genes have seven exonic regions; *BFP* is longer and contains the catalase-like domain which is missing in *BFPL* due to a truncation of exon 7. *BFPL* has a much longer second intron compared to *BFP.* Image constructed using Gene Structure Display Server.

apart from being up regulated in *nin* root hairs 5 dpi with the rhizobia *S. meliloti* (previously described in Chapter 4).

The expression profiles of *BFP* and *BFPL are* compared in Table 5.1; Figure 5.6. In root hairs infected with rhizobia, *BFPL* does not appear to be expressed (including in the *nin* mutant), in contrast to *BFP* (Breakspear et al,2014; Guan thesis, 2012). In whole roots colonised by AM and arbuscules, *BFPL* is up regulated, but to a lesser degree than *BFPL* (Table 5.1). In contrast to *BFP,* there is no up regulation of *BFPL* in cortical cells adjacent to arbuscules (Table 5.1) (Gaude et al, 2011). The most striking difference is that, unlike *BFP, BFPL* is not only expressed during AM colonisation; it is up regulated during seed development as well (Table 5.2), suggesting that it has acquired an additional function.

5.2.4 *MtBFP* expression is correlated with the levels of AM colonisation and is deregulated in *nsp1*, *ccamk* and *ram1*

To quantify expression of *BFP* during mycorrhization, I used cDNA of *M. truncatula* roots that had been inoculated with *R. irregularis* and harvested during a time course experiment of between 0-21 dpi (cDNA provided by S. Roy from three biological replicates, procedure described in her PhD thesis; Roy, S., 2015). At 3 dpi, the roots had less than 1% colonisation, but no arbuscules (Figure 5.7A). Over the time course, *BFP* up regulation correlated with an increase in colonisation, until the last time point of 21 dpi, which represents a 63% arbuscule colonisation. This expression pattern was also confirmed in a whole root RNAseq time-course (Figure 5.7). The RNAseq data shows a fold change up regulation of

```
Medtr2g098490.1      1 MDPERSIEEQFSKLHPSLPLNTRIGIVGAGPSGISAAYALSRLGYNNITVLEKHHAVGGM
                       MD ERSI++QFSKLHPSLP NTR+GIVGAGPSG+SAAYAL+RLGY N+TVLEKHH VGGM
Medtr4g066130.1      1 MDTERSIDDQFSKLHPSLPENTRVGIVGAGPSGLSAAYALTRLGYKNVTVLEKHHTVGGM

Medtr2g098490.1     61 CESVEIEETESALEELDSHKLAIIDTSSGKYQDIKVADDYVSVMSLTLKIQEKVKNSGRF
                       CESVEIEET SALEELDSHKLA++D +SG+YQDIKVADDYVSVMSLTL+IQEKVKN GR
Medtr4g066130.1     61 CESVEIEETGSALEELDSHKLAVVDPTSGEYQDIKVADDYVSVMSLTLEIQEKVKNRGRI

Medtr2g098490.1    121 GVHAVSEVAADLTPEFLEHHGLKSIPNSVAFGYTASGYGFIQDMPYAYIHEFTRTSMAGK
                       GVHAVS++A+DLTPE+LE HGLKSIP SVA+GYTASGYGF+QDMPYAY+HEFTRTSMAGK
Medtr4g066130.1    121 GVHAVSDIASDLTPEYLECHGLKSIPKSVAYGYTASGYGFVQDMPYAYLHEFTRTSMAGK

Medtr2g098490.1    181 IRRFKGGYTSLWQKIAESLPIKLHCNTEVLAIRRNFDSVNVNVKSSNETETMEFDKIIVS
                       IRRF+GGYTSLWQKIAESLP+KL CNTEVLAI+RN D V V++K+SN  ETMEFDKII+S
Medtr4g066130.1    181 IRRFQGGYTSLWQKIAESLPLKLLCNTEVLAIKRNSDGVKVHIKNSNVVETMEFDKIIIS

Medtr2g098490.1    241 GNFPLKYGRTYRSAPSNCIDSEAEVMDASDIEKELFSKVEINDYYTTAFKIRGLDHMPVG
                       G+FPLKYG  YRS PS CI+ E EVMDAS++EK+LFSKV+ NDYYTT FKI+GL+H P+G
Medtr4g066130.1    241 GSFPLKYGSIYRS-PSTCIELEKEVMDASELEKDLFSKVQTNDYYTTVFKIKGLEHFPIG

Medtr2g098490.1    301 FYYFNEYMDDPSTIGNPVAMQKFYADTDIFLFWSYGNSFDIKGPTITELAIKAIKSIGGE
                       FYYF++YM+DP+TIGNPVAMQKFY D++IFLFWSYGNS DIKGP + ELA K ++++GGE
Medtr4g066130.1    300 FYYFDKYMEDPNTIGNPVAMQKFYDDSNIFLFWSYGNSVDIKGPAVKELARKTVEAMGGE

Medtr2g098490.1    361 VESFILQRRFKYFPHVSSQDMKNGFYEKLESELQGSRNTYYVGGLMAFELTERNSSYAMA
                       VESFILQR FKYFPHVSSQDMK+GFYEKLESELQGSRNTYYVGGLMAFELTERNSSYAMA
Medtr4g066130.1    360 VESFILQRNFKYFPHVSSQDMKDGFYEKLESELQGSRNTYYVGGLMAFELTERNSSYAMA

Medtr2g098490.1    421 LMCKNFASSSDLPVFPYTKSLFPLQTEFQRKEPKQLAELPGVQFPNLPTLNGYLKHWGTH
                       L+CKNFA+S+DLP FPYTK LFPLQTE Q+K PK+L ELP V+FPNLP+LN YLKHWGTH
Medtr4g066130.1    420 LICKNFANSNDLPTFPYTKDLFPLQTESQKKNPKELGELPEVRFPNLPSLNSYLKHWGTH

Medtr2g098490.1    481 PVTEDRTLYSWINEQGTVIGKRTYREQHLNASCIASKLLKSQKP----GDKVLLVYVPGL
                       P+T++RTLY+WINE+G  + +RTY EQH  +SC+A KL  SQKP    GD+VLLVYVPGL
Medtr4g066130.1    480 PITQNRTLYTWINEEGNPVCRRTYAEQHFYSSCVAHKLSTSQKPVIKPGDRVLLVYVPGL

Medtr2g098490.1    537 DFIDAFFGCLRAKVIPVPVIPPDPMQRSGQALLKIENIAKSCGIVAILSTVAYHSAVRAG
                       DFIDAFFGCL+AKVIPVP+IPPDPMQRSGQAL+KIENIAKSCGIVAILST+AYHSAVRAG
Medtr4g066130.1    540 DFIDAFFGCLKAKVIPVPIIPPDPMQRSGQALMKIENIAKSCGIVAILSTIAYHSAVRAG

Medtr2g098490.1    597 LVKNF--ITLKNGKSSARWPSLPWLHTDTWVNNSRSYALENLYDDQRESQSGDICFLQFT
                        +KN   IT K GKSSARWP+LPWLHTDTWV NS++  LE+L DDQ E Q  DICFLQFT
Medtr4g066130.1    600 SLKNLISITRKKGKSSARWPNLPWLHTDTWVKNSKTIVLEDL-DDQCEPQPDDICFLQFT

Medtr2g098490.1    655 SGSTGDAKGVMITHGGLIHNVKLMQSRYKSTSRTVLVSWLPQYHDMGLIGGLFTALVSGG
                       SGSTGDAKGVMI+HGGLIHNVKLMQ RYKSTSRT LVSWLPQYHDMGLIGGLFT+LVSGG
Medtr4g066130.1    659 SGSTGDAKGVMISHGGLIHNVKLMQRRYKSTSRTKLVSWLPQYHDMGLIGGLFTSLVSGG

Medtr2g098490.1    715 TALLFSPMTFIKKPLLWIETMSKYQATHSAGPNFAFELVVRRLESSDKDKLQNLDLSSMI
                       +A LFSPMTFIKKP+LW+E +SKYQATHSAGPNFAFEL++RRLES+ KDK+QNLDLSS++
Medtr4g066130.1    719 SAFLFSPMTFIKKPMLWLEIISKYQATHSAGPNFAFELLIRRLESN-KDKIQNLDLSSLV

Medtr2g098490.1    775 FLMVAAEPVRQKTLKRFLELTGPYGLSQKAMAPGYGLAENCVFVSCAFGEGNPIFVDWQG
                       FLMVAAEPVR KTLKRF+ELT P+GLSQK MAPGYGLAENCVFVSCAFGEG PI VDWQG
Medtr4g066130.1    778 FLMVAAEPVRHKTLKRFIELTTPFGLSQKVMAPGYGLAENCVFVSCAFGEGMPIIVDWQG

Medtr2g098490.1    835 RVCCGYIHPGNADVDIRIVDPDGIEELQEDGKEGEIWISSPSAGIGYWGKEELSQSTFQN
                       RVCCGY+HP + D+DIRIVD +  +EL EDGKEGE+WISSPSAG+GYWG+EELSQ TF+N
Medtr4g066130.1    838 RVCCGYVHPADTDIDIRIVDSETCKELHEDGKEGEVWISSPSAGVGYWGREELSQKTFRN

Medtr2g098490.1    895 QLPNHPGRFYTRTGDLGRIIDGKLFITGRIKDLIIVAGRNIYSSDVEKTVESSSELLRPG
                       +L N PGR YTRTGDLGRIID KLFITGRIKDLIIVAGRNIYSSDVEKT+E+SSE LRPG
Medtr4g066130.1    898 ELMNRPGRNYTRTGDLGRIIDQKLFITGRIKDLIIVAGRNIYSSDVEKTIETSSEFLRPG

Medtr2g098490.1    955 CCAVIGVPEETLSAKGISLPDGSDQVGLVVIAELRDGKPVSKDVVDDIQTRVAEEHGVNV
                       CCAVIGVPEE LSAKGIS+PDGSDQV LVVIAE+RDGK VSKDV++ I+ RVAEEHGV +
Medtr4g066130.1    958 CCAVIGVPEEILSAKGISIPDGSDQVALVVIAEVRDGKQVSKDVIEIIKMRVAEEHGVVL

Medtr2g098490.1   1015 ASVKLIKPRTISKTTSGKIRRFECLKQFADGTLNLVP---QPVLTKKKLVRSFTTGTCKE
                       ASVKLIKPRTISKTTSGKI+RFEC+KQF D TLNLVP   +P+LTKK ++ SF+T TC+E
Medtr4g066130.1   1018 ASVKLIKPRTISKTTSGKIKRFECIKQFTDETLNLVPLGTKPMLTKKSMIWSFSTVTCRE

Medtr2g098490.1   1072 GRTPRAQLANSTP----------------------------------------------
                        + PR QL  S P
Medtr2g098490.1   1085 ----------------------------------------------ITSPRIGNKEIM
                                                                    I S RI   +I+
Medtr4g066130.1   1138 KATQKLSDFLGTPVAAIDVFTASCIQELASFSEDLLSKTRPQTERSIPIPSKRISKNDIL

Medtr2g098490.1   1097 EFLKRLISEQAGIPVSKISVTDNMSTYGMDSISVVKATQKLSDFLGVTVAAIDVFSASCI
                       EFLKRLISEQ G+ V KISVTDN+++YG+DSI VVKATQKLSDFLG  VAAIDVF+ASCI
Medtr4g066130.1   1198 EFLKRLISEQTGVAVDKISVTDNLTSYGIDSIGVVKATQKLSDFLGTPVAAIDVFTASCI
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Medtr4g066130.1   1258 QELASFSEDLLSKTQPQLSNNPSDVPEVDIDCTEPVVEVSKSRKLGIRSLQFLALIYISI

Medtr2g098490.1   1217 LVVSPAYLSITTFQIFIASFGKSAYGIPLSNYIFSLALAPLSWILCIASTCICISFFGNS
                       ++ SPAYLSIT F     S  KS  G+P  NYIFSL  APL+WILCIASTC+CIS FG+S
Medtr4g066130.1   1318 MLASPAYLSITAFLNSSLSASKSVAGVPWLNYIFSLIFAPLAWILCIASTCVCISLFGSS

Medtr2g098490.1   1277 FLRPNYALTPEMSIYSIAFVKWWALYKSQEISSKVLATHLKGTVFLNYWFEILGARIGSS
                        +  NY    ++SIYS  FVKWWALYK+QEISSKVLATHL+GTVFL YWFE+LGARIGSS
Medtr4g066130.1   1378 LVGLNYEHASDISIYSTDFVKWWALYKTQEISSKVLATHLRGTVFLKYWFEMLGARIGSS

Medtr2g098490.1   1337 VLIDTVDITDPSLVSIGDEAVISEGVLVQSHEVKNGILSLHPIRIGRNSSIGPYAVIQKG
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Medtr4g066130.1   1438 VLLDTVDITDPTLVSIGDEAVVAEGVLVQSHEVKNGILSLHPIKIGKCSSIGPYAVVQNG

Medtr2g098490.1   1397 SIIKEGAEIQPLQKVEGGQHVLKTAKL---NNNAVLLVTMTKTESDAIYHFLGIYLVAFV
                       S+I E  E+  LQKV  G+HVLK+ KL   + NA L    ++T+ D IYHF+GIYLV F+
Medtr4g066130.1   1498 SVIGESVEVHALQKVAEGEHVLKSDKLKSIDKNADLPAINSETQYDTIYHFMGIYLVGFL

Medtr2g098490.1   1454 SSLAAAITYFMYTWFFQKPASIQSFSFVCICGAFHWIPFTITAYATMFSEVQSNPIAFAI
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Medtr4g066130.1   1558 SSLAAAIAYFLYINFSNQPPSLQHFSFVCICGAFHWIPFTVIAYATMFSEVPSNPITFAI

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Medtr4g066130.1   1618 TFTIAYLLHGLILIALTAVFTRLLIHNQKQTKFKTWLQCRLNISCHLRCAKLLSGTEAFC

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Medtr4g066130.1   1678 VYLRLLGAKIGKHCSIRAINPVSNPELMSIGAGVHLGDFSKIITGFHSSNGYTSGKIEVQ

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Medtr4g066130.1   1738 DNSVTGSQSLILPGSLIQKNVILGALSLAPMNSTLQEGGLYIGSQSNAA--NLATTLHKQ

Medtr2g098490.1   1693 RIEEMDIDYKKIVANLAANLAVTTMNAKARYFHRIGVSGKGHLKIYNKLEGIPMHKIFHP
                         +++     +      ++L +      A     + +S    L +   L+      IF
Medtr4g066130.1   1796 DSQKVTFTLTRKWYQTFSSLFIQPFLQTALPHFLLAISIYAPLNLIFHLKNTQKVPIFWL

Medtr2g098490.1   1753 GKSYPIIVRHSNSLSADDDARI----DARGAALRIFSDEPATDSSDSPPPTLID---LTL
                         + I+     +L       +    + +G  + I+S     DS+     TLI    + +
Medtr4g066130.1   1856 FPLFWILSGVLAALLCVIAKWVIIGRNKKGEKVPIWSKRIIFDSTWQAIRTLIGDYFMEM

Medtr2g098490.1   1806 KTGNAFYARTLADFASWLVCGLAAREELVKSAPHVREAVWNSLRHADSYAEMHYY-SNYC
                          G+  +   +      +   +          P + +         D+    H Y  +
Medtr4g066130.1   1916 TCGSFMFVTWMKMMGVDVNNDVYVDSMGALLNPEMVKIEKGGCVEKDALFFGHLYEGDEG

Medtr2g098490.1   1865 RLMRFED---GQQMYVKFKLRPHDTSISEDKGKVNPTGI-LPPETGAIARDENDSRPLLF
                        L++F +   G+  +V  +       + E++  V    + +  E    ++ E
Medtr4g066130.1   1976 GLVKFGEIKVGENGFVGSRAMVMPGVMLENEANVGALSLAMKDEIVRSSKKE--------

Medtr2g098490.1   1921 LANDFQNRVSSSNGVSYVFQIQVRPVPDDTQGREVALDCTKPWDENEFPFTDVGEINLNE

Medtr4g066130.1         ------------------------------------------------------------

Medtr2g098490.1   1981 NIPMEDSQKLEFNPYLKSNELDTITATSSTQSASIDHGRSLIYEICQHVRNRQPLPEAWR

Medtr4g066130.1         ------------------------------------------------------------

Medtr2g098490.1   2041 NLVQQSNVKVDLSCCPIASSAPLPEKEPLLKKKATPALTLTRTWYQTFSALFIQPLLQTI

Medtr4g066130.1         ------------------------------------------------------------

Medtr2g098490.1   2101 LPHMVIGLAAFVPLNMVVYFKDVKKLPLHWLLPFFWILSGFIAALSCVIAKRVLVGKRKL

Medtr4g066130.1         ------------------------------------------------------------

Medtr2g098490.1   2161 GETIPIWSKKIVFDSTWQAIRTLVGDYFMDITNGSFLSVIWMKMMGAEIEMDGVYVDSNG

Medtr4g066130.1         ------------------------------------------------------------

Medtr2g098490.1   2221 AMLNPEMVKIERGGCIGREALLFGHIYEGEEGGMVKYGEIKIGEDGFVGSRAVVMPGVEV

Medtr4g066130.1         ------------------------------------------------------------

Medtr2g098490.1   2281 ECEASLASLSLAMKGEIIRSR 2301
```
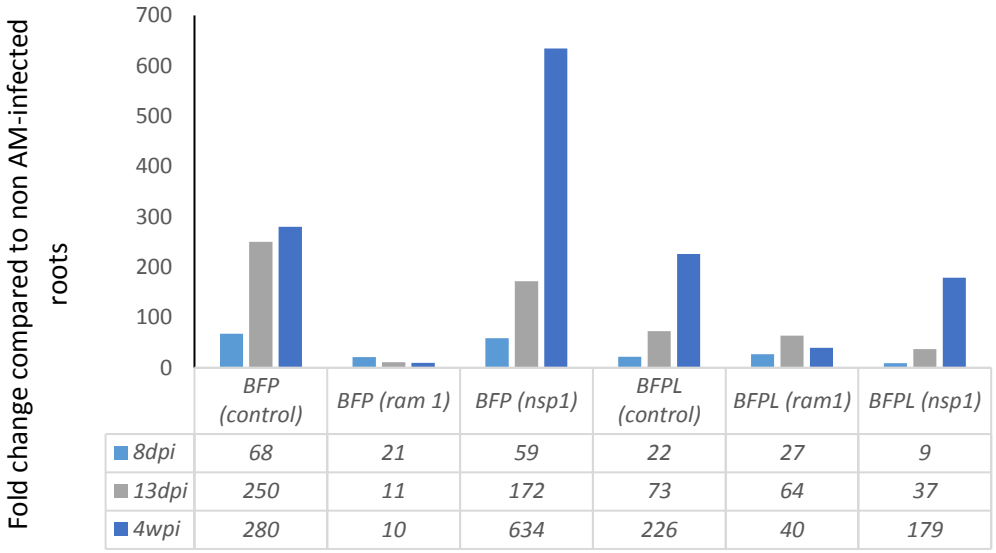

Figure 5.5 **Amino acid alignment of *BFP*** (Medtr2g098490.1) **and *BFPL*** (Medtr4g066130.1)

Alignment constructed using Geneious software



	BFP (control)	BFP (ram 1)	BFP (nsp1)	BFPL (control)	BFPL (ram1)	BFPL (nsp1)
8dpi	68	21	59	22	27	9
13dpi	250	11	172	73	64	37
4wpi	280	10	634	226	40	179

Figure 5.6 **Fold change expression of *BFP* and *BFPL* in *ram1* and *nsp1* during AM colonisation**

RNAseq fold changes compared to those in non-colonised roots during a mycorrhizal time course. Data courtesy of L. Luginbuehl

BFP that is dramatically increased between 8 and 13 dpi, and then increases more gradually between 13dpi and 28dp.

As *BFP* shows AM-specific expression, I wanted to ascertain whether its induction is dependent on the common symbiotic pathway. *MtDMI3* (Doesn't Make Infections) is a common symbiotic gene that is essential for the infection of both AM fungi and rhizobia. *NSP1* is a GRAS transcription factor lies downstream of the common symbiotic pathway. It is essential for nodulation (Heckmann et al, 2006) and has also been implicated in the mycorrhizal symbiosis, particularly in regard to its role in strigolactone biosynthesis (Liu et al., 2011). I carried out a qRT-PCR analysis using cDNA from *dmi3* and *nsp1* roots inoculated with *R. Irregularis* (4wpi) (supplied by S. Roy), to see if *BFP* expression is dependent on the presence of these genes. As expected for *dmi3* mutants, the plants were defective for AM colonisation (data not shown). In WT and *nsp1* roots, *BFP* was up-regulated compared to non-inoculated WT controls (Figure 5.7B).

The qRT-PCR analysis determined that *BFP* is not induced in *dmi3* and, therefore, is dependent on the common symbiotic pathway (Figure 5.7B). In the *nsp1* mutant, *BFP* was significantly up regulated compared to the uninoculated WT controls (Figure 5.7B).

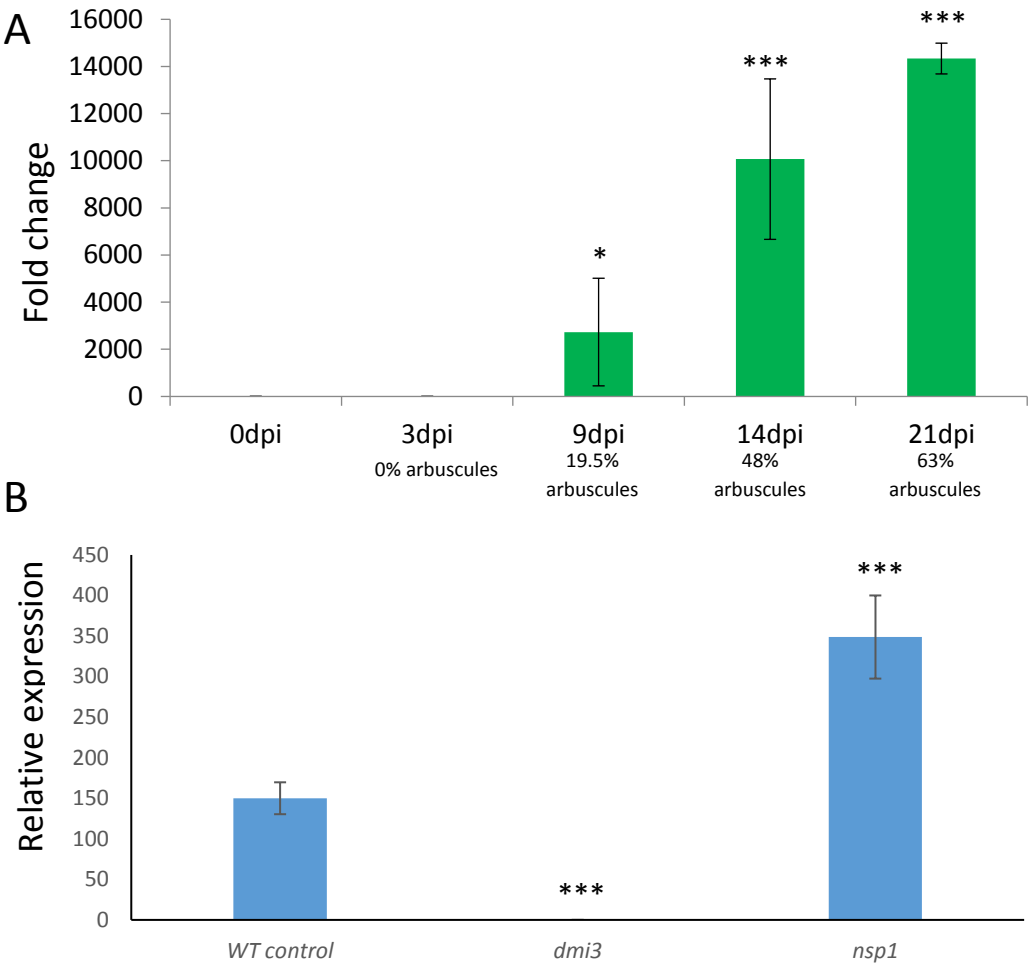


Figure 5.7 ***BFP* expression in *R. irregularis* colonised roots**

Fold change expression of *BFP* during a mycorrhizal time course **[A]**.

Relative expression of *BFP* in the common symbiotic mutants *dmi3* and *nsp1* during AM colonisation at 4wpi, compared to AM colonised WT roots **[B]**.

*p=≤0.05; ***=p≤0.001. Percentage arbuscule colonisation values (S. Roy-thesis)

During the course of this project, RNAseq data for AM-inoculated *nsp1* has recently become available (courtesy of L. Luginbuehl). This data confirms my qRT-PCR data in the *nsp1* background, with *BFP* showing a two-fold increase in expression 4 wpi (Figure 5.7B). However, this increased expression compared to WT inoculated roots was not observed at 8 dpi and 13 dpi. *BFPL* had lower expression in *nsp1* than in WT at all three time points (Figure 5.6).

RAM1 is a mycorrhizal specific GRAS transcription factor that is required for normal hyphopodia and arbuscule formation (Gobbatto et al, 2012). As an AM-specific gene, it is reasonable to hypothesize that *BFP* could be dependent on *RAM1* for its expression. Referring to the RNAseq data in the *ram1* mutant indicates that *BFP* expression is negatively influenced by the absence of *RAM1.* However, *BFP* is still up regulated in the *ram1* background, which means that its expression is not completely dependent on the presence of *RAM1* (Figure 5.6). The same is also true for *BFPL* expression.

5.2.5 *bfp-1* and *bfp-2* have normal AM colonisation levels

The initial AM phenotypes observed in the *bfp* alleles showed a subtle yet significant decrease in the number of arbuscules formed (Chapter 4; Figure 4.6). This assay used the in-house *R. irregularis* inoculum that is grown on chive roots. As explained in Chapter 3, this inoculum is very potent as it typically contains chive nurse plants that germinate during the experiment. If *BFP* is required to produce a secreted signalling molecule, the presence of nurse plants could mask a potential phenotype by providing the signals that the mutants lack through connected AM hyphae. To test this possibility I carried out a mycorrhizal time course using commercial spores to prevent any such effects. Two alleles (*bfp-1* and *bfp-2*) and WT plants were inoculated with *R. irregularis* spores and scored at four time points: 2, 4, 6, and 8 wpi. Each root was scored for the percentage of arbuscules, vesicles and internal hyphae (colonisation) present. In this assay, only *bfp-1* was significantly reduced in arbuscules, and only at 4 wpi (Figure 5.8). The arbuscules looked like those of the WT control plants, as did all the AM structures. The mean arbuscule percentage was reduced by 23% at 4 wpi, compared to the 32% reduction seen at 6 wpi in the initial mycorrhizal assay (Figure 4.6). Therefore, this result does not confirm the earlier findings, and mutations in *BFP* seem to have no clear effect on AM colonisation.

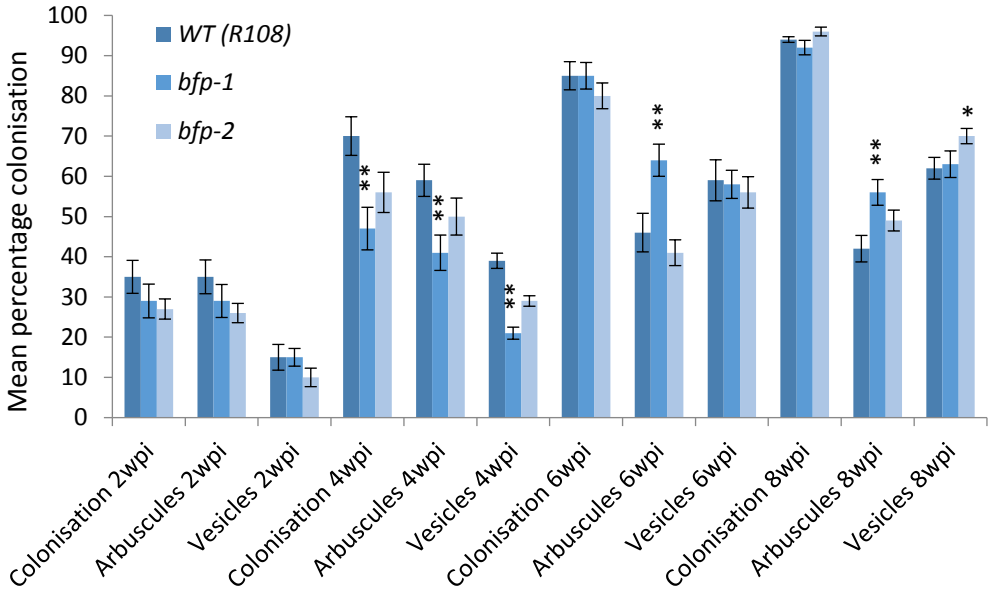


Figure 5.8 **AM phenotype in *bfp-1* and *bfp-2* mutants over a time course**

Plants were inoculated with *R. irregularis* spores and assessed for AM structures at 2, 4, 6 and 8 wpi. *P≤0.05 **p≤0.01 Bars represent standard error of the mean.

5.2.6 RNA interference (RNAi) of *BFPL* does not influence the *bfp-1* phenotype

Although *BFPL* lacks the C-terminal catalase/AOS-like domain, its close homology to *BFP* suggests a related biochemical function. Therefore, it seemed possible that the presence of *BFP* in legumes may be able to compensate for loss of *BFP*, and may be the cause of the inconsistent mycorrhizal phenotype.

To test this hypothesis, it would have been ideal to find a stable mutant for *BFPL*, and cross it with *bfp* allele to create a double mutant. Unfortunately, no *BFPL Tnt1* insertion lines were available in the Noble mutant database, and were not found using reverse genetic screening (X. Cheng, personal communication). Instead, an RNAi silencing assay was used to specifically knock down *BFPL* both in the WT and *bfp-1* backgrounds using hairy root transformation. Colonisation by *R. irregularis* was then scored. The *BFPL* RNAi knockdown in WT roots showed no difference in AM colonisation levels compared to the control plants (WT transformed with an empty vector; Figure 5.9). Both *bfp-1* transformed with an empty vector control, and *bfp-1* transformed with the *BFPL* RNAi

construct had a significant reduction in AM colonisation for all fungal structures scored: arbuscules, vesicles and internal hyphae (P<0.05; Figure 5.9). They both had a 25% reduction in the percentage of arbuscules in the roots, and both had reduced (20-29%) hyphal colonization. The only difference that could be attributed to knockdown of *BFPL* was a slight decrease in the percentage of vesicles in the double mutants compared to *bfp-1* (P=0.02). This experiment provides evidence that *BFP* plays a role in AM colonization, and that, along with *BFPL,* could have a role in vesicle formation. However, because the phenotype of the knockdowns was unexciting, the expression of *BFPL* was not confirmed in the RNAi roots. For this reason, we cannot draw any conclusions from this.

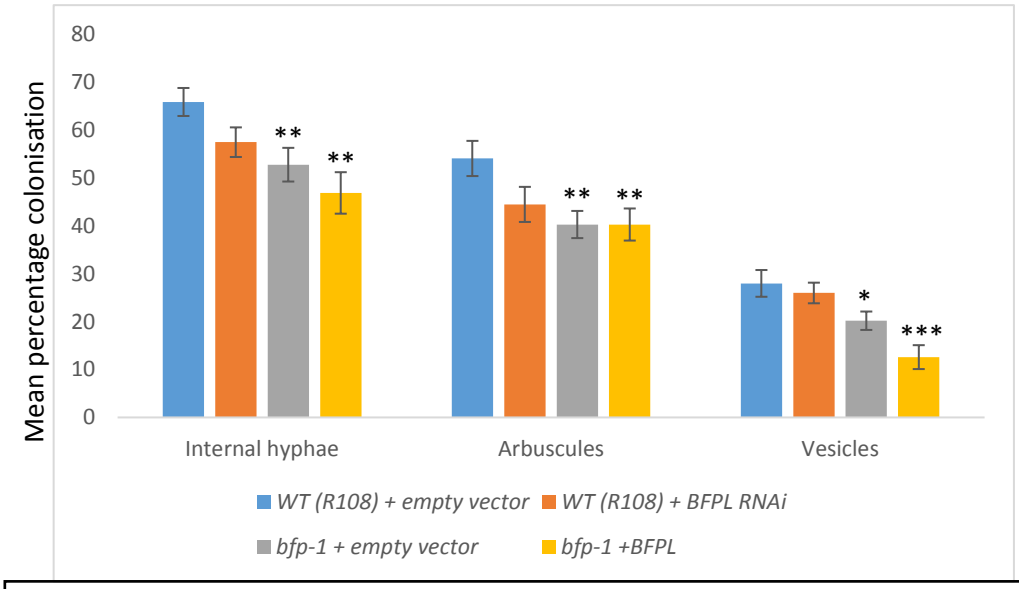


Figure 5.9 **AM phenotype of *bfpl* RNAi knockdown compared to a stable *bfp* mutant.**

A *bfpl* RNAi construct was transformed in WT (R108) and also in the stable *bfp-1 Tnt1* insertion allele. WT controls were transformed with an empty vector, as were some *bfp* mutants (*bfp-1*= empty vector). Both WT (R108) and *bfp-1* plants were transformed with the *BFPL* RNAi knockdown construct.

Plants were inoculated with *R. irregularis* and checked at 4 wpi. *p≤0.05 **p≤0.01 ***p≤0.001. Error bars represent standard error of the mean.

5.2.7 AM phenotyping of new *bfp Tnt1* alleles

Tnt1 transposon insertions are generally considered to create knock out mutations as the 5.3 kb transposon will produce premature stop codons providing it inserts in an exon. However, *BFP* is a large protein (2301 amino acids in length) with two major domains that could have independent biochemical functions. The *bfp-1* and *bfp-2* alleles both have insertions in exons encoding the C–terminal catalase/AOS domain. One possibility is that these alleles could produce transcripts that encode a truncated protein with a fully functional AMP-binding domain, resulting in the subtle and variable phenotype observed. This idea seems supported by the existence of BFPL which appears to be a version of BFP lacking the catalase-like/AOS domain.

To test the hypothesis that *bfp-1* and *bfp-2* are weak alleles that can produce partially functional proteins, I searched the Noble *Tnt1* mutant database and found two new alleles with insertions that are positioned further upstream of *bfp-1* and *bfp-2*. The first (*bfp-3*), is an exonic insertion positioned within the AMP-binding domain, and the second (*bfp-4*), is an exonic insertion situated between the AMP-binding and acyl-carrier protein domain (Figure 5.11). Homozygotes for these lines were isolated using PCR (Figure 5.11). Unfortunately there were not many homozygous seed, and only 50% of those germinated so only one time point could be assayed for mycorrhization (4 wpi). AM colonisation was measured by percentage of arbuscules, vesicles and internal hyphae (colonisation). There was no difference seen in the quantitative mycorrhizal phenotypes for either *bfp-3* or *bfp-4* relative to WT (Figure 5.12). Although some arbuscules looked like they may be underdeveloped, there were also many normal-looking arbuscules (Figure 5.10).

ImageJ was used to quantify the mean area of arbuscules in *bfp-3* and *bfp-4* compared to WT controls. At least 40 arbuscules were measured per allele; representives from five individual plants. The *bfp-3* mutant showed a significant reduction in mean arbuscule size compared to WT plants ($p=0.01$), however *bfp-4* was the same as WT. These results should be treated with caution, as not all arbuscules quantified would have been in the same cortical layer.

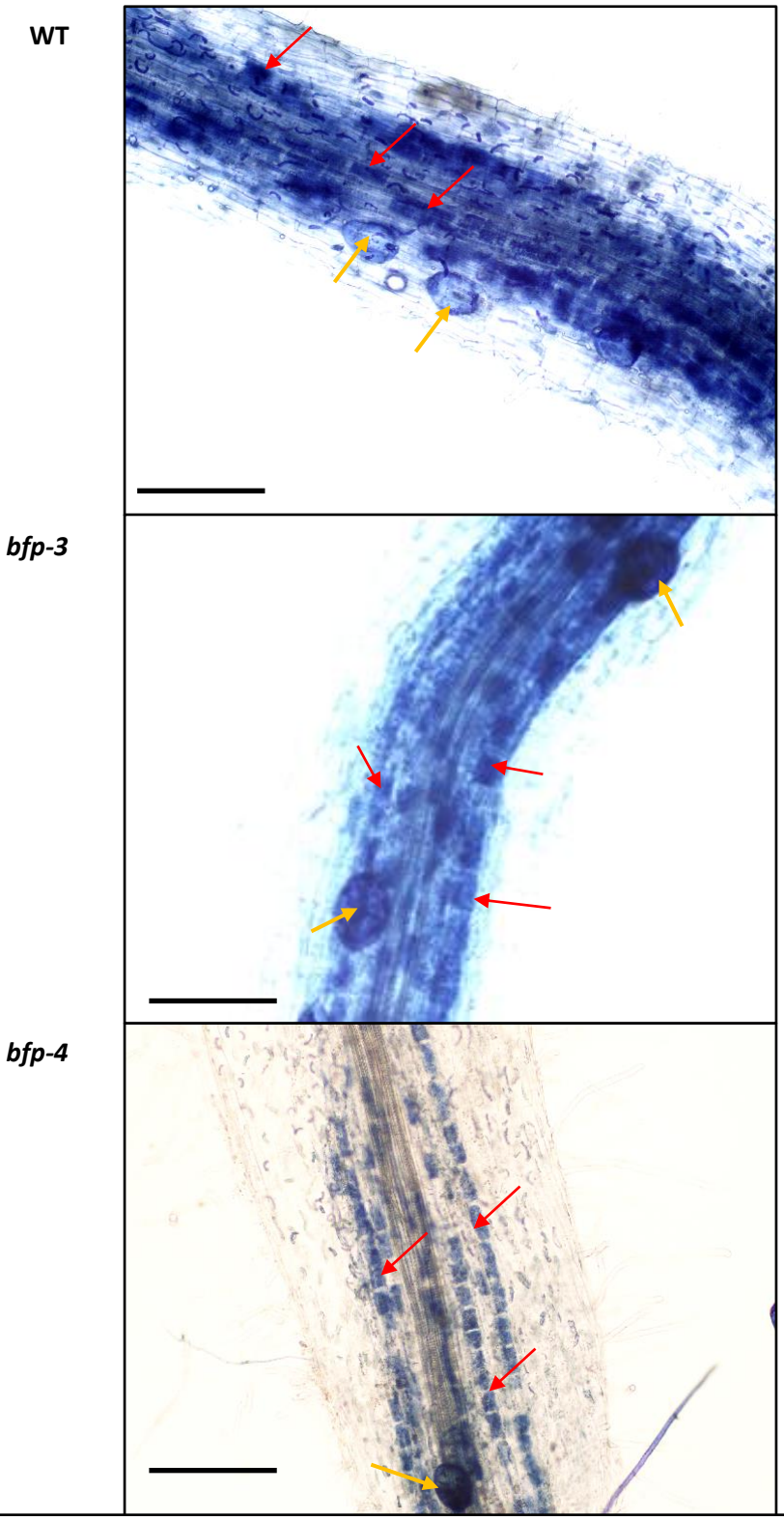


Figure 5.10 **The *Mtbfp-3* and *Mtbfp-4* mutant alleles have normal AM structures in colonised roots**

After 4wpi with AM inoculum, WT (R108) plants had roots with many arbuscules (red arrows) and several vesicles (orange arrows). The *bfp-3* and *bfp-4* mutant lines were also abundant with these AM structures. Images are representative of most roots observed. Scale bars=500µm

5.2.8 The rice *BFP* orthologue is colonized normally by *R. irregularis*

Mycorrhizal phenotyping assays for *M. truncatula bfp* mutants have proved inconclusive. Without a stable *bfp-bfpl* double mutant, it is not possible to conclude whether *BFPL* acts redundantly in the *bfp* mutant background. Because *BFPL* is restricted to legumes, it seemed appropriate to study a *bfp* mutant in a non-leguminous plant. I chose rice, as I was able to find two transposon mutants for *OsBFP* in the *Tos17* insertion mutant flanking sequence tag database (https://tos.nias.affrc.go.jp/).

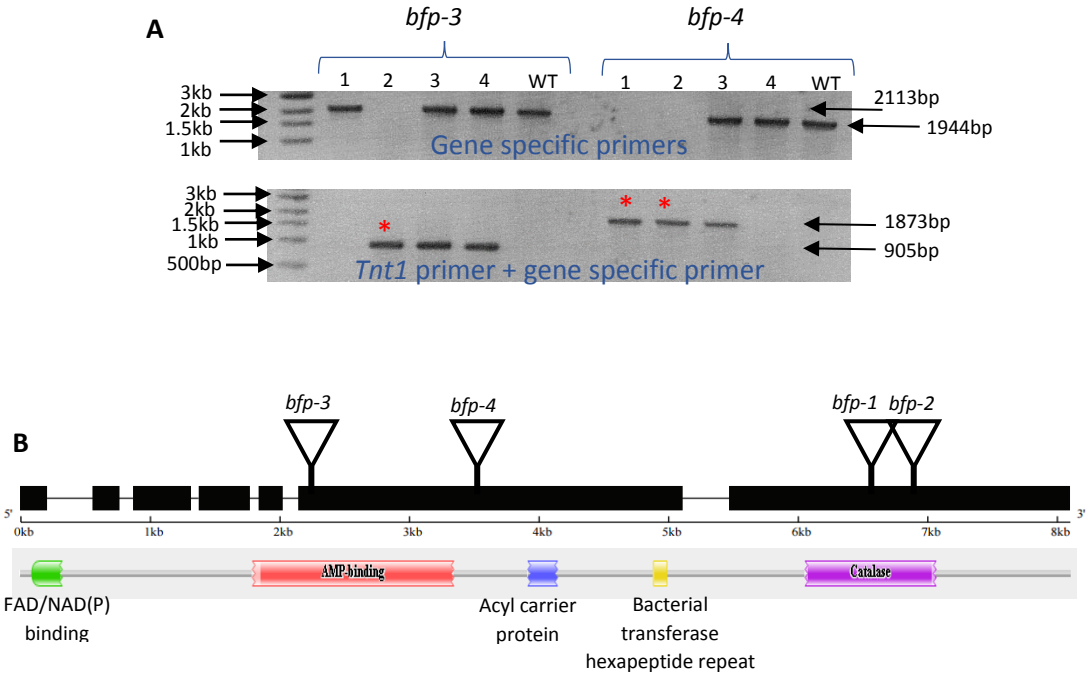


Figure 5.11 **Identification of *Tnt1* insertion mutants for *BFP***

Upper gel shows amplification products for gene-specific primers that flank the *Tnt1* insertion in *bfp-3* and *bfp-4.* Lower band shows amplification products for *Tnt1* insertion junctions using a gene-specific primer and a *Tnt1*-specific primer **[A]**.

Positions of the four *bfp1* Tnt1 insertion (top) and their positions relative to the major protein domains (bottom). *bfp-1* and *bfp-2* have insertions in the catalase-like domain; *bfp-3* in the AMP-binding domain; *bfp-3* is between the AMP-binding and the acyl-carrier protein domains. Protein domain analysis and image from Pfam **[B]**.

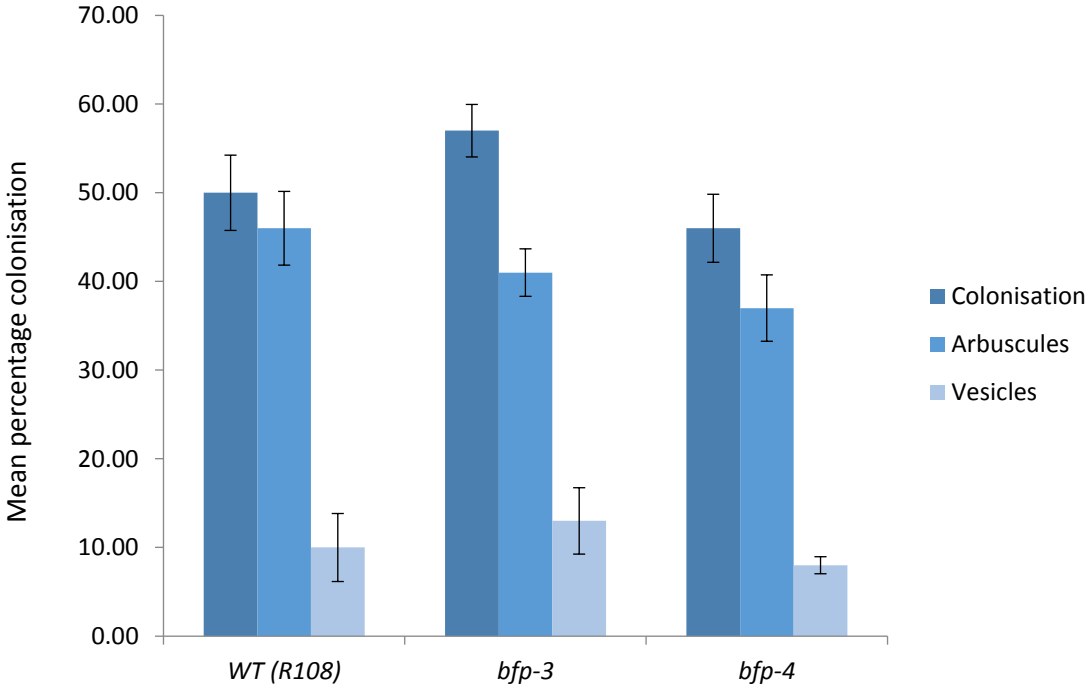


Figure 5.12 **AM phenotype in *bfp-3* and *bfp-4* mutants at 4wpi**

Plants were inoculated with *R. irregularis* spores and assessed for AM structures at 4 wpi. Bars represent standard error of the mean.

There is one *BFP* orthologue in rice (Os04g39780; Figure 5.3), which is up regulated in rice roots during the AM symbiosis (U. Paszkowski, personal communication). Like *MtBFP,* it has six introns and the same predicted protein domains (Figure 5.13). A BLAST search of the *OsBFP* genomic sequence against the rice *Tos17* insertion mutant flanking sequence tag database yielded two lines, NC06602 (*Osbfp-1*) and NE7031 (*Osbfp-2*). *Osbfp-1* has an insertion situated at 1707bp from the start site, just inside an intronic region in the AMP-binding domain, and the *osbfp-2* insertion is at 3765bp in an exon, positioned downstream of the AMP-binding domain before the first ACP domain (Figure 5.13). I obtained the seeds from The National Institute of Agrobiological Sciences in Japan and inoculated with *R. irregularis* spores, alongside WT (Nipponbare) for initial phenotyping. Homozygous lines for both mutant alleles were isolated using PCR with gene specific primers to detect WT alleles and a combination of a gene specific and *Tos17* primer to detect the transposon insertions in *OsBFP* (Figure 5.14).

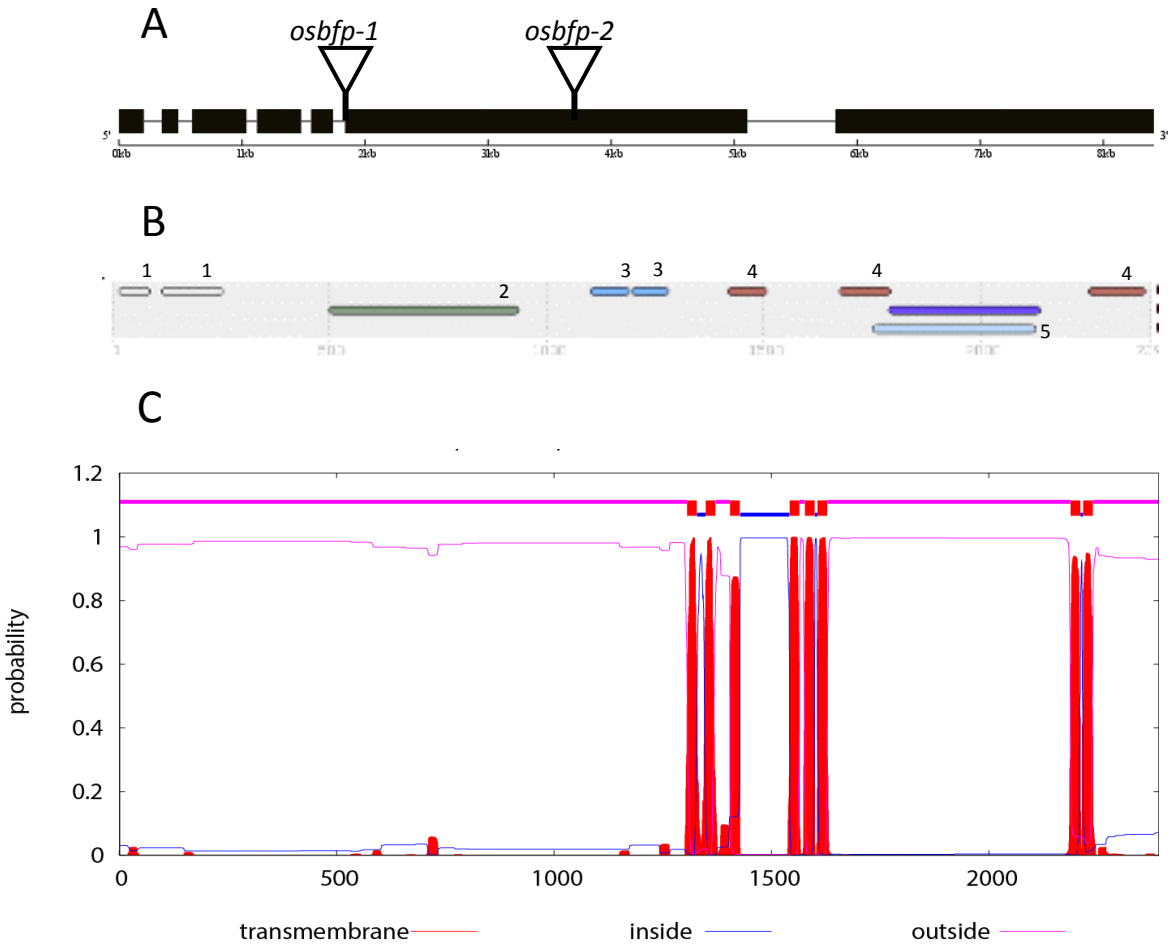


Figure 5.13 **Gene structure of *OsBFP* with *Tos 17* transposon insertion sites.**

Positions of *Tos 17* insertions in *OsBFP* **[A]**

Predicted protein domains (InterPro) **[B]**:

7. FAD/NAD(P) binding
8. AMP-dependent synthetase/ligase
9. Acyl carrier-like protein with phosphopantetheine binding site
10. Trimeric LpxA-like protein
11. Catalase-like/AOS

Predicted transmembrane domains (THMMM version 2.0) **[C]**

Exon structure figure form Gene Structure Display Server.

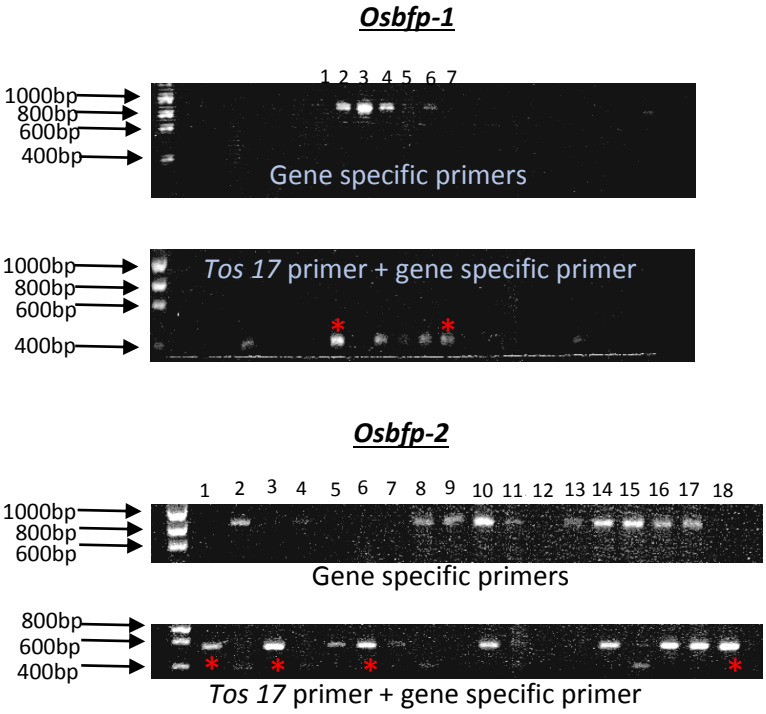


Figure 5.14 **Identification of *Tos 17* insertion mutants for *OsBFP***

Upper gels for each allele (*Osbfp-1; Osbfp*-2) show amplification products for gene-specific primers that flank the *Tnt1* insertion. The lower gels show amplification products for *Tos 17* insertion junctions using a gene-specific primer and a *Tos* 17-specific primer. Red asterisk indicates a line homozygous for the *Tos 17* insertion.

Unfortunately, the spores used in this experiment were too old to be functional and, therefore, at 6 wpi, the control plants did not show any signs of colonisation. The six week-old plants were re-inoculated with fresh spores. When checked ten weeks later, the WT controls had very variable colonisation and thus the mutants were unable to be quantified in any meaningful way. Neither the WT or *Tos 17* lines had many arbuscules, and were highly colonised with vesicles, as they were observed at a very late time point. As expected, the roots were colonised and the fungal structures present looked the same in both the WT and mutant lines. This experiment should be repeated using more replicates from the bulked seed I have proKusterduced.

5.2.9 The closest fungal homologue to BFP and BFPL belongs to R. irregularis

It was recently discovered that there are no genes that encode for a fatty acid synthase in the *R. irregularis* genome (Wewer et al, 2014). These are large, multi-domain proteins required for the biosynthesis of *de novo* fatty acids. *R. irregularis*, as with other AM fungi, are obligate biotrophs; they require a plant host to complete their life cycle. It suggests that AM fungi have lost genes that enable them to lead an independent lifestyle. The lack of lipid synthases in the *R. irregularis* genome suggests that lipid biosynthesis is one role that AM fungi have become dependent on their plant hosts for.

I was interested to see whether *BFP*, with its possible role in lipid modification, had a similarity to any fungal genes. The reasoning was that if I could find similar proteins in some non-obligate fungi that are not present in AM fungi, it would suggest that *BFP* could be involved in synthesising a specific fatty acid that is required by the AM fungi. To investigate this possibility, I carried out a search for BFP and BFPL homologues in the NCBI database of predicted fungal proteins using BLASTP. Surprisingly, the top hit for both proteins belongs to *R. irregularis* (RirGEXX53363.1). It has an amino acid length of 1637, smaller than BFP and BFPL. For BFP, the *R. irregularis* amino acid sequence has a 52% coverage and 30% identity, for BFPL a 61% coverage and 21% identity. This suggests that it is unlikely that this annotation is the result of a plant BFP gene in the composite root, as the identity is too low. The predicted protein domains are very like those of *BFP*: the protein consists of a FAD/NAD(P)-binding domain, an AMP-dependent synthetase/ligase domain, and two ACP domains. The domains are arranged in the same order as in the *BFPs*. The differences are that the *R. irregularis* protein contains two

ACPs (instead of one), no Lpx domains, and there is a thioesterase domain (IPR006683) at the C-terminal region instead of a catalase/AOS-like domain (Figure 5.1). This domain is found in enzymes such as cytosolic long-chain acyl-CoA thioester hydrolases, which catalyse the hydrolysis of long chain fatty acyl-CoA thioesters (Hunt et al, 2012). This would appear to be more than a coincidence. In fact, using the *R. irregularis* BFP homologue to BLASTP against all genomes highlights that plant BFP and BFPL proteins are the top hits, followed by some fungal and oomycete pathogens. This suggests that these this protein has a possible evolutionary link with BFP.

Figure 5.15 **Top fungal BLASTP hits of BFP and BFPL include *R. irregularis* and *S. punctatus* protein with similar predicted domain structures**

The BFP and BFPL amino acid sequences were analysed in NCBI using BLASTP to determine the most homologous fungal proteins. *R. irregularis* (RirGEXX53363.1) was the top hit. The fourth was a protein from *S. punctatus* (SPPG08952), a known AM pathogen.

1)FAD/NAD(P) binding domain

2)AMP-dependent synthetase/ligase

3)Acyl carrier-like protein/phosphopantetheine attachment site

4) Trimeric LpxA-like

5)Catalase/AOS-like domain

6)Thioesterase domain

7) Amino oxidase (flavin containing amine oxidoreductase)

The fourth hit from this BLASTP search was an interesting protein from *Spizellomyces punctatus,* a fungal pathogen of AM spores (SPPG08952). The predicted domains of this protein is intriguing, as it appears to have domains common to both BFP, BFPL, and the *R. irregularis* protein. It is 2845 amino acids long and includes all the predicted domains in the *R. irregularis* protein, as well as a catalase-like domain (as in BFP) and the amino oxidase in BFPL. Like the *R. irregularis* protein, it does not contain any predicted Lpx domains (Figure 5.15).

The second and third BLASTP hits were for *Botryobasidium botryosum* and *Sporisorium scitamineum.* Neither of these proteins have a predicted domain structure that is similar to BFP, BFPL or the two fungal proteins, and only hit on the AMP-binding domain, as did all the hits after the *S. punctatus* protein.

The BLASTP hits from BFP, all align with the AMP-binding domain, and appears to be the common element in all the outputs of this analysis. I did the same analysis, but only using the AMP-binding domain of BFP, which yielded the same results as the full BFP sequence. A transcriptome of another AM fungal species, *G.rosea,* has recently become available (Tang et al, 2015). By using tblastn with the *R. irregularis* protein sequence, I discovered that *G. rosea* also has this BFP-like protein, with the same predicted domain structure as *R. irregularis*.

5.2.9.1 BFP and BFPL may have evolved from a microbial genome

The fact the the AMP-binding domain is the common domain of all the fungal BLASTP hits, prompted the question as to whether this domain was originally plant-derived. An initial BLASTP against *A. thaliana* yielded no proteins with any similarity, despite the fact that it has many AMP-binding domains of this type in its genome. This result could point to the mycorrhizal-specific nature of BFP. A closer inspection of the BLASTP of BFP against all species in the database indicates that, immediately after all the BFP and BFPL proteins, the next hits belong to mycobacteria, aligned to the AMP-binding domain. This result prompted a further search of the BFP AMP-binding domain against all prokaryotes. The first, second and fourth hits were *Mycobacterium kansaii* proteins, predicted as long chain acyl CoA synthetases. The other top hits include two from the marine diazotrophic bacteria, *Crocosphaera watsonii,* as well as several marine algae. The identities of these range from 38-41%. This is slightly more homologous than that of

the *R. irregularis* BFP-like protein which has a 37% identity to just the BFP AMP-binding domain.

The presence of a fungal or prokaryotic protein domain in plants could have potentially occurred from either horizontal gene transfer or endosymbiosis. Two AM endosymbionts have been identified: the endobacter *Candidatus Glomeribacter gigapsorarum (CaGg),* and those endobacteria belonging to the *Mollicutes*. A BLASTP against both these endobacteria using NCBI BLAST yielded hits only aligned to the AMP-binding domains for BFP, the *R. irregularis* protein*,* and *S. punctatus* protein (22-25% identity). The *Mollicutes* hit putative AMP ligase/synthetase proteins in *Mycoplasma.* The top hits for *CaGg* were the AMP-binding domain of putative non-ribosomal peptide synthases (NRPS). These proteins consist of repeats of AMP-binding, PP and condensation domains. The AMP-binding domains of these proteins consist of amino acid adenylation domains, which are not present in the domains of BFP, BFPL, the *R. irregularis* and *S. punctatus* proteins.

5.2.9.2 BFP and BFPL have similarities to non-ribosomal peptide synthases (NRPS)

The main distinction between BFP and BFPL compared to the *R. irregularis* and *S. punctatus* proteins is the absence of the three Lpx domains. The output from the Conserved Domain Database (CDD) in NCBI, predicts the presence of the three Lpx domains as an 'NRPS terminal domain'. In BFP, the catalase domain appears to interrupt this putative domain, almost as if it had been inserted later (Figure 5.16).

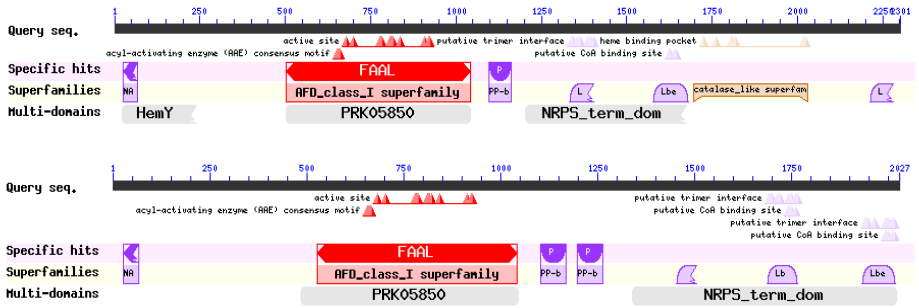


Figure 5.16 **BFP and BFPL are putative NRPS terminal domains**

The Conserved Domain Database in NCBI predicts that the three Lpx domains in BFP and BFPL as non-ribosomal peptide synthese (NRPS) terminal domains.

Using the domain architecture tool in Interpro, I searched the database for proteins that have three Lpx domains, like the BFP and BFPL proteins. There were several proteins with the predicted domain structure of AMP-binding domains, followed by a PP, and then three Lpx domains. In plants, these were only represented by BFP and BFPL. There were representatives in bacteria and fungi, either classed as uncharacterised proteins, or putative CoA synthetase-like proteins.

Although the AMP-binding domain is dominant in the BLASTP searches, I wanted to find out whether the Lpx domains in BFPL, which may constitute a NRPS terminal domain, could also have originally evolved from bacteria or fungi. I did a BLASTP search using just the three Lpx domains of BFPL. After the plant BFPL homologues, the first five hits were from cyanobacteria (the first a NRPS), followed by several other bacteria. Interestingly, there were no hits in either the *R. irregulais* proteome or the *G. rosea* transcriptome. It would be interesting to discover whether these AM fungi have proteins which contain these NRPS-like terminal domains.

5.2.9.3 The catalase-like domain in BFP and *S. punctatus* may be plant-derived

The predicted AOS prediction of the catalase-like domain of BFP, suggests that it is likely to be plant derived. However, the *S. punctatus* protein also has one. Initially, I blasted the amino acid sequence of this domain against *A. thaliana* to determine whether this domain is similar to other known AOS domains. Interestingly, there was no significant similarity to any *A. thaliana* proteins, which suggests that this domain has specifically evolved for the AM symbiosis. Also, blasting the domain against the whole fungal kingdom, only resulted in a hit with the *S. punctatus* protein. A reverse blast of the *S. punctatus* catalase domain against all legumes put BFP proteins as top hits. When blasting against all plants, however, the top hits are predicted oxylipins.

5.3 Discussion

5.3.1 *BFP* is a highly conserved gene expressed specifically during AM interactions

In this chapter, I have shown how *BFP* is an AM-specific gene that is highly conserved in the plant lineage. The gene is a fusion of two main domains that could potentially serve different functions: an AMP-binding synthetase/ligase and a catalase/AOS-like domain. This is highlighted by the fact that *BFPL* has lost the catalase/AOS protein sequence, but

is still expressed. Although I have not observed a mycorrhizal phenotype for *bfp,* it probably performs an important role in AM interactions because it is unlikely that any organism would retain such a large gene (>8000bp) unless its role provided a strong selective advantage. This idea is supported by the fact that plants that can no longer form mycorrhizal associations (e.g. *A. thaliana* and *L. angustifolius*) have lost *BFP,* and that primitive plant species, such as the liverwort *M. paleacea* still have retained it.

5.3.2 *BFPL* is a legume-specific homologue of *BFP* with a potential role in AM symbiosis

I investigated whether *BFPL* could function redundantly with *BFP* in AM interactions in *M. truncatula. BFPL* shows a very similar AM expression pattern to *BFP* with the key difference being that *BFPL* is also expressed in seeds. However, *BFPL* is not as strongly up regulated in mycorrhizal tissues (Table 5.1; Figure 5.6).

I used an RNAi silencing approach to knock down *BFPL* in the *bfp-1* background, which indicated that silencing *BFPL* has no obvious effect on AM colonisation (Figure 5.9). However, the results of this experiment are equivocal. While the presence of a *Tnt1* insertion may be enough to completely knock out any protein function, we cannot rule out that the *bfp-1* mutant we used in this experiment could still produce a protein fragment containing a functional AMP-binding synthetase/ligase and Lpx transferase. In light of this, the *bfp-3* allele that has an insertion in the AMP-binding domain, which was not available when the experiment was carried out, may have been a better choice as a background for the *BFPL* RNAi silencing approach. It is generally accepted that most legume species diverged subsequent to a genome-wide duplication approximately 60 million years ago (Young et al, 2011). The evolutionary fate of duplicate genes can result in one of many potential outcomes including gene loss or neo- and sub-functionalisation (Zhang, 2003). This genome-wide duplication event is thought to have been important in the evolution the legume-specific symbiotic associations with nitrogen-fixing bacteria. The appearance of *BFPL* in the legume clade could suggest a possible role in the legume-rhizobia symbiosis. However, *BFPL* has no expression in either infected root hairs or nodulated roots, and the fact that *BFPL* has not been lost in *L. angustifolius*, a legume that is unable to form AM associations, suggests that it fulfils another role in legumes. In *M. truncatula*, *BFPL* is induced in developing seeds between 20 and 36 days after pollination. The fact that *BFPL* has much lower expression relative to *BFP* during AM

colonisation, suggests that *BFPL* may be in the process of neo-functionalisation, with a role in seed development. *RAM2,* which may be required for cutin monomer signalling events at the root surface during AM symbiosis, also has a role in seeds. The *ram2* mutant produces darker seeds that are permeable to water-solubilized dye. This is similar to the *gpat5 A. thaliana* mutants and is, presumably, caused by the seeds having lower amounts of cutin (Murray et al, 2013). It would be interesting to see whether *BFPL* is expressed in the seeds of other legumes.

The RNAi assay of *BFPL* in the WT background exhibited no difference in arbuscule and hyphae colonisation compared to the empty vector control roots. This suggests that *BFPL* is not essential for mycorrhizal associations in *M. truncatula*. However, the RNAi assay in the *bfp-1* background did yield an almost 40% decrease in the number of vesicles, compared to *bfp-1* transformed with an empty vector control (p=0.02). This is worth noting, as vesicles are filled with lipid droplets and probably utilised as lipid storage organs (Declerck et al, 2005). It would be interesting to check this phenotype in a *bfp/bfpl* double mutant, and also properly in the rice *bfp* mutants. If the reduction in the number of vesicles is confirmed, it could suggest a role for BFP and BFPL in the synthesis of membrane lipids or storage lipids in AM-derived vesicles.

5.3.3 *BFP* expression in AM colonised roots is dependent on DMI3

In whole roots, *BFP* expression was not detected before arbuscules formed (Figure 5.7A). However, since vesicles were not quantified in the roots used in this analysis, the expression of BFP could be more closely related to the appearance of vesicles. The lack of up regulation at early time points suggests that either *BFP* is not induced during the early signalling or infection events of mycorrhizal associations, or that its induction is so low that it cannot be detected in whole roots. Both *BFP* and *BFPL* show increased expression during the course of AM colonisation (Figure 5.6; Figure 5.7A). Both genes, therefore, are likely to be involved in either the ongoing communication between plant and fungus or the physiological changes occurring during AM colonisation.

BFP is dependent on *DMI3*, which indicates that it lies downstream of the common symbiosis pathway (Figure 5.7B). One of the outputs of the common symbiosis pathway during the AM symbiosis is the up regulation of *RAM1,* and *BFP* and *BFPL* are not as highly up regulated in *ram1* roots compared to WT roots during AM colonisation (Figure 5.6). This partial dependence on *RAM1* is likely to reflect the reduction of AM structures

in the roots of the *ram1* mutant, all which were reduced compared to WT, particularly at the later time point. There were no instances of mature nodules in any of the *ram1* roots; all were small and underdeveloped (L. Luginbehl, personal communication). Equally, the lower induction of *BFP* and *BFPL* may due to indirect control of gene expression by this AM-specific transcription factor.

It was surprising to find that *BFP* was more than two-fold up regulated in AM-colonised *nsp1* roots compared to WT at 4 wpi (Figure 5.7B). This phenomenon was independently confirmed by the RNAseq data at the same time point (Figure 5.6). RNAseq data on many AM genes show this type of pattern in *nsp1;* a reduced expression compared to WT, followed by increased expression at 4 wpi (L. Luginbuehl-personal communication). This could indicate that an absence of *NSP1* produces a delayed response to the AM fungus, and could reflect the apparent delayed AM entry seen in *Ljnsp1* mutant roots (Takeda et al, 2013). The induction of *BFP* at the later time point could represent the root overcoming the delay in colonisation and rectifying it by increased formation of arbuscules.

5.3.4 BFP has a likely role in AM interactions

Quantitative analysis of AM structures in the host roots has so far proved inconclusive. Two experiments showed a decrease in colonization, while a third showed a decrease only a single time point of three tested, and the fourth experiment showed no difference. The AM phenotyping of *bfp Tos17* mutants in rice was incomplete because of a shortage of available mutant plants and difficulties with the inoculum. All that could be gleaned from this experiment is that rice *bfp* mutants do have the ability to form arbuscules and vesicles. As discussed in Chapter 3, once AM fungi are able to penetrate the root, normal colonisation can sometimes occur in some AM mutants, which can mask an early phenotype. This experiment needs to be repeated with the progeny from the *Osbfp* lines at different time points.

5.3.5 *BFP* predicted protein domains indicate a role in lipid metabolism

The predicted protein domains of BFP sugges a role in lipid modification. The AMP-dependent synthetase/ligase is known to activate fatty acids; a requirement to perform

in further metabolic processes. The ACP domain includes binding sites for a phosphopantetheine prosthetic group that acts as a swinging arm to move a fatty acid to different enzymatic regions to modify the chain. The Lpx domains may then add GlcNac residues, or some other sugar to the fatty acid. The catalase-like domain is predicted to be an allene oxide synthase, which suggests it may use a hydroperoxy fatty acid as a substrate potentially creating an epoxide, similar to the action of AOS in JA biosynthesis (Figure 5.17).

Notably the Lpx domains are associated with the transmembrane regions of the protein. The MurG protein, although it has no transmembrane regions, is associated with the membrane, most likely through a concave hydrophobic path surrounded by basic residues, which is consistent with its use a hydrophobic (lipid) acceptor (Ha et al, 2000). Similarly, the association of the Lpx domains with the transmembane regions of BFP fits its acceptance of hydrophobic substrates.

Lipids are a large, structurally diverse group of molecules that perform many different functions, including intercellular and systemic signalling, and communication with other organisms. Profiling of fatty acids in mycorrhizal roots has shown that levels of palmitic acid (16:0) and oleic acid (18:1) increase with increased levels of AM (Stumpe et al, 2005). Another study identified that the presence of arbuscules from *R. irregularis* and *Scutellospora calospora* (an AM fungus that produces few vesicles) colonising *Plantago lanceolate* was directly related to the amount of C16:1 fatty acids present (Aarle and Olsson, 2003). This indicated that C16:1 lipid accumulation could be used to determine arbuscule frequency in AM colonised roots.

Recently, it was discovered that the AM fungus *R. irregularis* is missing fatty acid synthase genes (Wewer et al, 2014). During root colonisation, the fungus will require large amounts of fatty acids for storage and synthesis of new membrane in the arbuscule (Gaude et al, 2012). The obligate nature of AM fungi could be reflected in their inability to produce their own *de novo* fatty acids and relying on their host for this.

In addition to important roles in nutrition, lipid signalling has also recently been shown to have a role in plant-fungal interactions including AM colonisation. In *M. truncatula, RAM2,* which encodes a glycerol-3-phosphate acyl transferase (GPAT), is required for early signalling and establishment of the symbiosis. It is necessary for the production of cutin monomers that promote the formation of AM hyphopodia on the root surface. The addition of C16 aliphatic fatty acids to *ram2* mutant roots is enough to re-establish

the formation of hyphopodia. This role may not be restricted to symbiotic fungi, as the pathogenic oomycete *Phytophthora palmivora* appears to require the presence of *RAM2* for appressoria formation (Wang et al, 2012), however, another study did not observe difficulty of *P. Palmivora* in *ram2* mutants (Huisman et al, 2015).

It has also been shown that the fungal pathogen *Ustilago maydis* responds to the presence of cutin monomers to induce filamentation and appressoria formation on a hydrophobic surface (Mendoza-Mendoza et al, 2009). Likewise, the rice blast fungus

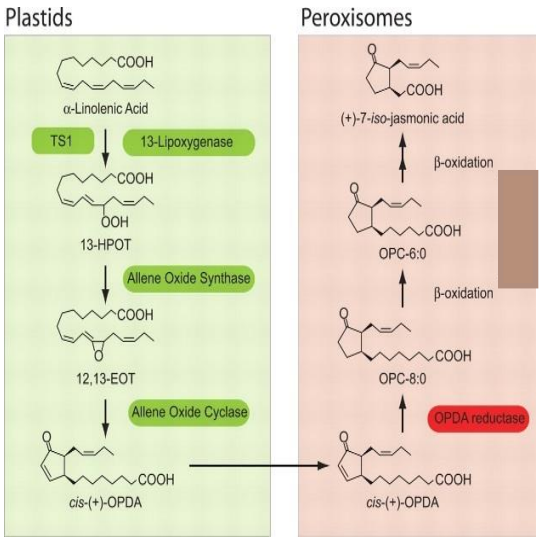


Figure 5.17 **The biosynthesis of jasmonic acid in plants.**

Reactions in the octadecanoid pathway from the oxidation of α-Linolenic acid in plastids to the production of jasmonic acid (JA) in peroxisomes (Image adapted from Acosta et al (2009)

Magnaporthe grisea, which infects leaf tissue, relies on cutinases to degrade the surface cutin. Without these cutinases, appressorium maturation and penetration peg formation are abnormal. However, the application of syntheitic cutin monomers can restore virulence, suggesting that cutin is acting as a signal (Skamnioti and Gurr, 2007).

Another class of lipids involved in plant-microbe interactions are the oxylipins which are lipid-based signalling molecules that have been implicated in plant-microbe associations. They are biologically active compounds produced by the oxidation of polyunsaturated fatty acids (PUFAs), namely linoleic acid (18:2), α-linolenic acid (18:3) and roughanic acid (16:3). Oxylipins
formed in plants include hydroperoxy-, hydroxy-, oxo- and
epoxy-fatty acids, divinyl ethers, volatile aldehydes and the plant

hormone, jasmonic acid (JA) (Grechkin, 1998). The diversity of oxylipins can be further increased by the esterification in complex glycerolipids (glycolipids, phospholipids, and neutral lipids) and their conjugation to amino acids and other metabolites (Mosblech et al, 2009). They are thought to play important roles in defence as signalling molecules, wound healing promoters, or as constituents of cutin (Blee, 2012). Oxylipins are formed from PUFAs that are released by lipases from membranes, and oxidised by lipoxygenases (LOXs) to form hydroperoxides in the 'LOX pathway' (Dave and Graham, 2012). These hydroperoxides are substrates for special cytochrome P450 (CYP74) enzymes to produce oxylipins. CYP74s are atypical cytochrome P450s in that they do not require molecular oxygen or NAD(P)H-dependent cytochrome P450-reductase. Instead, carbon-oxygen bonds are formed using an acyl hydroperoxide, both as a substrate and oxygen donor (Stumpe et al, 2005). This can occur either through the 9- or 13-LOX pathway, depending on which carbon position of the fatty acid is oxidised.
The best studied plant oxylipin is the hormone jasmonic acid (JA), which is synthesised in the octadecanoid pathway. The first step in this pathway is the oxidisation of α-linolenic acid by 13-LOX to make a fatty acid hydroperoxide called 13*S*-hydroperoxy-octadecatrienoic acid (13-HPOT). This intermediate compound is used in several enzymatic pathways. The first committed step in JA synthesis is the dehydration of 13-HPOT by a CYP74 known as allene oxide synthase (AOS; Figure 5.1). The product of this reaction, the unstable allene oxide 12,13-oxido-9,11,15-octadecatrienoic acid (12,13-EOT), is a substrate for allene oxide cyclase (AOC), which produces pufa (OPDA). These reactions take place within a plastid and then OPDA is transported into the peroxisome, where it undergoes a reduction step and several oxidation steps and modifications to produce JA (Mosblech et al, 2009; Yan et al, 2012).

JA is generally associated with its role in defence against necrotrophic pathogens and herbivory. However, in both *Hordeum vulgare* (barley) (Hause et al, 2002) and *M. truncatula* (Stumpe et al, 2005), JA levels are higher in roots colonised by *R. irregularis*. In tomato, the JA-deficient *suppressor of prosystemin mediated responses 2* (*spr2*) mutant has lower levels of root colonisation by the AM fungus *Glomus fasciculatum*, which can be reversed by methyl jasmonate (MeJA) application (Tejeda-Sartorius et al, 2008). Also, a knock down of the JA biosynthetic gene allene oxide cyclase (AOC) in *M. truncatula* resulted in a decrease in JA levels and *R. irregularis* colonisation (Isayenkov et al, 2005). The stimulation of JA biosynthesis by repeated leaf wounding in *M. truncatula* also resulted in an increase in mycorrhiza in *R. irregularis* colonised roots (Landgraf et al,

2012). JA also effects ectomycorrhizal (EM) colonisation. In spruce seedlings inoculated with the EM fungi, *Laccaria laccata* and *Pisolithus tinctorius,* initial EM colonisation was significantly accelerated after JA treatment (Regva et al, 1997; Regva and Gogala, 1996). Also, the significant increase in dry root weight from *Laccaria laccata* colonisation was further increased by JA application (Regva et al, 1997).

However, there are conflicting reports on JA's role in AM interactions in different plant species. The tomato mutant *jasmonate insensitive* (*jai*), which is deficient in the JA receptor, displayed higher levels of *R. irregularis* colonisation (Herrara-Medina et al, 2008). This study observed that systemic application of MeJA resulted in a reduction of mycorrhiza. On the other hand, there was no difference in AM colonisation of a rice JA-deficient biosynthesis mutant, *constitutive photomorphogenesis 2* (*cpm2*), compared to WT plants (Gutjahr et al, 2015). When the authors added JA to WT rice roots, AM colonisation decreased, and this decrease correlated with the expression level of the defence gene *PR4*. It was postulated that the suppression of AM colonisation is mediated through defence induction. These conflicting AM responses to JA are difficult to reconcile. It is possible that JA acts as an external signal for AM colonisation, but induces defence when perceived internally.

Some other plant oxylipins formed in the 9-LOX pathway have shown essential roles in plant defence against microbial pathogens (Blee, 2002; Borrego and Kolomiets, 2012). For example, maize *Zmlox3* mutants have increased resistance to several fungal pathogens (Gao et al, 2007; Isakeit et al, 2007), however mutants have increased susceptibility to *Aspergillus flavus* and *Aspergillus nidulans* (Gao et al, 2009).

Studies are starting to reveal that other oxylipins may also have roles in the AM symbiosis. Transcriptomics on tomato roots colonised with both *R. irregularis* and *Glomus mossae* infections have detected the regulation of genes involved in the biosynthesis of both 9- and 13-LOX oxylipins (Lopez-Raez et al, 2010). Split root AM experiments in tomato plants infected with *R. irregularis* had increased expression of 9-LOX biosynthesis genes in the particular areas of the root with AM colonisation. However, this altered expression was not seen in JA-deficient plants (Morcillo et al, 2013). Silencing the expression of AOS3 (a key enzyme in the 9-LOX pathway) in potato during *R. irregularis* colonisation reduced the amount of AOS3-derived products, increased the percentage of AM colonisation, and caused an increase in the expression of in 13-LOX biosynthesis genes and amounts of JA. These studies indicate that the 9-

and 13-LOX pathways have a role in regulating mycorrhizal symbiosis (Morcillo et al, 2016).

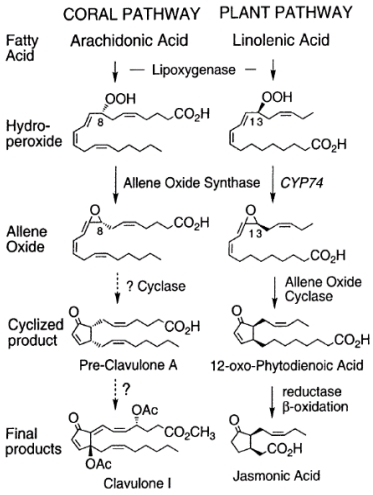


Figure 5.18 **Comparison of the biosynthetic pathways that utilise allene oxide synthase in plants and coral**

The jasmonic acid pathway in plants has been well-defined, whereas the conversion form arachadonic acid to protoglandins in marine invertebrates has yet to be determined (Tijet and Brash, 2002)

BFP is a fusion protein as it consists of two distinct protein domains. Examples of fusion proteins are found in many proteomes, for example, there is an example in the coral, *Plexaura homomalla,* which consists of a lipoxygenase and a cytochrome P450 allene oxide synthase (AOS). This protein forms part of the arachidonic acid pathway to produce prostaglandins in coral. The fusion protein appears to perform two biosynthetic roles in the pathway, with the initial production of a hydroperoxide by the lipoxygenase, followed by the conversion to allene oxide by the AOS. This coral pathway has many parallels with the JA biosynthetic pathway in plants, where a fatty acid utilises a lipoxygenase and AOS to produce an allene oxide (Figure 5.18) (Tijet and Brash, 2002). The AOS domain in coral has been well studied due to its striking similarity to catalase core proteins. It was found that the catalase fold in the coral AOS has been converted from having hydrogen peroxide-binding specificity, to being able to bind to fatty acids instead (Oldham et al, 2004). Furthermore, it was deduced that a single mutation in the active of the coral AOS could induce catalase activity (Tosher et al, 2006).

It is tempting to speculate how the domains in BFP function together to predict a particular lipid product. There is no doubt that BFP is involved in the modification of

fatty acids, and the AOS-like domain suggests an oxylipin is produced. However, oxylipins are not the only lipids implicated in signalling in plants, and it is possible that BFP may help to make other types of lipids. The three Lpx domains suggests the addition of GlcNac or other sugar moieties. It suggests that the product may be a complex glycolipid that undergoes oxidation. This could be involved in signalling between the AM fungus and plant host within the root during colonisation, or outside the root prior to the physical interaction. The apparent absence of fatty acid synthase in the AM fungi, and the requirement of the production of new membrane at the peri-arbuscular interface, would require the synthesis of large amounts of lipids for membrane deposition.

5.3.6 BFP may have originally evolved from a bacterial or fungal genome

I looked for BFP homologues in fungi based on the idea that BFP may act to complement a specialized fungal lipid biosynthetic protein which could be missing in certain AM species. Instead I found that *R. irregularis* had a very similar protein. The significance of this is not clear, but it appears that the AMP-binding domain in *R. irregularis* is more similar to BFP and BFPL plant proteins than in any other fungal or bacterial species. It appears that BFP has greater homology to an *R. irregularis* gene than any in *A. thaliana,* as well as to the fungal AM parasite *S.punctatus.* This suggests that BFP could be more similar to fungal genes than plant genes, and could provide a product (e.g. lipids) that is required for the fungus during AM interactions.

Other than BFP and BFPL plant proteins, BLASTP outputs of BFP only have homology to the AMP-binding domain. Apart from other BFP and BFPL proteins, BLASTP hits focus solely on the AMP-binding domain, also predicted to be a FAAL. These preferentially hit on certain actinobacteria and cyanobacteria, although they have similar homology to fungal proteins, particulary *R. irregularis.* This includes some NRPS proteins, but often these are short putative fatty acyl CoA ligases. The C-terminal end of BFPL, which constitutes three Lpx domains, has domain architecture similarity to C-terminal NRPS domains. Blasting the amino acid sequence of these domains suggests that this is also more homologous to both fungal and bacterial proteins, some of which are predicted to have NRPS C-terminal domains, and some which do not. This evidence suggests that BFP has evolved from either bacteria or fungi, with the subsequent addition of a catalase/AOS-like domain.

CHAPTER 6

NF-Ys with roles in nodulation and mycorrhization

6.1 Introduction

The *M. truncatula* genome possesses eight NF-YAs, nineteen NF-YBs and eleven NF-YCs. The most well studied of these is NF-YA1 (previously known as HAP2.1) which is important in the legume-rhizobia symbiosis. MtNF-YA1 is required for the early stages of rhizobial infection as well as the maintenance of the nodule meristem (Combier et al, 2006). The *Mtnf-ya1* mutant exhibits aberrant infection threads that have thinner cell walls, which results in them becoming swollen and bulbous during rhizobial progression. Many infection threads appear to abort prior to reaching the cortex, resulting in fewer nodules. The nodules that are present are small, round and lack a meristem. Promoter: GUS expression analysis of *NF-YA1* indicates that it is expressed mainly in the presence of the encroaching rhizobia through the root hair and cortical cells and into the nodule primordium. In the fully developed nodules, expression is restricted to the meristem and infection zone (Laporte et al, 2013). Studies have shown that *NF-YA1* transcripts are spatially regulated in the nodule by post transcriptional reduction of mRNA transcripts by miR169 and uORF1p, a small peptide produced from alternative splicing of the 5' leader sequence (Combier et al, 2008).

It has been shown that MtNF-YA1 can form a trimeric complex with MtNF-YB16 (orthologue of LjNF-YB1), and MtNF-YC2 *in planta,* and they individually associate with the same CCAAT box on the *ERN1* promoter (Baudin et al, 2014). The *Phaseolus vulgaris* orthologues of these (PvNF-YA1/PvNF-YB7/PvNF-YC1) can also form a complex *in planta,* indicating that this could be an evolutionary conserved NF-Y complex for nodulation (Baudin et al, 2015). All three genes are under the regulation of the transcription factor Nodule Inception (NIN), a master regulator of the nodulation pathway that sits downstream of the common signalling pathway (Marsh et al, 2007; Soyano et al, 2013). No nodulation phenotype has been observed for the *Mtnf-b16 (Ljnf-yb1)* mutant knockdown, although this gene is highly expressed in nodules (Soyano et al, 2013; Roux et al, 2014), nor when knocked down in combination with its closest homologue, *MtNF-*

YB18 (Baudin et al, 2015). This highlights how not all subunits in a complex will yield an obvious phenotype when transcription is reduced. An RNAi knock down of the *P. vulgaris PvNF-YC1* (orthologue of *MtNF-YC2*) shows a similar phenotype to the *nf-ya1* mutant: aborted infection threads and underdeveloped nodules (Zanetti et al, 2010).

Although the *Mfnf-ya1* mutant has abnormal infection threads, a double knock down of *MtNF-YA1* and *MtNF-YA2* enhances this phenotype (Laloum et al, 2014). Although a nodulation phenotype has been observed in the *PvNF-YC1* knock down*,* this phenotype was not replicated when its *M. truncatula* orthologue, *MtNF-YC2* was knocked down. Only when both *MtNF-YC2* and its close homologue *MtNF-YC1* were both knocked down could a nodulation phenotype be observed, resulting in fewer, under-developed nodules (Baudin et al, 2015). Phylogenetic analysis and genetic studies show that *MtNF-YC1* and *MtNF-YC2* are paralogues that diverged during the legume-specific gene duplication approximately 58 million years ago (Young et al, 2011; Laloum et al, 2012).

The three NF-Y subunits in the conserved complex have also been associated with the cell cycle or cell division. Knock down of *PvNF-YC1* resulted in reduction of cell cycle genes (*CDC2* and *CDC25;* Zanetti et al, 2010). The over-expression of *LjNF-YA1* (orthologue of *MtNF-YA1*), and *LjNF-YB1* (orthologue of *MtNF-YB16*) lead to the formation of lateral root-like structures indicating that they are positively involved in the enhancement of cell division. In addition, over expression of *LjNF-YA1* produced extra cell division in the lateral root primordia and proximal regions. This phenotype was more pronounced when *LjNF-YA1* and *LjNF-YB1* were over expressed together; there were shorter intervals between lateral roots and a greater number of cell divisions in lateral root primordia (Soyano et al, 2013).

In Chapter 4, I described a reverse screen of *M. truncatula* genes which are up regulated during mycorrhizal associations. Three of these, *CBF1* (*MtNF-YC6*)*, CBF2* (*MtNF-YC11*) and *CBF3* (*NF-YB7*) also have expression in root hairs during early rhizobial infection (Breakspear et al, 2014; Chapter 4 Figure 4.3). I was able to isolate one *Tnt1* insertion allele for *CBF1* and three independent alleles for *CBF3.* None of these mutants yielded a mycorrhizal phenotype. However, the *cbf1* mutant and two of the *cbf3* alleles showed a significant reduction in the number of nodules in *S. meliloti* infected roots (Chapter 4 Figure 4.7). The third allele (*cbf3-3*) also displayed reduction in nodules with a p value of 0.06, just below the 0.05 significance threshold. This chapter describes the further

investigation of the potential role of *CBF1, CBF2* and *CBF3* in nodulation. This is in collaboration with the Kuster lab. They have previously published detailed AM expression analyses of *CBF1* and *CBF2,* and are concentrating on the role of these genes during mycorrhization. It has been agreed that my work, which investigates the potential role of these genes during rhizobial infection, will supplement their research with a view to publishing these results together. *CBF3* has not yet been published and both the Murray and Kuster labs have a shared interest. The Kuster lab will do detailed AM phenotyping and expression analyses. This collaboration is beneficial and has enabled us to share resources. For example, we have provided homozygous *Tnt1* mutant alleles for *cbf1* and *cbf3,* and they have provided us with promoter:GUS constructs for all three genes.

6.2 Results

6.2.1 *CBF1* and *CBF2* encode highly homologous tandemly duplicated genes

In the *M. truncatula* genome, *CBF1/NF-YC6* (Medtr2g081600) and *CBF2/NF-YC11* (Medtr2g081630) are situated next to each other on chromosome two and are very homologous, sharing a 96% amino acid identity (Figure 6.1A). To study the relationship between the *M. truncatula* NF-YCs, I did a phylogenetic analysis of all NF-YC subunits (Figure 6.2). Like *NF-YC1* and *NF-YC2, CBF1* and *CBF2* appear to be the result of a duplication event. However, unlike *NF-YC1* and *NF-YC2,* which are found on separate chromosomes, *CBF1* and *CBF2* are closely linked (16.6 kbp apart) and are more homologous to each other than they are to any other legume NF-YC orthologues, suggesting they arose from a tandem duplication event (Laloum et al, 2012). This, and the fact that *CBF1* and *CBF2* have highly similar expression patterns in roots colonised by AM fungi, suggests that they are likely to display some level of genetic redundancy, and might participate in the same heterocomplex.

To further investigate the similarity in expression between *CBF1* and *CBF2*, I compared the nucleotide sequences from both genes 1500 base pairs (bp) upstream of the transcriptional start site (TSS) using the BLASTN function on the NCBI web server. The output from this analysis clearly identified that the 243bp immediately upstream of the TSS had strong homology, whereas the rest of the sequence could not be aligned at all. This is remarkable since promoter regions between genes, even those belonging to the

same family, are usually poorly conserved. This suggested that this 243bp sequence is the promoter sequence containing the important *cis* regulatory motifs, possibly in the UTR. I then used Geneious software to align these sequences, which have an 87% homology (Figure 6.1B).

A

```
CBF1      1 MRQAGAYSGILNGGIGRTGPHSLPLARIKKIMKNSSEDVKMISGVAPIVFSKACELFIEE  60
            MRQAGAYSGI+NGGIGRTGPHSLPLARIKKIMKNSSEDVKMISGVAPIVFSKACELFIEE
CBF2      1 MRQAGAYSGIVNGGIGRTGPHSLPLARIKKIMKNSSEDVKMISGVAPIVFSKACELFIEE  60

CBF1     61 LTRRSWIMAIDAKRRTLNKEDVASAVIATDIFDFLITLVSNSD-STDDTTVMQMETMNSS 119
            LTRRSWIMAIDAKRRTLNKEDVASAVIATDIFDFLITLVSNSD STD TT+MQME++
CBF2     61 LTRRSWIMAIDAKRRTLNKEDVASAVIATDIFDFLITLVSNSDSSTDATTLMQMESI--- 117
```

B

```
CBF1      1 GAGTCAACAAGTCAAATTTTTATTTTGACACAGTCAACAAGTCTAATAAAAAAATACACA  60
            GAGTCAACAAGTC A  TTTTATTTTGAC CAGTCAACAA TCTAAT AAAAAA ACACA
CBF2      1 GAGTCAACAAGTCTAGATTTTATTTTGACTCAGTCAACAAATCTAATTAAAAAA-ACACA  59

CBF1     61 CAT--ATTAATTTATTCATTTCTAATAATCTCAAATTCTCAATTAGCCGGCAAAAGCAGG 118
             AT    TAATTTATTCATTTCTA TAA        TCTCAATTAGCCGGCAAAAGCAGG
CBF2     60 GATTCTCTAATTTATTCATTTCTATTAA--------TCTCAATTAGCCGGCAAAAGCAGG 111

CBF1    119 CATAGCCGGCAATGTAAAAAGAGTGAAAGCTTCAACTTAATCGCTATTTATTAATTTGTT 178
            C TAGCCGGCA TGTAAAAAGAGTGAAAGC TCAACTTAATC CT  T ATTAATTTGT
CBF2    112 CTTAGCCGGCAGTGTAAAAAGAGTGAAAGCATCAACTTAATCACTGATAATTAATTTGTA 171

CBF1    179 TATATATAACACTGAAATTAAGACTCATAATCCAACTCAAAAACAGAGAAAAATAAAGAA 238
            T TATATAACACTGAAATTAA ACTCATAATCCAACTCAAAAACAGAGAAAAATAAAGAA
CBF2    172 TTTATATAACACTGAAATTAACACTCATAATCCAACTCAAAAACAGAGAAAAATAAAGAA 231

CBF1    239 ACAAT 243
            ACAAT
CBF2    232 ACAAT 236
```

Figure 6.1 **Alignments of *CBF1* and *CBF2* predicted protein sequences and promoters**

CBF1 and *CBF2* protein sequences share a 96% amino acid identity **[A]**. To determine the promoter sequences of *CBF1* and *CBF2,* sequences 1,500bp upstream of both their transcriptional start sites (TSS) were compared using the NCBI nucleotide BLAST tool. Only 243bp immediately upstream of the TSS were comparable. These promoter regions share an 87% sequence identity. Neither promoter contains a CCAAT-box motif, either in the forward or reverse orientation **[B].** Alignments were performed using Geneious software.

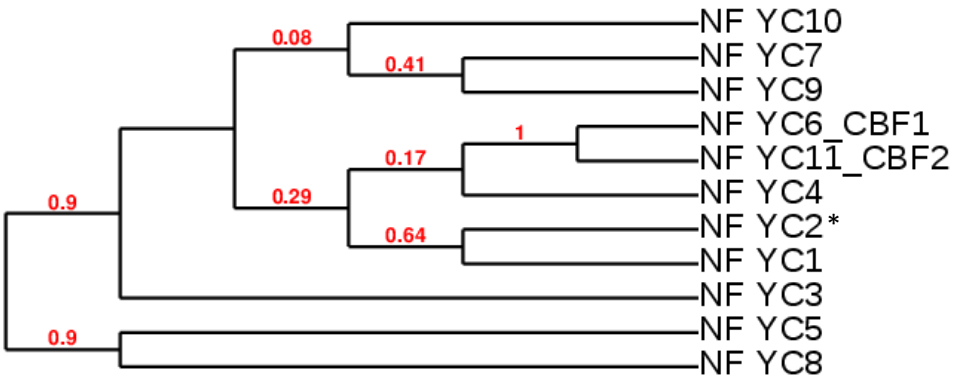
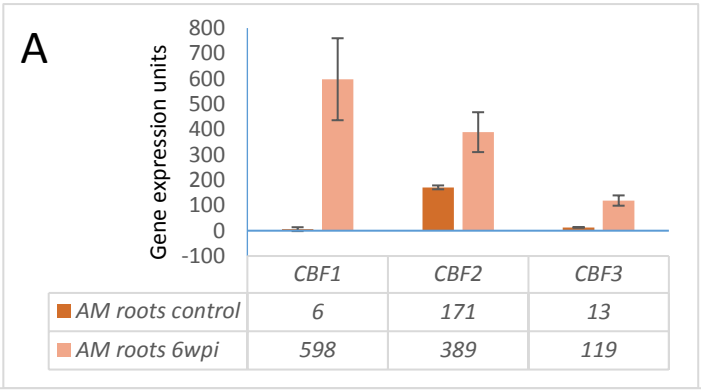


Figure 6.2 **Phylogenetic tree of *M. truncatula* NF-YC subunits**

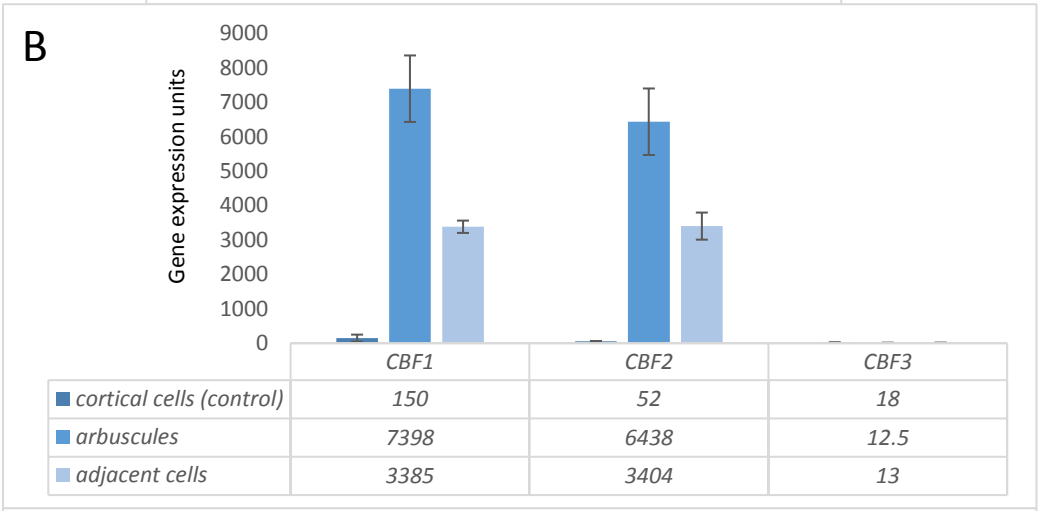
A tree was constructed based on protein sequences using the Phylogeny.fr server. Red numbers refer to branch support values for each branch. *CBF1* and *CBF2* are paralogous genes. *induced by rhizobial infection (Breakspear et al, 2014)

6.2.2 *CBF1* and *CBF2* have overlapping expression during AM colonisation

At the start of my PhD, the gene expression data available for roots colonised with AM fungi was limited to data for partially dissected roots (Gomez et al, 2009). Since then, microarray analyses have been done using laser capture microdissection (LCM) on specific cell types, at different stages of colonisation (Gaude et al, 2011; Hogekamp et al, 2013). This revealed that both *CBF1* and *CBF2* are expressed in whole roots, arbuscules and adjacent cortical cells (Figure 6.3A, B). The expression of these two *NF-YC*s is almost identical across both LCM-based experiments. *CBF1* and *CBF2* are both up regulated in cortical cells when the root contains either AM hyphae or arbuscules, suggesting that they are responding to the presence of the fungus rather than any particular developmental feature. This expression pattern matches the promoter:GUS analysis which showed that the transcripts of both genes overlap, preceding the path of fungal colonisation from the epidermis to the cortex (Hogekamp et al, 2011). By 28dpi, when the root is well colonised with arbuscules, epidermal expression ceases. *CBF1* had stronger GUS staining in arbuscules compared to adjacent cells, whilst in *CBF2* they were comparable.

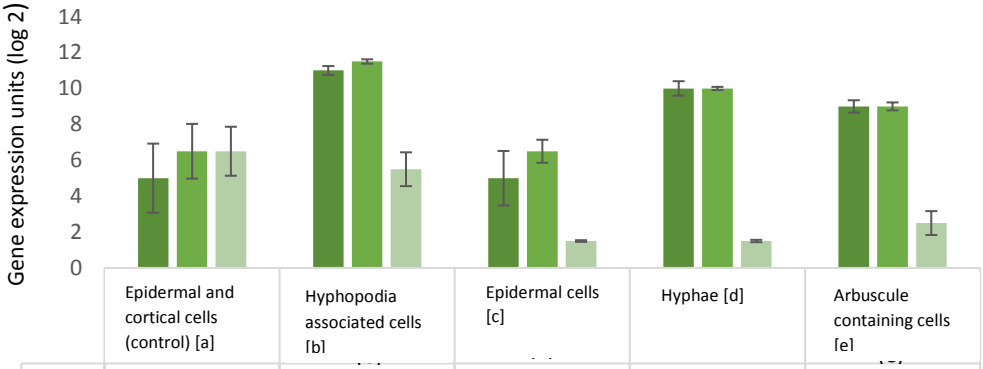


	CBF1	CBF2	CBF3
AM roots control	6	171	13
AM roots 6wpi	598	389	119



	CBF1	CBF2	CBF3
cortical cells (control)	150	52	18
arbuscules	7398	6438	12.5
adjacent cells	3385	3404	13

C



	Epidermal and cortical cells (control) [a]	Hyphopodia associated cells [b]	Epidermal cells [c]	Hyphae [d]	Arbuscule containing cells [e]
CBF1	5	11	5	10	9
CBF2	6.5	11.5	6.5	10	9
CBF3	6.5	5.5	1.5	1.5	2.5

Figure 6.3 ***CBF* expression during AM colonisation**

Microarray data taken from the Medicago Gene Expression Atlas (MtGEA). (Gomez et al, 2009) **[A].** LCM data (Gaude et al, 2011) **[B].** LCM of **(a)** epidermal and cortical cells from non-colonized areas of mycorrhizal roots; **(b)** epidermal and adjacent cortical cells from areas with visible appressoria **(c);** epidermal cells from root areas containing mature mycorrhizal structures; **(d)** cortical cells from roots containing hyphae; **(e)** cortical cells from roots containing arbuscules (Hogekamp et al, 2013**) [C].** Bars show standard error of the mean.

6.2.3 The *cbf1* mutant may have a defect in the AM symbiosis

The mycorrhizal promoter:GUS expression analyses of the two NF-YCs (*CBF1* and *CBF2*) was published by the soon after I studied the phenotypes of the *cbf1* mutant (Hogekamp et al, 2011). The root hair infectome data (Breakspear et al, 2014) suggested that in addition to a possible role in the AM-symbiosis, these genes might function in nodulation. A collaboration was formed with the Kuster group, on the understanding that my work on nodulation could complement their research on these genes during mycorrhization.

6.2.4 The *cbf1* mutant has an inconsistent nodulation phenotype

The initial nodulation assay of *cbf1* revealed a 36% reduction in nodule number three weeks after rhizobial inoculation (Figure 4.7A). I repeated this assay using a homozygous sibling line (*cbf1(3)*) and was unable to confirm the phenotype (data not shown). Since nodule number can vary greatly between experiments, I then repeated this assay another four times, each time using several different sibling lines. In these subsequent assays, nodules were classified as white (non-fixing) and pink (nitrogen fixing) nodules. Two of these assays are shown and highlight the inconsistency of the nodulation phenotype of *cbf1* (Figure 6.5). It is notable that the significant reduction in nodule number initially found for *cbf1(11),* (Figure 4.7A), was not confirmed (Figure 6.5B).

The different phenotypes observed in the same sibling line *cbf1(11),* suggests that the reduction in nodules is not a consequence of a lack of *CBF1* transcription. This was explored further by a nodulation assay in a *cbf1* segregating population. A quarter of the plants did not survive, suggesting that a lethal background mutation was segregating within the *Tnt1* line. The mean number of nodules in the plants homozygous for the *cbf1* mutation, and in the heterozygotes were no different from the plants with no transposon insertion *CBF1* (Figure 6.4).

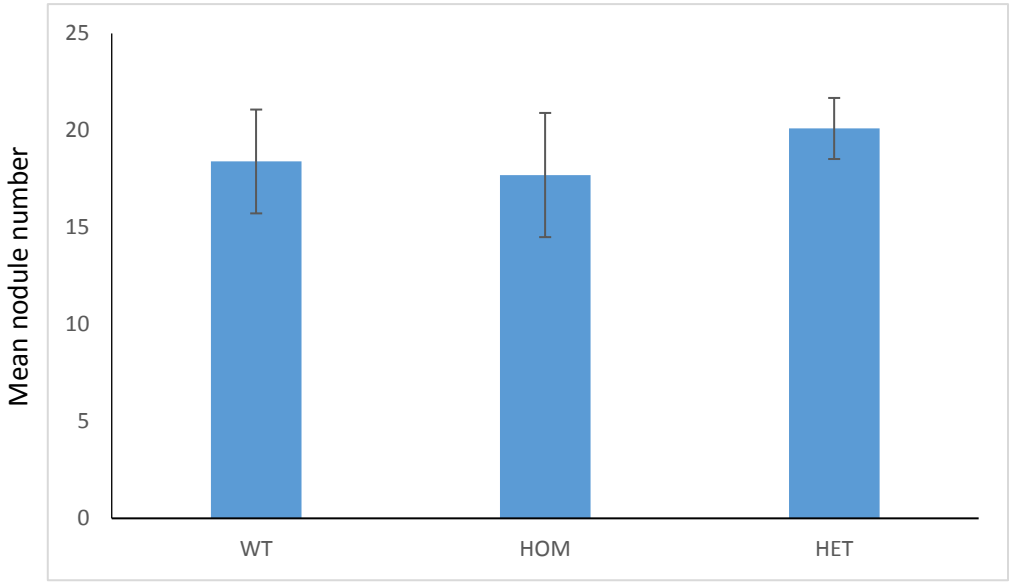


Figure 6.4 **Nodulation assay of *cbf1* segregating population**

Nodules were quantified 3wpi with *S. meliloti* (n=74). No difference in nodule number was observed, showing that the phenotype does not co-segregate with the knock out of *CBF1*.

6.2.5 Expression of CBF1, CBF2, and CBF3 in nodulation and AM interactions

6.2.5.1 CBF1 and CBF2 have overlapping expression during nodulation

Microarray data available in the lab indicated that all the CBFs (NF-Ys) that I am studying show expression in root hairs during early rhizobial infection. However, this study was limited to very early events in the epidermis and could not discriminate whether the expression is in all root hairs or just those infected by rhizobia. As part of the collaboration, I used promoter:GUS constructs for all three CBFs (acquired from the Kuster lab) to study their expression pattern during nodulation. Both *CBF1* and *CBF2* had promoter activity that was tightly linked to infection. Using the *lacZ*-expressing strain of *S. meliloti* 1021, with X-Gal staining to visualise the bacteria, I could identify GUS expression in the root hairs concomitant with the infection of rhizobia in the extending infection threads (Figures 6.5A, B; 6.6A, B). This appeared to follow the course of rhizobial infection from the epidermis to the inner cortex, with GUS expression also occurring in the adjacent epidermal and cortical cells (Figures 6.5C, D;

6.6C). In the nodule primordia, it was very strong for both *CBF1* and *CBF2*, and was restricted to the area where rhizobia would be present, and not the nodule periphery (Figures 6.5E; 6.6D, E). In mature, differentiated nodules, GUS expression was highest in the meristematic zone, and was less strong in the infection zone (Figures 6.5F; 6.6F). The only difference in expression was in lateral root tips: *CBF1* consistently showed GUS expression, whereas none was detected for *CBF2* (Figure 6.8).

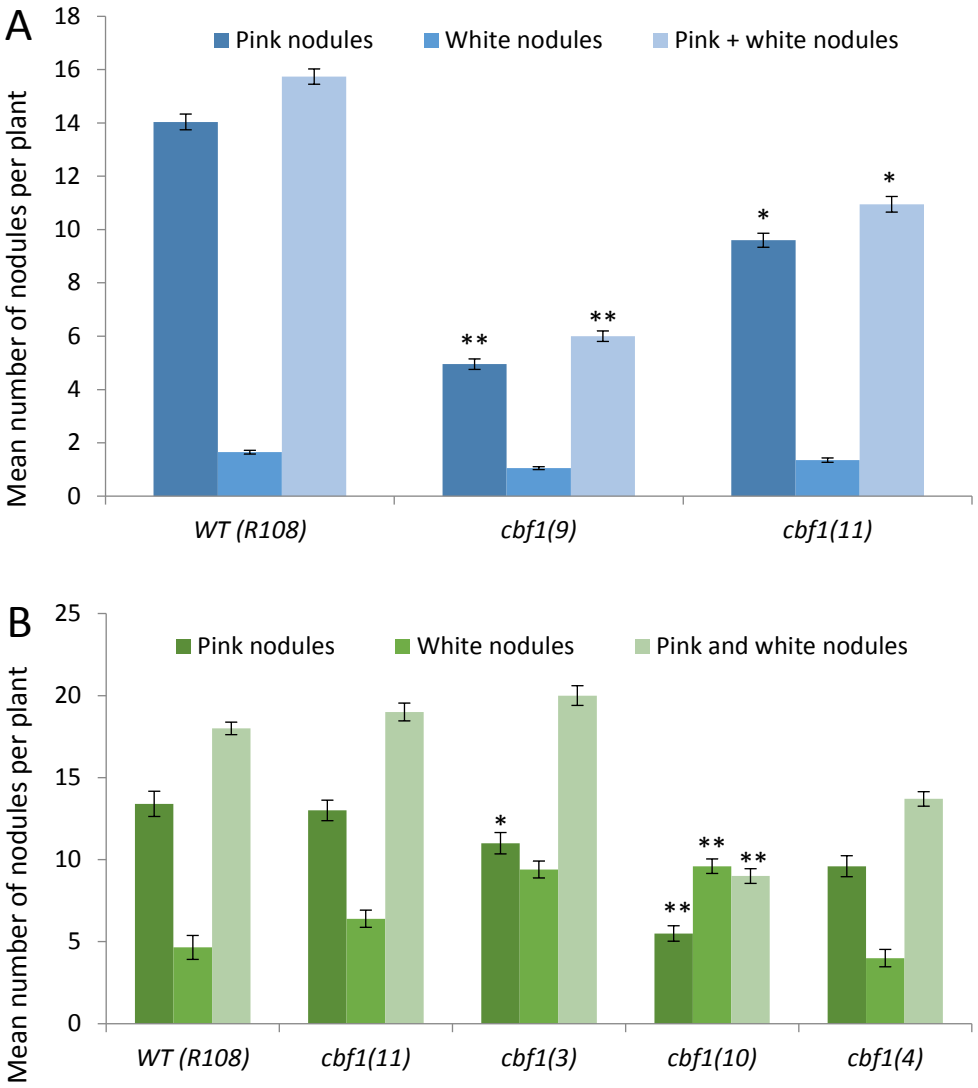


Figure 6.5 **Nodulation phenotype of different *CBF1* sibling lines**

Two separate nodulation assays with *CBF1* sibling lines **[A, B].** Three weeks after inoculation with *S. meliloti,* plant roots were checked for the number of white or pink nodules. Pink nodules are correlated with nitrogen fixation. The numbers in brackets refer to the different sibling lines from the original R1 seed. Number of plants ≥20. Bars represent the standard error of the mean. *p=≤0.05 **p=≤0.01

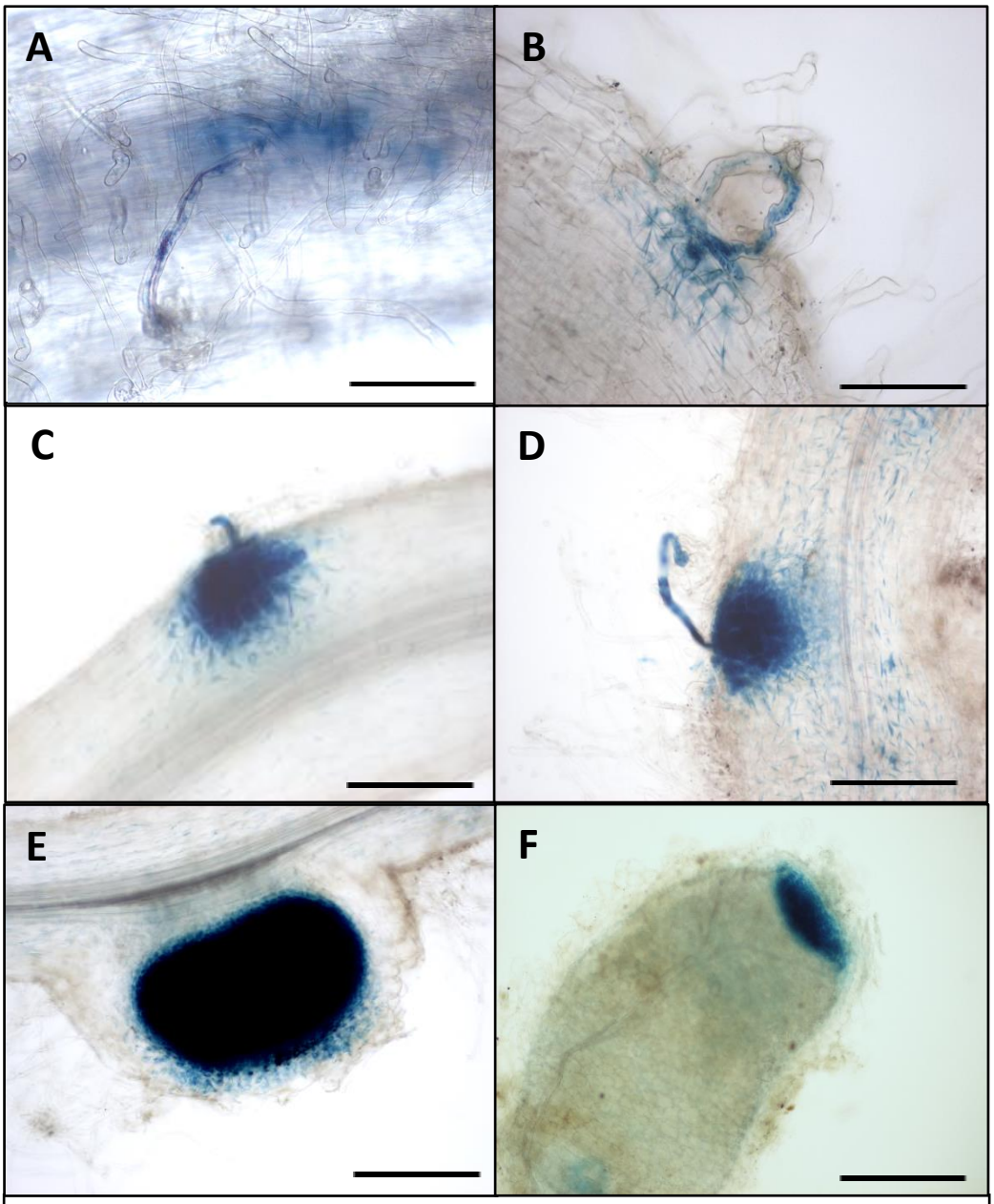


Figure 6.6 **Expression of *CBF1* during root hair infection and nodule development**

M. truncatula A17 WT hairy roots containing prom:GUS expression (blue). Roots inoculated with *S. meliloti* 1021 strain expressing *lacZ* were stained using X-Gal (magenta) **[A]**. *CBF1* is expressed in root hairs with rhizobial infection **[A-D]**. Expression spreads to nearby epidermal and cortical cells **[C, D]**. Nodule primordia **[E]**; mature nodule **[F].** Images were taken at 7dpi **[A-E]** and 26dpi **[F]**. Scale bars = 500µm **[A-D];** 250µm **[E, F]**

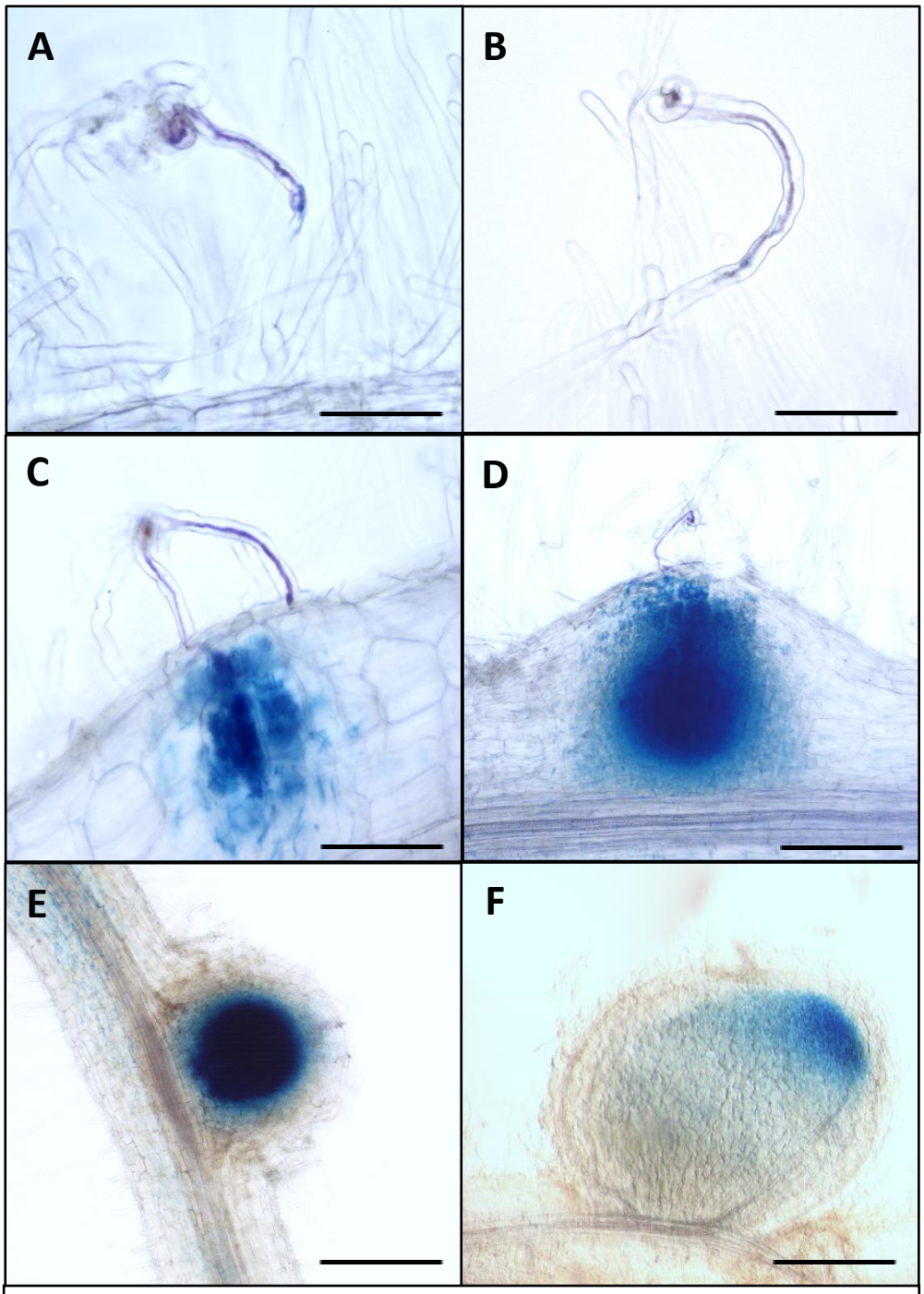


Figure 6.7 **Expression of *CBF2* is correlated with infection of *S. meliloti***

M. truncatula A17 WT hairy roots expressing prom*CBF2*:GUS. Roots inoculated with *S. meliloti* 1021 strain expressing *lacZ* and stained using X-Gal (magenta) **[A-D]**. *CBF2* has transcript accumulation in root hairs closely associated with rhizobial infection **[A, B]**. This spreads initially to nearby epidermal and cortical cells **[C]**, then is present throughout the nodule primordia **[D, E].** It is restricted to the meristem and infection zone of mature nodules **[F]**. Scale bars 1000µm **[A-C]**; 500µm **[D]**; 250µm **[E, F]**.

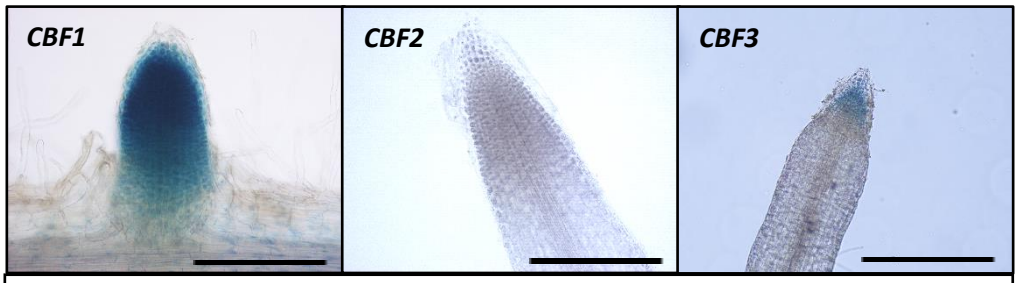


Figure 6.8 **Expression of the *CBFs* in lateral root tips**

M. truncatula A17 WT hairy roots promoter:GUS expression using the *CBF1* promoter **[A],** *CBF2* **[B]** and *CBF3* **[C]** in lateral root tips. Scale bars = 250µm.

6.2.5.2 *CBF3* is induced by LCOs

Compared to only eight NF-YAs in *M. truncatula,* the NF-YB family has greatly expanded to nineteen members (Figure 6.9). To date *LjNF-YB1* (orthologue of *MtNF-YB16*), is the only NF-YB subunit that has been implicated in symbiosis (Soyano et al, 2013). According to microarray data publicly available on MtGEA, *CBF3* is the only *M. truncatula* NF-YB that has expression in roots during AM colonisation. However, five out of the nineteen NF-YBs are not on the gene chip, including *NF-YB16*, so there may be more. Although *CBF3* appears to not be as highly expressed in AM colonised roots as *CBF1* and *CBF2*, it is nine fold induced (Figure 6.3A). However, the LCM data indicates that it is not expressed in cells containing arbuscules or associated with hyphae (Figure 6.3B, C). It therefore, does not appear to be directly associated with the presence of AM fungi in the same way as *CBF1* and *CBF2.* A promoter:GUS assay for *CBF3* during mycorrhization did not yield any GUS expression (Kuster, H.-personal communication).

Nod-LCOs		nsMyc-LCOs		sMyc-LCOs		s-nsMyc-LCOs	
6h	24h	6h	24h	6h	24h	6h	24h
9	1.8	3	1	3.5	1	5.6	1
s= sulphated; ns=non-sulphated (Czaja et al, 2012)							

Table 6.1 **Fold change expression of *CBF3* in response to LCOs**

Most interesting is the expression of *CBF3* in response to lipochitooligosaccharides (LCOs), the initial signalling molecules from the rhizobial and AM fungal symbionts to the plant host (Czaja et al, 2012-Table 6.1). *CBF3* is induced by both Myc and Nod LCOs after 6 hours of treatment, an effect which disappears by 24 hours. There is a greater fold induction in response to Nod-LCOs, and this strong response was also seen in root hairs in response to purified Nod factors, showing the highest expression of *CBF3* across all tissues and treatments in the public database (Breakspear et al, 2014). Also, the sulphated/non-sulphated mix of myc LCOs induces *CBF3* more than either one individually (Czaja et al, 2012). This early response to LCO treatment is unique to any *M. truncatula* NF-Ys, and sets *CBF3* apart. On this basis, the Kuster lab will revisit the AM *CBF3* promoter:GUS experiment, concentrating on very early responses.

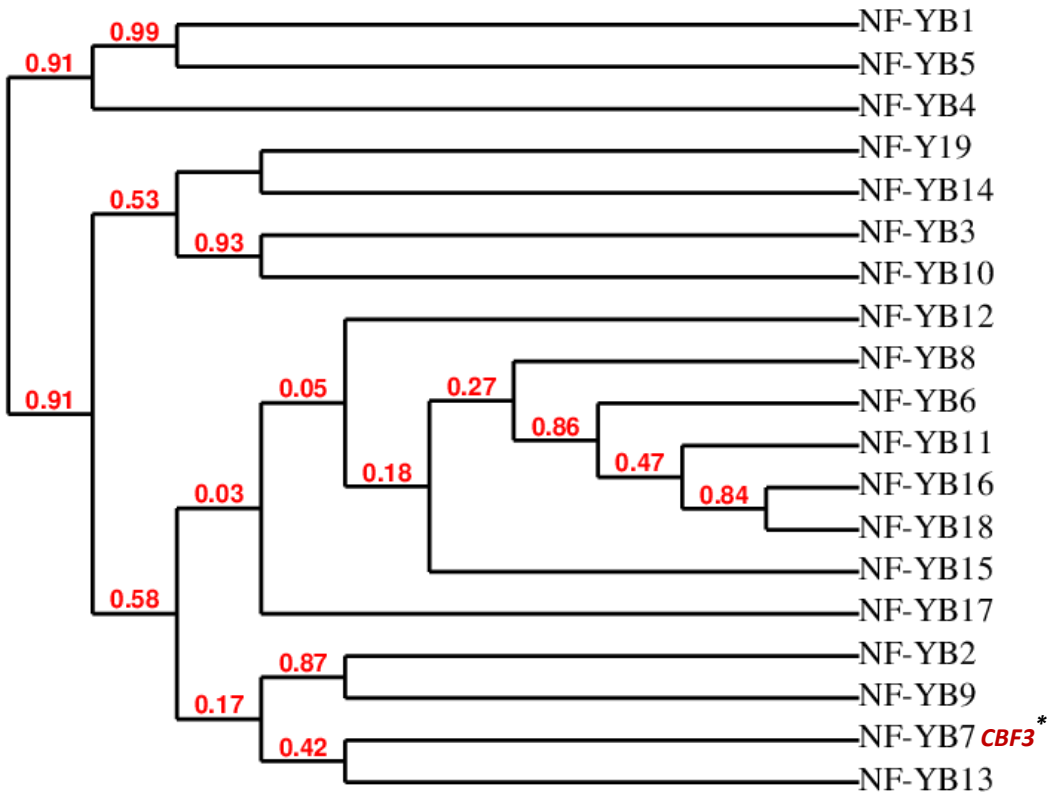


Figure 6.10 **Phylogenetic tree of *M. truncatula* NF-YB subunits**

A cladogram was constructed based on protein sequences using the Phylogeny.fr server. Red numbers refer to branch support values. * induced by rhizobial infection (Breakspear et al, 2014).

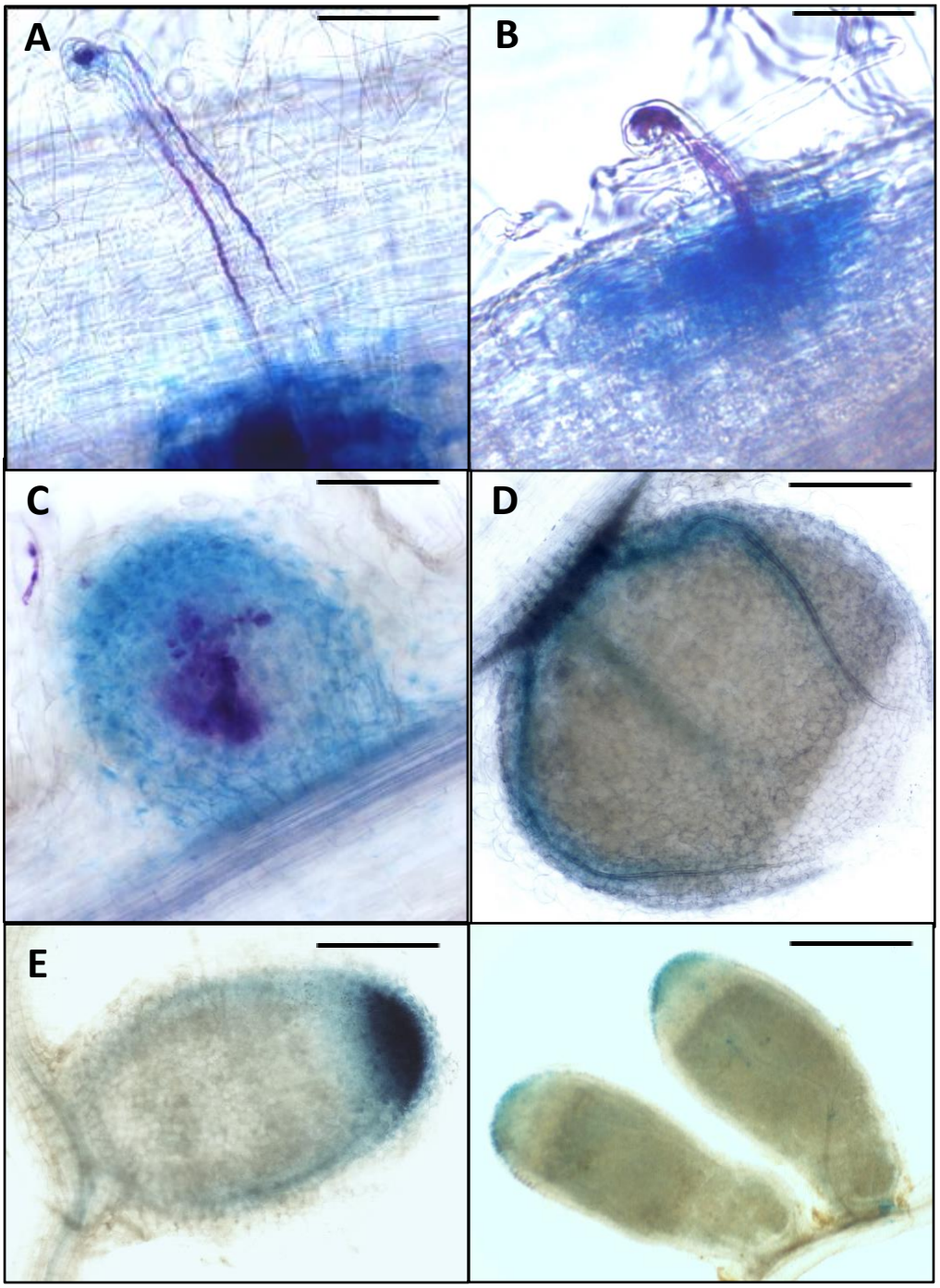


Figure 6.10 **Expression of *CBF3* in early rhizobial infection and nodule development**

M. truncatula A17 WT hairy roots prom:GUS expression (blue). Roots were inoculated with *S. meliloti* 1021 strain expressing *lacZ* (magenta). *CBF3* is expressed in root hairs undergoing rhizobial infection **[A, B]**. Expression spreads to underlying epidermal and cortical cells **[A, B]**. In nodule primordia, expression is diffuse over the whole structure **[C]**. In intermediate sized nodules (2wpi), expression is restricted to the nodule vasculature and in the root vasculature that immediately contacts the nodule **[D]**. As nodules mature and start to differentiate, *CBF3* transcription polarises to the meristematic and infection zones (26dpi) **[E, F]**

6.2.5.3 *CBF3* is expressed during rhizobial infection

I used a *CBF3* promoter:GUS construct provided by the Kuster lab to observe expression during rhizobial infection and nodule organogenesis. The assay showed that its expression during early infection is directly comparable to that of *CBF1* and *CBF2;* GUS expression was detected in root hairs containing infection threads (Figure 6.10 A, B).

Once rhizobia reached the base of the root hairs, expression occurred in the epidermal cells that were closely associated with the infected root hair (Figure 6.10 A, B). Like the other *CBFs,* the expression moved down into the cortex immediately below the infected root hair closely associated with the presence of rhizobia (Figure 6.10A, B). During nodule development, the expression of *CBF3* diverges from that of *CBF1* and *CBF2.* In the nodule primordia, where the expression *CBF1* and *CBF2* is still tightly linked to the presence of rhizobia, in *CBF3,* expression is diffuse over the whole nodule tissue, even where rhizobia are absent (Figure 6.10C). During nodule growth, *CBF3* expression is confined to the nodule vasculature and that of the root which is directly associated with it (Figure 6.10D). When the nodule matures and begins to differentiate, expression polarises towards the apex of the nodule; moving away from the vasculature and settling in the meristematic and infection zones (Figure 6.10E, F).

6.2.6 The nodulation phenotypes of CBF3

6.2.6.1 The *cbf3* mutants have a delay in nodulation

CBF3 exhibits non-specific AM expression and the *cbf3* mutant does not exhibit a mycorrhizal phenotype. On the other hand, the preliminary nodulation phenotype described in Figure 4.7, its rhizobial-induced root hair expression, and strong Nod-LCO response suggests a role for *CBF3* in nodulation. To confirm the nodulation phenotype, another phenotyping experiment was done at 2 wpi. For this experiment there were only seed available for *cbf3-2,* and *cbf3-3*. Both alleles had a significant reduction in the number of nodules at this time point (Figure 6.11A, $p<0.05$, $n\geq15$). Interestingly, *cbf3-2*, which had the weakest phenotype previously had the greatest reduction (50%), and *cbf3-3* had a 30% reduction. It is possible this variation in the strength of their nodulation could be due to some residual expression of the gene which could lead to the production of a partly functional protein. To test this possibility reverse transcriptase (rt) PCR was used, which showed that transcription of *CBF3* is absent in all three mutant alleles (Figure 6.12).

Once I obtained seed from bulking the *cbf3* alleles, I carried out a nodulation time course from 1-3 weeks post inoculation (wpi). This was to identify whether the reduction in nodules was consistent over time. This experiment was attempted three times, as I was unable to replicate the phenotype I had already secured in the two initial assays. Initially, I considered that the lack of phenotype could be due to fungal contamination, as the WT controls had a lower number of nodules than expected, so was considered unrepresentative, and some roots had an orange colour. On the second assay, the only reduction observed was for *cbf3-2* at one week after inoculation. However, at this time point, it is often difficult to distinguish nodule primordia from lateral root primordia. To address this issue, on the third attempt, I used the *lacZ*-expressing *S. meliloti* 1021 strain which enabled me to identify with certainty the nodule primordia, as I could visualise the infecting bacteria. This assay (n=≥17) showed a significant (50%) reduction in nodule primordia for *cbf3-1* and *cbf3-2* at 1wpi, compared to WT controls. However, at two and three weeks, the number of nodules present was no different than the control plants (Figure 6.11B)

6.2.6.2 The *cbf3* mutants have fewer infection threads after rhizobial inoculation

Due to the strong expression of *CBF3* in root hairs during rhizobial infection, and its early response to Nod-LCOs, I carried out an assay to score infection thread formation in the *cbf3* mutants. WT, *cbf3-2* and *cbf3-3* plants were inoculated with lacZ expressing *S. meliloti* 2011. At 6 dpi with *S. meliloti*, roots were stained with X-Gal and infection events were counted using the following categories: a) infection foci (entrapment of bacteria within the curled root hair; b) infection threads within the root hair cell; c) ramifying threads (those that have grown past the epidermis and bifurcated). These quantitative observations were to establish whether infection was disrupted at particular developmental time points. A significant reduction in the number of infection threads per plant compared to controls was seen in both alleles (Figure 6.13A), and the number of infection foci was reduced in *cbf3-3*. All the observed infection events were comparable to WT plants, with no signs of aborted threads, such as seen in the *nf-ya1* mutant (Laporte et al, 2014).

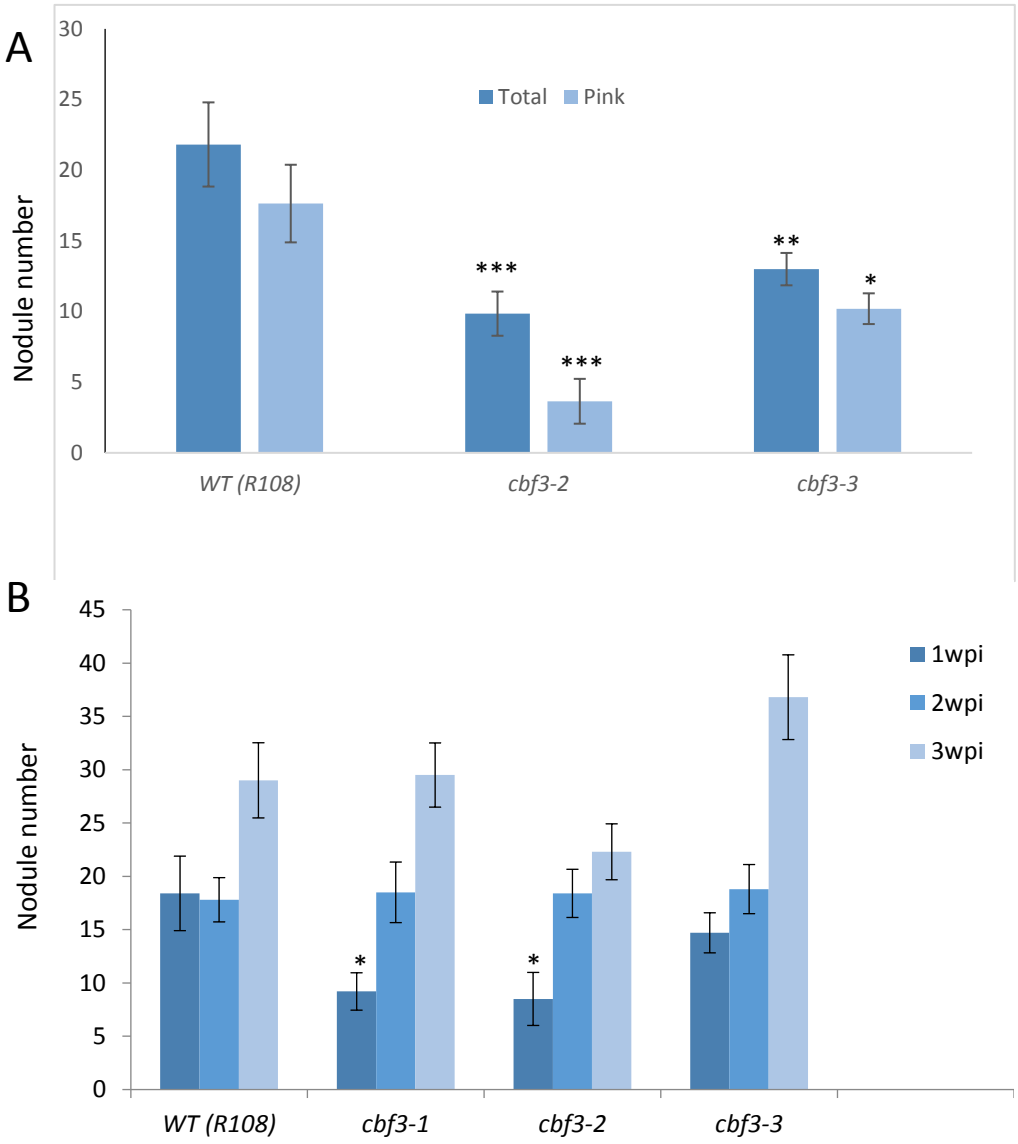


Figure 6.11 **Nodulation phenotype of *cbf3*.**

Plants were inoculated with *S. meliloti* 1021 in three separate repeat experiments. Total number of nodules and pink nodules were counted after 2wpi with two *cbf3* alleles (n=≥15) **[A]**. A three week time course with all three alleles only showed a reduction in nodulation at 1wpi **[B].** Results are from the third experiment, and are representative of all three biological repeats. Bars depict the standard error of the mean. *=p≤0.05 **=p≤0.01 ***=p≤0.001

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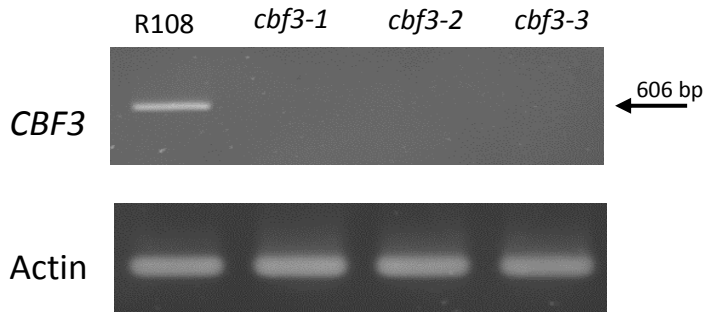

Figure 6.12 ***CBF3* is not expressed in *Tnt1* insertion alleles**

RT-PCR of full length *CBF3* mRNA and Actin (housekeeping gene) in wild type R108 and the *cbf3* mutant alleles. RNA extracted from AM colonised roots.

To confirm the infection thread phenotype of *cbf3*, the infection thread assay was repeated with all three alleles. After an inconsistent phenotype had been shown in the later nodulation assays, it was of paramount importance that I discover whether the infection thread phenotype is robust and reliable. For this reason, twice as many roots were scored than previously (n=20) when seed from all three alleles were available. This experiment showed a dramatic reduction in infection events for all *cbf3* alleles (Figure 6.13B). These reductions were far greater than the initial infection thread assay. Student's t-test was used to determine significance in both instances and it yielded much lower p values in the second experiment, presumably due to using twice the number of plants per allele. The results of this repeat experiment confirm that *CBF3* is required for the normal number of infection events during rhizobial symbiosis; this phenotyping protocol is thorough and robust. However, despite the reduced number of infections and delay in nodulation, the *cbf3* mutant eventually yields a WT number of normal-looking nodules.

6.2.6.3 Overexpression of *CBF3* has no effect on nodule number

NF-Ys work in a complex, requiring three different subunits. It follows that over expression of just one subunit may not enhance transcriptional effects and yield a phenotype. However, two recent papers show how both a wheat NF-YA and a *Picea wilsonii* NF-YB that were over expressed in *Arabidopsis* had an impact on stress tolerance (Ma et al, 2014; Zhang et al, 2015). With this in mind, a *CBF3* over expression construct, using the *Medicago* ubiquitin promoter, was made using Golden Gate cloning and introduced into *M. truncatula* A17 via *A. rhizogenes* hairy root transformation to create a composite plant. Plants were inoculated with *S. meliloti* and nodules counted 3 wpi. There was no difference in nodule number or nodule morphology between the plants expressing the over expression construct and the control expressing an empty vector (Figure 6.14).

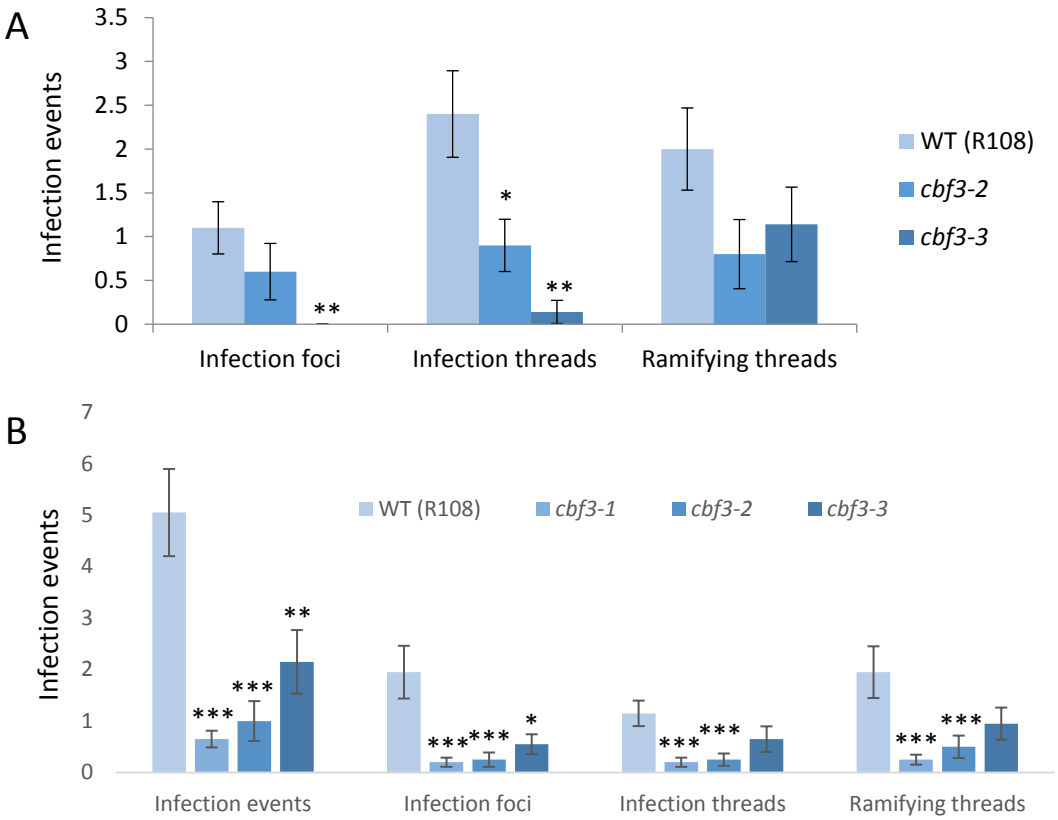

Figure 6.13 **Infection thread phenotype of *cbf3* insertion alleles**

M. truncatula WT R108 and *cbf3 Tnt1* insertion alleles were inoculated with *S. meliloti* 2011 strain expressing *lacZ* and scored at 6dpi. Two independent experiments were done, the first with 2 alleles (n=10) **[A]** and the second with 3 alleles (n=20) **[B]**. Roots were stained using X-Gal (blue) and infection events were quantified into three groups: infection foci, infection threads in root hairs, and ramifying infection threads in the cortex. Bars show standard error of the mean. *=p≤0.05; **=p≤0.01; ***p=≤0.001.

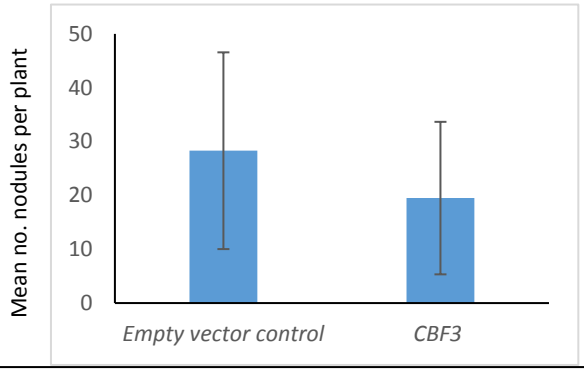

Figure 6.14 **Effect of overexpression of *CBF3* on nodulation in transgenic hairy roots**

M. truncatula plants were transformed with prom*UBQ-CBF3.* Plants were inoculated with *S. meliloti* and nodules counted 3wpi (n=≥26). Bars depict the standard error of the mean.

6.2.6.4 Expression of all *M. truncatula* NF-Ys during symbiosis

The *CBFs* studied in this thesis have expression in both AM and rhizobial infection, and require other subunits to complete a functional heterotrimeric complex. To get a more complete picture of which *M. truncatula* NF-Ys might act in which symbiosis and with which partners I compiled a table of expression data for all available family members based on the root hair infectome (Breakspear et al, 2014) and the LCM RNAseq data from the different zones in *M. truncatula* nodules (Roux et al, 2014). This table was published in the article 'Identification of a core set of rhizobial infection genes using data from single cell-types' (Chen et al, 2015 appendix ; Table 6.2).

During root hair infection *NF-YA1, NF-YA2, CBF3*, and *NF-YC2* are all up regulated, including in response to Nod factors. This fits with the stronger infection thread phenotype reported when both *MtNF-YA1* and *MtNF-YA2* are knocked down, compared to *MtNF-YA1* alone (Laloum et al, 2014). AN RNAi knockdown of *NF-YC2* did not yield an infection phenotype unless its paralogue, *NF-YC1* was also suppressed. The nodule data for Table 6.2 is summarized in Figure 6.14. The NF-Ys already implicated in nodulation that I have introduced, all stand out as being extremely highly expressed in nodules

(Roux et al, 2014-Figure 6.14). The homologues of each subunit are also expressed in the same nodule zones (Table 6.2). *NF-YA1* and *NF-YA2* are both predominantly expressed in the meristem and infection zones, *NF-YB16* and *NF-YB18* in the meristem and distal infection zone, and *NF-YC2* and *NF-YC1* are ubiquitous throughout the nodule. These expression patterns indicate that in the nodule the predominant NF-Y complex may consist of *MtNF-YA1/B16/C2*, and their homologues. The expression of *CBF3* in the nodule is very low compared to the other reported *NF-Y* subunits we know are required for normal nodulation (*NF-YA1, NF-YA2, NF-YC1, NF-YC2*), as well as *NF-YB16* and its homologue *NF-YB18*. *CBF2* is also expressed at a very low level in nodules. Unfortunately there is no nodule RNAseq data available for *CBF1*, but given the homology and overlapping promoter-GUS expression during both AM colonisation and rhizobial infection, *CBF1* and *CBF2* are likely to be comparable.

6.3 Discussion

In this chapter, I examined the symbiotic roles of three NF-Y genes, *CBF1* (*NF-YC6*)*, CBF2* (*NF-YC11*)*,* and *CBF3* (*NF-YB7*). In collaboration with the Kuster lab, I have studied their role in nodulation, to complement their efforts researching the mycorrhizal aspect. As discussed above, all three *Medicago* genes that have been implicated in nodulation, *NF-YA1*, *NF-YB16*, *and NF-YC2*, have close homologues. Both *NF-YA1, NF-YA2* and *NF-YC2, NF-YC1* are functionally redundant, but it is not known whether the same is true for *NF-YB16, NF-YB18. CBF1* and *CBF2* are tandemly duplicated genes. Duplicated genes are initially surplus to requirements and, therefore, have the flexibility that allow mutations to occur without deleterious effects to the organism. The conserved amino acid sequences of *CBF1* and *CBF2* suggest that they perform a similar function. The close homology of *CBF1* and *CBF2*, and their overlapping expression patterns, and the

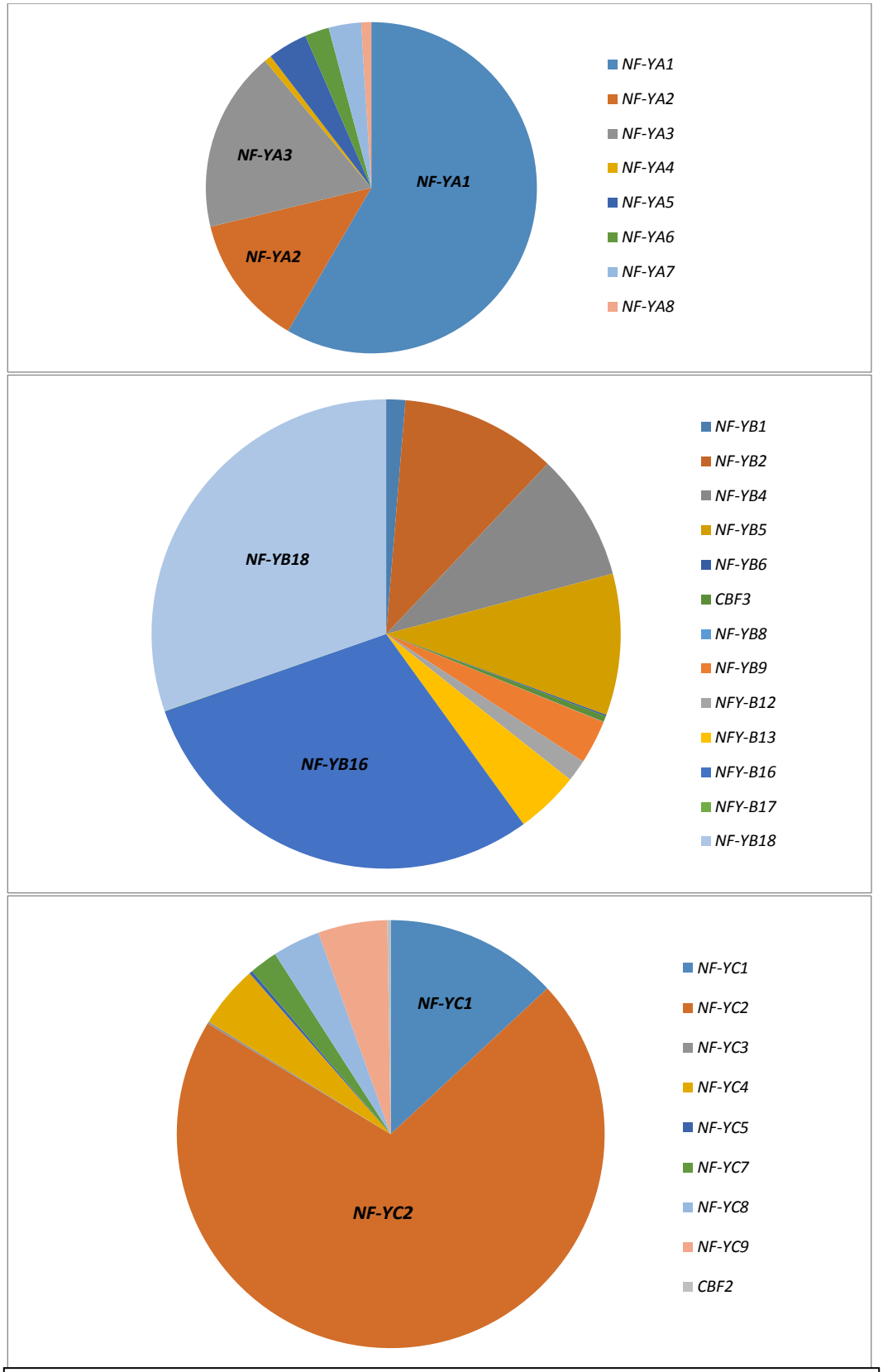

Figure 6.14 **Proportion of expression of *M. truncatula* NF-Y subunits in nodules**

The total number of RNAseq transcripts of each NF-Y subunit in root nodules (data used from Roux et al, 2015) was used to show which NF-Y genes are potentially the most influential in nodules, according to expression. Proportion of expression is shown in each subunit.

compensatory expression of *CBF2* in the *cbf1* mutant, suggests that *CBF2* can complement, to some degree, the *cbf1* mutant. Although there is no clear reduction in AM-colonisation in the *cbf1* mutant, other phenotypes (the reduction in expression of the arbuscule-specific phosphate transporter gene *PT4*, and reduced shoot growth) indicate that there is a negative impact on normal AM symbiosis, perhaps due to reduced nutrient acquisition. However, a non-inoculated control was not used, so the effect might be independent of the symbiosis, and since this data is from a single mutant allele, the AM phenotypes themselves could be due to a background mutation(s) and should be considered preliminary. A second allele would help resolve this issue. Nonetheless, it appears that the NF-Y complex has a role in AM infection (Soybean reference).

Despite *CBF1* having expression in infected root hairs, the initial nodulation phenotype of *cbf1* could not be repeated consistently. A segregating population analysis indicates that the phenotypes observed are not due to a mutation in *cbf1.* It is possible that it could be the result of a mutation in a background gene. We know that *CBF2* has increased expression in *cbf1* compared to WT roots during AM colonisation (H. Kuster-personal communication), but we don't know how consistent this is. This, along with their close sequence homology, suggests that *CBF2* plays a redundant role in the *cbf1* mutant. A double knock out of *CBF1* and *CBF2* would allow us to discover whether they have a nodulation phenotype and, therefore an important role in nodulation. This issue is complicated by the fact that *CBF1* and *CBF2* are closely linked, which means that obtaining a *cbf1 cbf2* double mutant through traditional crossing methods is not feasible. CRISPR/Cas9 technology may be the best approach to produce a double mutant.

The promoter:GUS expression analysis of *CBF1* and *CBF2* are comparable to each other during both nodulation and AM colonisation. This is consistent of the high conservation between the promoter sequences. The conservation is surprising, and may reflect the presence of conserved *cis* regulatory motifs that could act as promoter elements. Since this sequence is immediately upstream of the start site, all or part of it is likely transcribed as a 5' UTR. It is therefore possible that this region encodes upstream open reading frames (uORFs) such as in *NF-YA1*, or forms complex secondary structure to regulate translation. It would therefore be interesting to dissect the role of this upstream sequence.

In both symbioses the expression is tightly associated with the presence of the symbionts. This suggests that *CBF1* and *CBF2* may have comparable roles during the infection of AM fungi and rhizobia. The promoter:GUS expression observed during AM colonisation (Hogenkamp et al, 2011) agrees with the LCM data available on the gene atlas, where both genes have up regulated transcripts in cells that either have, or are adjacent to fungal structures. Similarly, the promoter:GUS expression I observed during rhizobial infection concurs with both the published data on root hairs and nodule expression.

CBF3 (*NF-YB7*) expression appears to be very similar to that of *CBF1* and *CBF2* during rhizobial infection, but not during AM infection or in mature nodules. *CBF3* is not expressed in arbuscules and the *cbf3* mutant has no mycorrhizal phenotype. However, there is up regulation in epidermal cells when visible appressoria are present, suggesting an early response to AM (Figure 6.3C).

The two initial nodulation assays with the *cbf3* mutants gave a consistent reduction in nodules per root; but this phenotype was not robust (Figure 6.11). However, the infection thread phenotype was consistent over two independent experiments (Figure 6.13). The second experiment, which used three different mutant alleles and 20 plants per allele, showed a strong reduction of infection events in *cbf3*. This shows that *CBF3* is required for the normal stages of early infection.

The key to understanding the role that *CBF3* plays in symbiosis may be its very early expression in response to LCOs. No other *M. truncatula* NF-Y gene on the gene chip has a response to LCOs at the 6 hour time point in whole roots (Table 6.1-Czaja et al, 2012). In root hairs, *CBF3* is responsive to 10nM Nod factors (LCOs) at 24 hours after inoculation (Breakspear et al, 2014). In that experiment, the up regulation is accompanied with the up regulation of four NF-YAs, (*NF-YA1*, *NF-YA2, NF-YA4, NF-YA5*), and *NF-YC2* (Table 6.2). This makes these subunits the best candidates as interacting partners with *CBF3* during rhizobial infection. Unfortunately, there is no root hair expression data for either *NF-YB16* or *NF-YB18* in response to Nod factors, as they were not represented on the gene chip. However, an RNAseq analysis on whole roots has shown that these NF-YBs are also responsive to Nod factors by 6 hpi (Larrainzor et al, 2015).

Over expression of *CBF3* did not change nodule number (Figure 6.13). I believe that, had I had enough time, observing the infection thread phenotype in the over expression lines would have been more informative, as this is the where I see a phenotype in the

mutant. The only similarity in expression pattern of *CBF3* for both AM and rhizobial symbiosis is the response to LCOs at very early time points, which suggests it might have an analogous role in early AM fungal infection. Considering the robust infection in the *cbf3* mutant, a transcriptomics study of root hair expression at early time points of rhizobial infection may have illuminated what genes *CBF3* regulates.

			Root Hairs [1] UP/DOWN=significantly changed compared to control (p≤0.05)					RNAseq normalized[2]	Percentage of transcripts expressed in each nodule zone[2]				
gene symbol	**Mt4.0v1**	**Affymetrix probeset**	**1 dpi**	**3 dpi**	**5 dpi**	***skl***	**NF**	**total reads LCM**	**Meristem**	**DZII**	**PZII**	**IZ**	**ZIII**
NF-YA1	Medtr1g056530.1	Mtr.43750.1.S1_at	UP	UP	UP	UP	UP	33950.1	26.3	33.6	27.2	8.5	4.4
NF-YA2	Medtr7g10645.2	Mtr.1584.1.S1_at	UP		UP	UP	UP	7449.9	17.3	45.6	31.5	4.7	0.9
NF-YA3	Medtr2g041090.2	Mtr.42674.1.S1_at						10279.9	8.3	24.2	18.4	14.6	34.4
NF-YA4	Medtr2g099490.1	Mtr.51561.1.S1_at					UP	404.4	17.3	10.4	9.9	24.1	38.2
NF-YA5	Medtr3g061510.2	Mtr.40999.1.S1_at					UP	2264.1	22.9	24.2	22.2	13.7	17.1
NF-YA6	Medtr2g030170.1	Mtr.44133.1.S1_at						1371	25.3	24.5	15.2	18.4	16.6
NF-YA7	Medtr8g037270.1	Mtr.5583.1.S1_at						1845.3	5.6	6.4	5.1	13.5	69.5
NF-YA8	Medtr8g019540.1	Mtr.34979.1.S1_at						573.1	42	35.1	11.2	4.1	7.5
NF-YB1	Medtr2g056000.1	Mtr.40530.1.S1_at				DOWN	DOWN	375.5	20.8	20.9	17.7	24.2	16.4
NF-YB2	Medtr7g100650.1	Mtr.38058.1.S1_at						3075.4	16.3	13.1	27.7	26	17
NF-YB3	Medtr4g133952.1	Mtr.2632.1.S1_at						NA					
NF-YB4	Medtr4g052950.1	Mtr.42266.1.S1_at						2506	44.6	25.8	9.3	8	12.2
NF-YB5	Medtr4g112380.1	Mtr.14243.1.S1_at						2782	20.2	21.3	15.6	22.9	20
NF-YB6	Medtr1g088860.1	Mtr.28326.1.S1_at						29.7	56.7	40.1	3.2	0	0
NF-YB7	Medtr8g091720.1	Mtr.4282.1.S1_at	UP				UP	114.9	2.9	41.3	51.2	4.6	0
NF-YB8	Medtr4g133938.1	Mtr.4407.1.S1_at					DOWN	4.5	100	0	0	0	0
NF-YB9	Medtr1g072790.1	Mtr.13328.1.S1_at						863.2	76.5	10.2	2.5	4.6	6.2
NFY-B10	Medtr1g039040.1	Mtr.26183.1.S1_at						NA					
NFY-B11	Medtr1g083070.1	Mtr.36243.1.S1_at						0	0	0	0	0	0
NFY-B12	Medtr3g058980.1	Mtr.37614.1.S1_at				DOWN		431.2	11.8	23.6	9.5	18.6	36.5
NFY-B13	Medtr2g026710.1							1251.2	15.1	29.5	8.6	11.3	35.5
NFY-B14	Medtr5g095900.1							NA					
NFY-B15	Medtr8g093920.1	Mtr.46490.1.S1_at						NA					
NFY-B16	Medtr4g119500.1							8457.6	36.3	51.3	8	1.9	2.6
NFY-B17	Medtr5g095740.1	Mtr.39690.1.S1_x_at						7.1	12.8	78.3	9	0	0
NF-YB18	Medtr0392s0020.1							8659.4	36.8	54	7.9	1	0.3
NF-YB19	Medtr1g029100.1							NA					
NF-YC1	Medtr1g082660.1	Mtr.1034.1.S1_at						3183.3	13.7	15.7	18	22.6	30.1
NF-YC2	Medtr7g113680.1	Mtr.48660.1.S1_at	UP		UP	UP	UP	17175.1	21.2	34.6	12.3	18.8	13.1

NF-YC3	Medtr3g099180.1	Mtr.40976.1.S1_at						39.3	64.2	16.2	9.2	0	10.4
NF-YC4	IMGA_contig_49832_3.1	Mtr.9876.1.S1_at						1148.9	9.7	17.1	18.1	44.4	10.8
NF-YC5	Medtr5g088760.1	Mtr.29974.1.S1_at						61.4	37.5	15.3	9.1	13	25
NF-YC6	Medtr2g081600.1	Mtr.51511.1.S1_s_at						NA					
NF-YC7	Medtr4g059710.1	Mtr.18368.1.S1_at						518.3	35.3	30.3	15.7	7.1	11.6
NF-YC8	Medtr3g012030.2	Mtr.38060.1.S1_at						876.7	17.9	16.2	16.9	27.2	21.8
NF-YC9	Medtr3g085430.1	Mtr.10110.1.S1_at						1273.3	29.3	22.4	8.7	18.5	21.1
NF-YC10	Medtr2g023340.1							NA					
NF-YC11	Medtr2g081630.1	Mtr.16863.1.S1_at						62.6	64.6	26.1	9.3	0	0
Orange-*CBF3;* Blue-*CBF1;* Green-*CBF2;* Grey-not on chip; NA-data not available. Table adapted from Chen et al (2015) **1.** Breakspear et al, 2014; **2**. Roux et al, 2015													

Table 6.2 **Expression of all *M. truncatula* genes encoding CCAAT-box subunits in root hairs of inoculated plants and in nodules**

CHAPTER 7

Main Discussion

The study of the AM and rhizobial symbioses has important agronomical implications for potentially reducing the amount of artificial fertilizers applied to crops. These mutualistic interactions have analogous infection strategies and a suite of common genes. The association with AM fungi is ancient, and this co-evolution has produced a scenario where the AM fungi are dependent on their host to complete their life cycle. In this study, I used a forward and reverse screen approach to identify novel plant genes required for the AM symbiosis. Such studies are important to understand the complex nature of this interaction and how this knowledge can be utilised for agronomical benefits.

7.1 Forward and reverse screens identified mycorrhizal mutants

For the forward screen, I chose to use a relatively small (655 lines) *Tnt1* population which has been allowed to self fertilise over several generations, generating an estimated 8000 homozygous alleles within the whole population. This allowed me to carry out a forward screen in a relatively short space of time. The mutant lines I identified represented plant mycorrhizal genes that had previously been identified. This may be an illustration of the fact that mycorrhizal mutants that have yet to be identified display more subtle phentoypes, where a forward screen is not the most appropriate screening method.

The reverse screen shows that this can be a good way of targeting potential mutants. If a mutant allele for a candidate gene can be sourced, this is a quick way of identifying mutants. However, a phenotype may not be forthcoming (as with *BFP*), and this can make studying such a gene more challenging as it is more difficult to show that it has a role in AM associations.

Other types of screens could also be used. High-throughput sequencing allows the use of Genome wide associations studies (GWAS) to detect natural variation in different *M. truncatula* ecotypes could allow us to corrolate phenotypes with associated genotypes in a natural population. A GWAS population of 226 *M. truncatula* accessions has been produced (Stanton-Geddes et al, 2013). Using this for mycorrhization studies may prove difficult as variations in AM colonization are likely to be subtle between natural

ecotypes. Also the phylogenomics approach, which compares the genomes of AM host plants to non-hosts, can detect gene conservation. This has been done for *M. truncatula* and *M. palealeae* (Delaux et al, 2015; Bravo et al, 2016).

7.2 BFP is an evolutionary conserved protein with microbial origins

Although we have yet to elucidate a mycorrhizal phenotype for the *bfp* mutant, or determine BFP's role in the AM symbiosis, phylogenetic analyses strongly suggest that it is important for AM interactions. This is demonstrated by its conservation throughout AM plant hosts, including extant species of ancient liverworts, and also its loss in plants that can no longer form AM associations, such as *A. thaliana.*

BFP shows no homology to any other plant proteins, except the legume-specific BFPL, which appears to have resulted from a gene duplication of *BFP* that has been modified by the removal of the catalase-like domain. Without this domain, BFP and BFPL have closer homology to fungal and bacterial proteins. However, generally, these proteins share homology with either the AMP-binding or Lpx domains, but not both in a single protein. The two exceptions to this are the ones found in the AM fungi *R. irregularis* and *G. rosea,* and that of the AM pathogen, *S. punctatus* (Figure 5.15). They do not have Lpx domains, but share an AMP-binding domain, NAD/FADH domain and a PP domain. The significance of this is not clear, although it appears to be more than a coincidence. What is also interesting is that the *S. punctatus* protein contains predicted domains that are shared by both the *R. irregularis* homologue and BFP, apart from the lack of Lpx domains.

BFPL differs from BFP in that it has no catalase domain in between the three Lpx domains. The protein sequence of the three Lpx's of BFPL is annotated as a 'C-terminal NRPS domain' in the NCBI BLAST server. NRPS proteins are found in bacteria and fungi which synthesise peptide-containing secondary metabolites, such as antibiotics. It has been proposed that fungal NRPS's initially originated from bacterial genomes, based on their general lack of introns, suggesting that they were derived from bacterial operons (Eisfeld, 2009). Bacterial NRPS's are subject to horizontal transfer between bacteria, including plant endophytes (Nongkhlaw and Joshi, 2016).

Bacterial NRPS's are often long proteins with repetitive sequences, which consist of an AMP-binding domain with predicted amino acid adenylation domains. BFP has an AMP-binding domain that is homologous to fungal and bacterial domains that utilise fatty

acids as a substrate, not amino acids. A hybrid NRPS has been identified in the cyanobacterium *Cylindrospermum alatosporum* that synthesises the cytotoxic lipopeptide Puwainaphycin (Mares et al, 2014). It is possible that BFP derived from a similar protein. As it is the adenylation domain that determines the substrate, and BFP has closer homology to fatty acid synthases, therefore, it seems likely that BFP does modify a fatty acid.

If BFP is the result of horizontal gene transfer, this could have been directly from a bacterial genome, or it may have been transferred initially to AM fungi, and then subsequently to plants. The homology of the AMP-binding domains to *R. irregularis* strongly suggests either that they share a common ancestral gene, or that BFP originates from an *R. irregularis* gene that is no longer present in the *R. irregularis* genome. The most likely scenario is that AM fungi acquired a NRPS-like gene from a bacteria by horizontal gene transfer, possibly from an endophyte. This was then acquired by plants and altered by insertion of a catalase-like domain. However, I was not able to find any homologous protein within the two types of known AM endophytes. Another possibility is that fungi acquired the ancestral bacterial gene by endosymbiosis. Further analyses of NRPS-like genes in bacteria and fungi would need to be undertaken to explore which possibility is the most likely.

During bioinformatic analysis of the Lpx domains, I was unable to find a *R. irregularis* protein that had three Lpx domains; I only hit on proteins that contained one Lpx domain. It would be interesting to further research whether these terminal NRPS-like domains that contain three Lpx's are actually present in AM fungi. If not, it is possible that BFP has originated from a previous AM gene, which has since been lost in the fungus. For AM fungi, mycorrhizal associations are required for the completion of their life cycle. This suggests that they have lost genes whose functions are now performed by the plant host. It is possible that BFP serves such a purpose, and that the homology to the *R. irregularis* AMP-binding domain reflects that this gene was once part of some AM genomes.

Bioinformatic analyses suggest that the catalase-like domain is plant-derived. This may have been added to BFP after its integration into a plant genome, presumably to allow for a specialised function. What is surprising is that this plant-derived domain has somehow been passed to the soil dwelling fungus, *S. punctatus.* It raises the question as

to how the *S. punctatus* gene evolved, and whether the presence of the catalase domain is advantageous for the pathogenesis of AM fungi, whether as a biological function or as a way of mimicking a friendly host plant.

The *S. punctatus* gene may also be a product of horizontal gene transfer. Its protein product has homology to the AMP-binding and catalase domains of BFP. It shares the same predicted domains as the *R. irregularis* protein, but with an added catalase (which bears no homology to other fungal proteins and is closest to BFP). This could indicate that *S. punctatus* initially acquired this from *R.irregularis* by horizontal gene transfer, and further acquired the catalase domain from an AM host plant. *S. punctatus* is a soil-dwelling fungus; there is no evidence to suggest that it is also a plant endophyte. How this fungus was able to gain a plant-specific protein domain is intriguing.

The evolutionary origins of BFP that I have hypothesised, indicate that the catalase domain was added by plants subsequent to acquiring a prokaryotic gene, possibly via AM fungi. The domain architecture of BFPL could initially suggest that BFPL is a progenitor of BFP, as it has no catalase domain. However, BFPL is only present in the legume clade, which diverged 60 million years ago (Lagunas et al, 2015). It actually appears that BFPL is a product of the removal of the catalase domain, and has possibly reverted to a protein that is more similar to the progenitor of BFP.

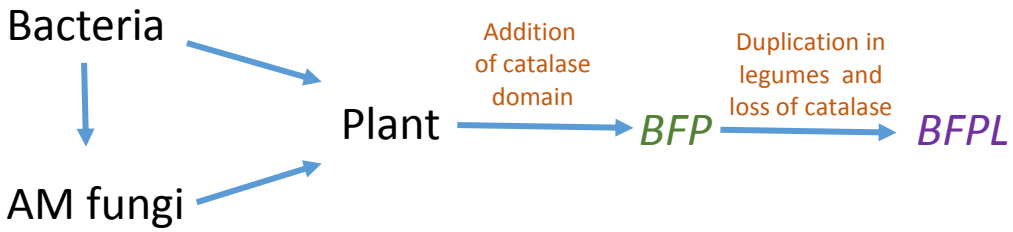

Figure 7.1 **Possible evolution of *BFP* and *BFPL* from a prokaryotic progenitor**

BFP seems likely to have originated from a bacterial gene. This may have directly transferred to plant genomes by endosymbiosis or horizontal gene transfer. Alternatively, this bacterial gene may have transferred initially to AM fungi either by endosymbiosis or horizontal gene transfer. This would probably have been taken up by plants through horizontal gene transfer during AM symbiotic associations. This seems the most likely due to the homology between BFP and *R. irregularis* AMP-binding domains. The addition of a catalase domain by the plant resulted in *BFP.* A duplication of *BFP* during the evolution of the legume clade allowed the removal of the catalase domain to produce *BFPL.*

7.3 The CBFs are NF-Ys expressed during both symbioses

7.3.1 *CBF1* and *CBF2* may have roles in the AM symbiosis

Promoter:GUS experiments suggest that *CBF1* and *CBF2* are transcribed during rhizobial infection concomitant with the presence of bacteria, analogous to that seen during AM colonisation (Hogekamp et al, 2011). This could suggest that these genes are playing a similar role in both symbioses. However, the root hair and nodule expression data (Table 6.2) indicate that the expression is very low, particularly compared to other NF-Y subunits (Figure 6.14). In contrast, expression in AM-infected tissues is relatively strong, and is particularly high in arbuscules. From this it appears that these genes are more important for mycorrhizal associations, but a role in nodulation cannot be ruled out. The *cbf1* mutant did not have an obvious AM or nodulation phenotype, and due to probable functional redundancy with CBF2 we were unable to study its role in the symbioses further. With only one *Tnt1* allele it was difficult to pin-point whether this was due to an insertion in a background gene. The issue is further complicated by the fact that *CBF1* and *CBF2* are tandem repeats, which means that a double *cbf1/cbf2* mutant through traditional crossing methods is not feasible. Recently, the use of the CRISPR/Cas9 approach to knock down genes has become more widespread. The Cas9 nuclease can site-specifically cleave double-stranded DNA at specific sites defined by specific guide RNAs. The subsequent activating of the double strand break repair machinery by non-homologous end joining results in deletions/insertions that disrupt the targeted sequence (Jinek et al, 2012). This would be the best approach of knocking down both genes, as it would result in a knock out, instead of simply a reduction in transcription (knockdown) when using RNAi silencing (Fire et al, 1998). The Kuster lab is currently following up the characterisation of this mutant.

A rhizobial infection phenotype in a *cbf1 cbf2* double mutant would indicate that these C subunits are good candidates as interacting partners in a NF-Y complex with *CBF3* and *NF-YA1/NFYA2.*

7.3.2 *CBF3* is LCO-responsive

CBF3 is significantly up regulated in root hairs 1 dpi after inoculation with rhizobia, but not at later time points. The expression is far higher in response to Nod factors (24 hours post treatment; Breakspear et al, 2014). Another, experiment using whole roots, shows that *CBF3* is induced at 6hrs post inoculation with Nod factors, but not at 24hpi. This suggests that the response to Nod factors is very early. *CBF3* is also responsive to Myc LCOs in whole roots at 6 hpi. The only specific cell types that show an induction of *CBF3* during AM infection are epidermal cells and nearby cortical cells where appressoria are present on the root surface. *CBF3* could be a common symbiosis gene whose role is to respond to the presence of LCOs and regulate early response genes. Mycorrhizal and nodulation assays in three *cbf3* mutant alleles have shown that it is not required for the later stages of either of these symbioses (Figures 4.5; 6.10). Despite the reduction in infection events (at 6 dpi), eventual nodule numbers are not affected. It is possible that *CBF3* is also important during AM early infection events. My collaborators were not able to detect expression in a pr*CBF3*:GUS assay during AM colonisation, despite its up regulation in colonised whole roots. This could illustrate a lack of *CBF3* expression, or a need to repeat the assay at early time points. A very early quantification of appressoria formation and initial penetration events could yield a phenotype in the *cbf3* mutants.

Although expression of *CBF3* is clearly apparent in nodules in the promoter:GUS assay, the actual transcripts in nodules are very low (Table 6.2). This, along with the formation of WT-looking nodules in the *cbf3* mutants strongly suggests that, unlike *NF-YA1, CBF3* does not have a role in nodule development or maintenance.

How does a robust infection phenotype lead to a leaky nodulation phenotype? If the reason were due to some kind of feedback control to compensate for the reduction in infection events, I would expect to see a greater number of attempted infections, as was reported for the *nf-ya1* mutant. In *nf-ya1,* there are many aborted infection threads which rarely reach the cortex. Probably as a result of positive feedback, the number of infection foci are actually greater than WT controls (Laporte et al, 2014). This was not seen in the *cbf3* mutants; the infections looked normal but there were fewer of them. Another hypothesis of this leaky phenotype is based on the assumption that B and C NF-Y subunits are interchangeable with their respective NF-Y counterparts. For example, an excess of a particular B subunit, may be able to partially complement the lack of a different B subunit. This assertion has not been confirmed, but due to the

interchangeable nature of these subunits during yeast hybrid experiments, this is a hypothesis worthy of consideration. If this were the case, in the *cbf3* mutant, there is likely to be other NF-YBs that are present in the root hair cytoplasm which could be co-opted by the NF-Y complex that *CBF3* is part of. The surplus of NF-YBs, that are constitutive in root hairs, could be in variable amounts depending on different enviromental factors, for example. This could reflect why there is was some variablilty in the initial infection thread assay. *CBF3* appears to be the only NF-YB induced in root hairs in response to Nod factors, reflecting its potential importance during early plant-rhizobial communication. However, *MtNF-YB16* is induced in root hairs in the presence of rhizobia at a much greater level than *CBF3* (N. Pauly; personal communication). It seems likely that the lack of a reduction in nodule number could be due to the subsequent presence of *NF-YB16.*

CBF3 is the only *NF-YB* that is induced in response to Nod factors at early time points, and is possibly the only B subunit expressed during this time. In the *cbf3* mutants this, effectively acts as a null mutant during this early response, producing a strong infection thread phenotype. The *L. Japonicus* homologue of NF-YB16, that is hypothesised to form a complex with *NF-YA1* and *NF-YC2 (LjNF-YB1)*, is expressed in infected root hairs (Soyano et al, 2013). This B subunit could take the place of *CBF3* when it is expressed in response to rhizobial infection.

7.3.3 Do the *CBFs* interact in the same NF-Y complex?

The promoter:GUS transcript accumulation of the *CBFs* during nodulation could implicate them in the same complex during the early infection stages. They are all expressed in root hairs and seem to follow the movement of rhizobia into the cortical cell layers. However, the *CBF3* response to Nod factors implicates *NF-YC2* as a better candidate, as it is the only NF-YC that is induced in root hairs in response to Nod factor (Table 6.2).

If all the *CBFs* were involved in both nodulation and AM symbiosis, it seems likely that they would fulfil similar roles in both symbioses and, probably interact with the same subunits. The fact that *CBF1* and *CBF2* are expressed in different cell types during AM, compared to *CBF3,* strongly suggests that they do not interact with *CBF3.* Also, the fact that *CBF3* is Nod factor-responsive, and that *CBF1/CBF2* are not again, makes interactions between them less likely.

7.3.4 Which genes does *CBF3* regulate during early infection?

The role of *CBF3* during infection has yet to be elucidated. Unfortunately, the planned experiment to perform transcriptomics on *cbf3* root hairs had to be abandoned. This data would have helped elucidate which genes that *CBF3* could be directly controlling, and which part of the infection process it influences. Despite the fact that I am left to speculate its role, the understanding of the roles of the NF-Ys known to have a role in nodulation, along with the understanding gleaned from the root hair infectome data, allow me to propose a plausible role for *CBF3.*

In mammals, NF-Ys regulate genes involved in cell cycle progression (Bolognese et al, 1999; Bhattacharya et al, 2003; Benatti et al, 2011). *NF-YA1, NF-YB16* and *NF-YC2* have all been implicated in cell cycle roles either in *M. truncatula,* or their orthologues in other legumes. When the *MtNF-YC2* orthologue in common bean, *PvNF-YC1,* was silenced, it prevented the normal induction of the cell cycle-related genes,*CDC2* and *CDC25* (Zanetti et al, 2010) an also resulted in smaller nodules. In *M. truncatula* the *NF-YA1* mutant also produces smaller nodules lacking a meristem (Laporte et al, 2013). The overexpression of *LjNF-YA1* results in extra cell divisions in lateral root primordia, which is enhanced when overexpressed with *LjNF-YB1* (Soyano et al, 2013). *CBF3* is up regulated in the root tip (X5 fold) (Watson et al, unpublished-MtGEA), which could include the root meristem, a site of extensive cell division (Figure 6.8). The role of cell cycle-related genes during nodule organogenesis seems clear, but what role then could *CBF3* have in rhizobial infection where no cell divison occurs?

The root hair infectome study highlighted the induction of many genes associated with the cell cycle (Breakspear et al, 2014). Specifically, the response to Nod factors in this study show that genes encoding both D-type and C-type cyclins are up regulated in root hairs. These cyclins are both associated with G_1 entry and progression in the cell cycle. Prior to the infectome study, the events preceding and during rhizobial infection have been studied in the outer cortical cells during infection thread formation. Nod factors can induce pre-infection thread (PIT) formation, (the cytoplasmic bridge which forms before infection thread progression) (van Brussell et al, 1992). PITs in the outer cortical cells appear to enter the cell cycle and arrest prior to mitosis (Yang et al, 1994). It has been proposed that the structures that form prior to infection thread formation in root

hair cells are equivalent to PITS (Fournier et al, 2008; Murray, 2016). This points to Nod factors triggering a mitogenic response in root hairs, inducing cell cycle responses from G_1 to S phase; arresting before actual cell division. It has previously been postulated that the cytoplasmic bridge of the PIT is analogous to the phragmosome, a structure seen in highly vacuolated cells prior to mitosis (Brewin, 1991). As *CBF3* is Nod factor responsive and NF-Ys implicated with cell cycle progression, a role in cell cycle responses prior to infection thread formation seems the most likely.

7.3.5 Which subunits interact with CBF3?

It has been shown that *MtNF-YA1, NF-YB16* and *NF-YC2* can form a complex *in planta* (Baudin et al, 2015). *MtNF-YA1* and *NF-YC2* are associated with rhizobial infection and are highly expressed throughout the nodulation process (Breakspear et al 2014; Roux et al, 2014). Based on gene induction in response to Nod factors in root hairs, it is plausible that *CBF3,* a B subunit, forms a NF-Y complex with *NF-YC2,* and one of the induced A subunits (*NF-YA1/A2/A4/A5*), possibly *NF-YA1.*

Based on the promiscuity of NFY subunits in different systems, I propose a scenario where NF-Y subunits are not restricted to just one NF-Y complex within the same developmental role or individual cell. In this scenario the NF-Ys are interchangeable and work in different combinations depending on the tissue and stage of development. Different NF-Y combinations could have different roles (e.g. early responses to Nod factor in the root hair, vs nodule meristem) and multiple combinations may be effective in a single cell at the same time. *NF-YA1*, for example, has very high expression levels from a few hours after inoculation with rhizobia, throughout nodule development and maintenance (Roux et al, 2014; J. Lilley and K. Scheissl, personal communication). It is plausible that these high transcripts allow *NF-YA1* to be part of more than one complex at any one time.

It is known that the NF-YA subunit is responsible for sequence-specific binding to CCAAT-box motifs on promoter/enhancer regions. However, there must be some other way that certain CCAAT motifs are targeted by NF-Y complexes. For example, *MtNF-YA1, NF-YB16* and *NF-YC2* preferentially target the third CCAAT motif of the five present in the *ERN1* promoter (Baudin et al, 2015). It is tempting to suggest that the combinations of different subunits available to plant NF-Ys may play a part in this

specificity. However, in mammals, a similar situation occurs where specific CCAAT motifs are targeted in different situations on the promoter/enhancer of the same gene (Dolfini et al, 2012). It is possible that nucleotide sequences flanking the CCAAT motif may have a role. It is also plausible that the deviant histones (NF-YB/NF-YC) have a part to play in this specificity. Their role in the complex seems to be to compete with their core histone counterparts in order to make certain genes transcriptionally active, as has been shown in other histone variants (Felsenfeld and Groudine, 2003).

7.3.6 What role do NF-Ys have in AM?

The expression of *CBF1*, *CBF2* and *CBF3* genes in mycorrhizal roots points to a role for the CCAAT-box transcription factors in the AM symbiosis. Evidence of cell division in cortical cells during AM colonisation have yet to be published. However, the pre-penetration apparatus (PPA) in epidermal and inner cortical cells are thought to be analogous to pre-infection threads (PITs). Nuclear enlargement and cytoplasmic aggregation form in similar ways, which results in the formation of membrane into tubular-like infection structures.

A role for *CBF3* in LCO responses and possible regulation of cell cycle cells to initiate the (PIT), could have an analogous role in AM, as *CBF3* is also responsive to Myc LCOs.

CBF1 and *CBF2* are expressed in epidermal cells, arbuscules and adjacent cells; all which form PPA structures. Promoter:GUS transcript accumulation of these genes indicates that they are induced in these cells on AM-hyphal contact before cell entry, which places them at the scene of PPA formation.

7.4 Conclusions and suggested further experiments

I have identified a novel NF-YB (CBF3) that is responsive to LCOs and required for the early stages of rhizobial infection. The lack of a nodule phenotype means that this gene would not have been identified in a normal forward nodulation screen. The root hair infectome data enables the study of early root hair responses in the presence of rhizobia. Given the apparent relationship between the pre-penetration apparatus and pre-infection threads, understanding the role of CBF3 in rhizobial-infected roots hairs may also help to further our understanding of the AM infection processes.

Due to the huge expansion of NF-Y subunits, and the conserved homology of their interacting domains, it is challenging to demonstrate that particular subunits form a complex for a particular function. The closest to achieving this goal in the symbiosis field was the study that demonstrated that *MtNF-YC2* and *MtNF-YB16* preferentially interact with each other in *N. benthamiana,* as opposed to interacting with the native subunits. They did this by observing nuclear localisation of *NF-YB16* in the presence of *NF-YC2* (Baudin et al, 2015). Most B and C subunits in *M. truncatula* are able to interact with each other in yeast, the exception being the *LEC-like* NF-YBs which are specifically expressed in seeds (Baudin et al, 2015). One approach to test my hypothesis that *CBF3* forms a complex with *NF-YC2* and *NF-YA1* would be to undertake a yeast 2-hybrid analysis using different combinations of the subunits and co-immunoprecipitation (CoIP) to show whether the interaction between the three subunits is possible *in planta*. If *NF-YC2* does form a dimer with both *NF-YB16* and *CBF3* in different complexes, like I propose, it would be interesting to do a competition experiment of some kind. For example, to replicate the *in planta* nuclear localisation experiment I described (Baudin et al, 2015), but add both B subunits with different coloured fluorophores. It would be interesting to see whether *CBF3* and *NF-YB16* are localised to the nucleus with *NF-YC2* equally, or whether there is a preferential interaction.

In this study I have developed a protocol for a mycorrhizal inoculum which is effective, cheap to produce, allows for large scale bulking and free from contaminating microorganisms. I have shown an AM –phenotype for a strigolactone mutant in *M, truncatula, Mtmax4,* for the first time. And, along with the forward screen, identified an allele of the ATP^{+}ase, *MtHA1,* for which I provided a detailed histochemical characterisation of the phenotype (Wang et al, 2014; appendices). I have identified a unique AM-specific gene that encodes a protein that points to a role in modifying fatty acids for mycorrhizal interactions.

I have also shown that two CCAAT-box transcription factors already implicated in AM interactions, *CBF1* and *CBF2* (Hogekamp et al, 2011), are expressed during rhizobial infection. This study has highlighted that these NF-YC subunits could possibly be common infection genes.

I have identified a new CCAAT-box transcription factor, *CBF3 (NF-YB7)* required for normal infection with rhizobia. This could interact with already identified symbiotic NF-Ys, or be part of a new complex. I propose that an NF-Y complex involving *NF-YA1, CBF3*

and *NF-YC2* form in the early response to Nod factors. Subsequently, *CBF3* could compete with *NF-YB16* in the complex which could explain the lack of nodulation phenotype at later stages (Figure 7.2).

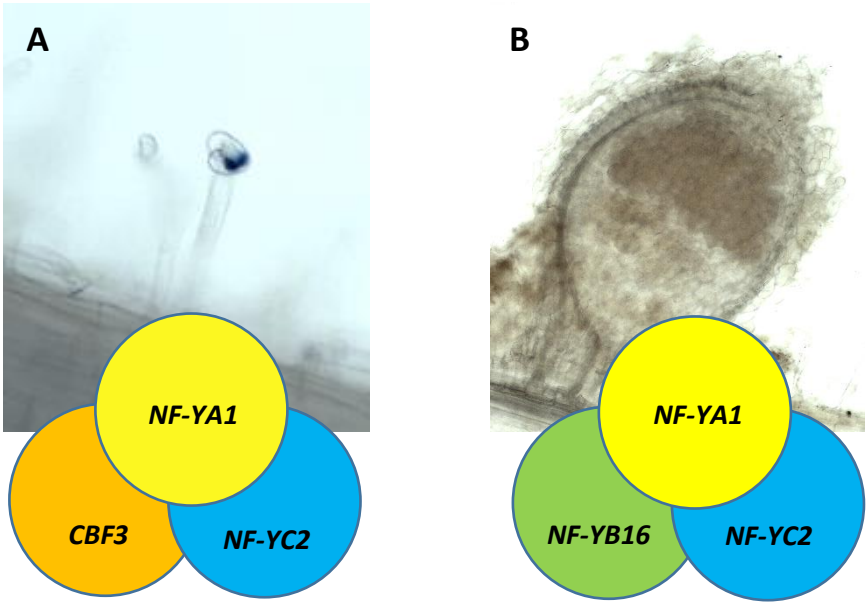

Figure 7.2 **Proposed NF-Y complexes involved in different stages of nodulation in *M. truncatula***

Based on expression data in root hairs, a complex involving *NF-YA1/CBF3/C2* is proposed to form in response to Nod factors (this study) **[A]**. During nodule development, *NF-YB16* forms a complex with *NF-YA1* and *NF-YC2* (Baudin et al, 2015) **[B]**.

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Publications

During the course of this thesis the following manuscripts have been published:

Murray, J.D., Cousins, DR., Jackson, KJ., Liu, C**.** (2013) **The Role of Cutin Monomers in Mycorrhization.** Molecular Plant, 6(5), 1381-1383.

I helped edit the paper prior to sending to the journal.

Wang, E., Yu, N., Bano, S. A., Liu, C., Miller, A. J., Cousins, D.Schultze, M. (2014). **A H + -ATPase That Energizes Nutrient Uptake during Mycorrhizal Symbioses in Rice and Medicago truncatula.** *The Plant Cell*, *26*, 1818–1830.

I provided images for Figure 6A, as well as some supplementarty figures.

Chen, D., Liu, C., Roy, S., Cousins, D., Stacey, N., & Murray, J. (2015). **Identification of a core set of rhizobial infection genes using data from single cell-types.** *Frontiers in Plant Science*, *6*(July), 575.

I provided a supplementary table of NF-Y expression in root hairs and nodules (Table 6.2 in this thesis).

Breakspear, A., Liu, C., Cousins, D. R., Roy, S., Guan, D. and Murray, J. D. (2015) **The Role of Hormones in Rhizobial Infection, in Biological Nitrogen Fixation** (ed F. J. de Bruijn), John Wiley & Sons, Inc, Hoboken, NJ, USA. doi: 10.1002/9781119053095.ch56

I wrote the section 56.4 – 56.7 concerning the role of jasmonic acid in nodulation.

In preparation:

A Plant Nonribosomal Peptide Synthetase-like Protein Expressed in Mycorrhiza in the *Medicago truncatula-Rhizophagus irregularis* Symbiosis

Donna Cousins, Jeremy D. Murray

This publication will cover work reported in Chapter 5 of this thesis, highlighting the evolutionary aspects of BFP in AM symbiosis.