Molecular characterization of plant mutants (*Medicago truncatula*) defective in mycorrhiza formation

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Abstract

Arbuscular mycorrhizas play a key role in plant growth by supporting the acquisition of mineral nutrients, especially phosphorus. Around 80 % of vascular plants exhibit this association. The major aim of research work was to isolate plant genes that are related to mycorrhizal association by using a mutant screening approach. Several plant genes involved in the arbuscular mycorrhizal association have been identified in the past decade. However these genes are also involved in the association of another type of symbiosis that is nodulation. In order to identify genes that are specifically involved in mycorrhiza formation, independent of nodulation, a transposon mutagenized population of *Medicago truncatula* was screened for reduced mycorrhizal colonization in the presence of normal nodulation. Two mutants showing reduced colonization were identified. In the presence of colonised host plants in the same growth compartment the number of intraradical hyphae and vesicles was normal, but arbuscules did not fully develop. This suggests that the block in colonization is not absolute. The genes affected in both mutant lines have been isolated. One mutant is affected in a GRAS transcription factor that is likely to act in the mycorrhiza-specific transduction of fungal signals. The other is affected in a mycorrhiza induced H^+-ATPase that is likely to be involved in generating an electrochemical gradient at the periarbuscular membrane to facilitate nutrient uptake at the fungus/plant interphace.

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Abbreviations

Abbreviations	Full	Notes
AM	Arbuscular mycorrhiza	
AMS	Mycorrhiza specific	
AM^{+}	Up-regulated during mycorrhizal association	
Arb^{-}	Absence of arbuscules	
Ard^{-}	Arbuscule development abnormal	
CCaMK	Calcium and calmodulin dependent protein kinase	
cm	Centimetre	Unit
CTAB	cetyltriethyl ammonium bromide	Chemical
dmi3	Does not make infection	Mutant
DNA	Deoxyribonucleic acid	
dNTP	Deoxy nucleotide triphosphate	
E. coli	*Escherichia coli*	
EDTA	Ethylene diaminetetraaceticacid	Chemical
EST	Expressed sequence tags	
Fd/incomp.arb	Fully developed/Incompletely developed	
FNB2 & FNB4	Fast-neutron-mutants	Mutants
g/l	Gram/litre	Unit
GAI	Gibberellic acid insensitive	
GUS	β-glucuronidase	Protein
H	Internal hyphae	
Hz	Heterozygous	
Ici^{-}	Absence of inner cortex invasion	
Incomp. arb	Incompletely developed arbuscule	
Kb	Kilo base pairs	
KOH	Potassium hydroxide	Chemical
LB	Liquid broth	
LHR	leucine heptad repeat	
M	Mutant	
M, mM	Molar, millimolar	Units
mm, nm	Millimetre, nanometre	Units
µM, nM	Micromolar, nanomolar	Units
M. truncatula	*Medicago truncatula*	Plant
Myc−	No fungal structures	
Nod^{+}/Fix^{+}	Nod/nitrogen fixation phenotype of mutant	

Nod−	Absence of nodules	
Nt	Non template control	
Pen−	Appressoria present, but no penetration	
PCR	Polymerase chain reaction	
Pi	Inorganic phosphorus	
ram1	Reduced arbuscular mycorrhiza	Mutant
Red	reduced	
RGI	Repressor of GAI	
RLC	Root length colonization	
RNA	Ribonucleic acid	
SCR	Scarecrow	
Tm	Melting temperature	
Tris	Trisodium citrate	Chemical
V	Vesicle	
v/v	Volume/volume	
Wt	Wild-type	
X-Gal	5-Bromo-4-chloro-3-indolyl-beta-D galactopyranoside	Chemical

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Author’s declaration

The work in this thesis is my own, except where otherwise stated. Mutagenized seeds were obtained by Noble foundation, Ardmore OK, USA. Primary mutant screening was done by Dr. Michael Schultze, while i had started my project from secondary mycorrhizal screening. I identified one mutant allele in a transposon mutagenized line of *M. truncatula*, the other allele was identified in John Inne’s Centre. I also identified a mycorrhiza related gene in another transposon mutagenized line.

Chapter 1

Introduction

1 Introduction

Plants need mineral nutrients for the improvement of their growth and development. Many different types of mineral nutrients like nitrogen, phosphorus, calcium, magnesium, sulphur, copper, zinc, iron, boron etc. are required for healthy plants. Most of these are present in the soil in available form, however a large part of some of the nutrients like phosphorus are present in the unavailable form in the soil because phosphorus forms complexes with other substances present in the soil. These complex phosphorus compounds are not soluble in water. Arbuscular mycorrhizal associations in plants play a key role to make the poorly soluble mineral nutrients especially phosphorus and some others available to the plants (Bolan, 1991). Plants provide carbohydrates to the fungus in return for these nutrients. Mycorrhizas also promote resistance of plants to root pathogens and abiotic stresses (Smith, 2003) and enhance drought tolerance (Subramanian et al., 1995). Decreased pathogen development in mycorrhizal root system is associated with accumulation of phenolics and plant cell defense responses (Cordier et al., 1998). Some fungi play an important role in sustainable agriculture by providing nutrients to the plants having mycorrhizal association (Jeffries, 1987). Soil texture is improved as a result of hyphal network spreading out from plant roots in soil by AM fungi (Bethlenfalvay and Schuepp, 1994).

1.1 Requirement of phosphorus for plants

Phosphorus is found in two forms in soil, which are organic and inorganic. A Few examples of organic phosphorus are plant residues, manures and microbial tissues. The amount of organic phosphorus in soil ranges from 5−90% of total soil phosphorus. Sources of inorganic phosphorus (Pi) include complexes of iron and aluminium phosphate, phosphorus absorbed on clay particles and apatite (the original source of all phosphorus). These organic and inorganic phosphorus compounds exhibit reduced solubility in water. The phosphate concentration in the soil solution can be less than 10 µM due to rapid adsorption of phosphate ions to clay and organic matter (Bieleski, 1975; Holford, 1997). Hence a lesser amount of phosphorus is available for absorption by roots, while the Pi concentration in the living plant cells is within millimolar range. Two ionic forms of phosphorus that can be absorbed by

plants are $H_2PO_4^-$ and HPO_4^{2-}. These phosphate ions have more reactivity with the soil particles and hence become part of soil particles by fixation process (Bache, 1964). The locking up of nutrients in the soil is the driving force for symbiotic relationship. This fixed form of phosphorus cannot be used by the plants. Mycorrhizal fungi help in the acquisition of phosphorus for the plants that support this symbiotic association (Bolan, 1991; Smith and Read, 2008). Organic sources of phosphorus (e.g. phytic acid and nucleic acids) are also acquired by mycorrhizal fungi to convert it to the available form for the plants (Jayachandran et al., 1992). The phosphatase enzyme released by the extraradical arbuscular mycorrhizal hyphae breaks down the bonds which are present between various elements of the organic phosphorus (Joner et al., 2000). Hence the majority of the plants having mycorrhizal association are better in growth as compared to non-mycorrhizal plants (Feng et al., 2003 ; Smith and Read, 2008; Tarafder and Marschner, 1995).

1.2 Mycorrhizal symbiosis

AM symbiosis increases the area of soil from which the plants can access mineral nutrients as the fungal hyphae grow into areas which the plant roots have not accessed (Smith and Read, 2008). After the germination of fungal spores, some morphological changes (eg, fan like structures of hyphae and increase in hyphal length) occur in the hyphae which increase the possibility of contact between hyphae and host roots. The biochemical (chemical composition) and topographical properties (configuration of the surface) of the host root cell wall help in the formation of AM fungal appressoria (Giovannetti et al., 1993; Nagahashi and Douds, 1997). The fungal hyphae grow through the plant roots by passing through the root epidermal, exodermal and cortical cell layers to reach the inner cortex (Fig 1.2). The arbuscules, the symbiotic functional units are formed in the cortex (Fig. 1.1). The host plasma membrane invaginates and proliferates around the arbuscule (Alexander, 1989; Bonfante-Fasolo, 1984; Gianinazzi- Pearson, 1996; Harrison, 1999). These studies suggested a 3.7-fold increase in host plasmalemma as a result of which the periarbuscular membrane is formed. In this way a new apoplastic space is created between periarbuscular membrane and the arbuscule, called the periarbuscular space (Bonfante and Perotto, 1995; Harrison, 1997) (Fig. 1.1). This creates a symbiotic interface which helps in the transport of nutrients to the plant roots and

photosynthates from plant to the fungus. Schoknecht and Hattingh (1976) and Cox et al. (1980) suggested that the phosphate transport between the fungus and the plant occurs at the periarbuscular membrane. The movement of phosphorus from the soil to the plant occurs through fungal hyphae (Pearson and Jakobsen, 1993; Sanders and Tinker, 1971; Smith and Gianinazzipearson, 1988) and the phosphate is translocated as polyphosphates. Before flowing outward from arbuscule to the peri-arbuscular space, the polyphosphates are degraded to phosphate (Cox et al., 1980; Solaiman et al., 1999). Different stages of development of arbuscular mycorrhizal symbiosis are shown in figure 1.2.

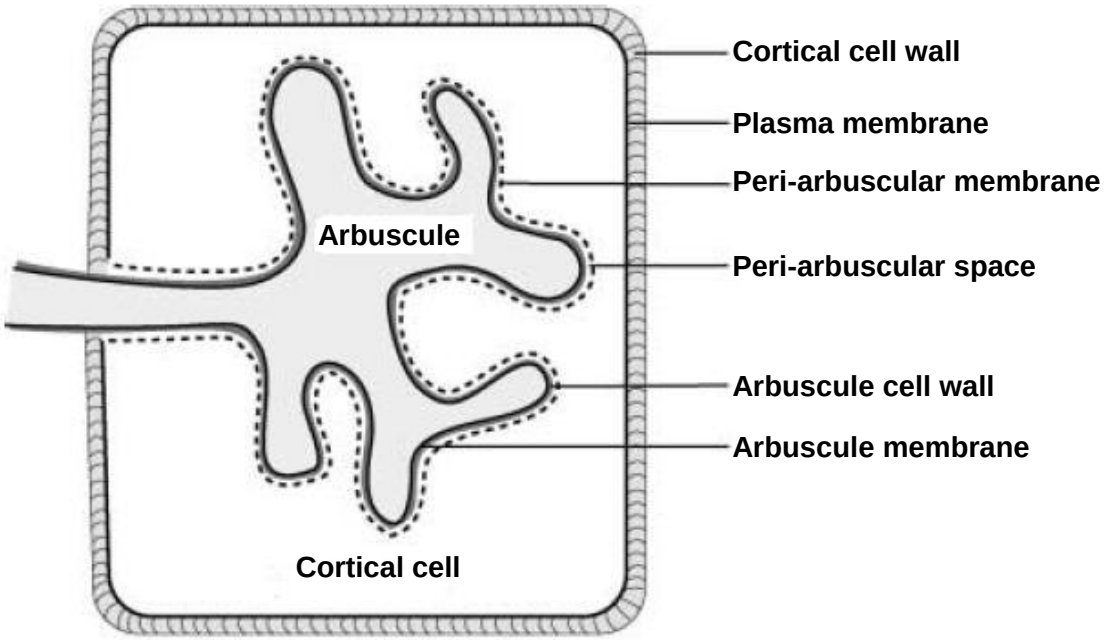


Fig. 1.1: Development of arbuscule in root cortical cell. Formation of peri-arbuscular membrane by the invagination of plasma membrane of plant cortical cell. Figure from Harrison et al. (2002).

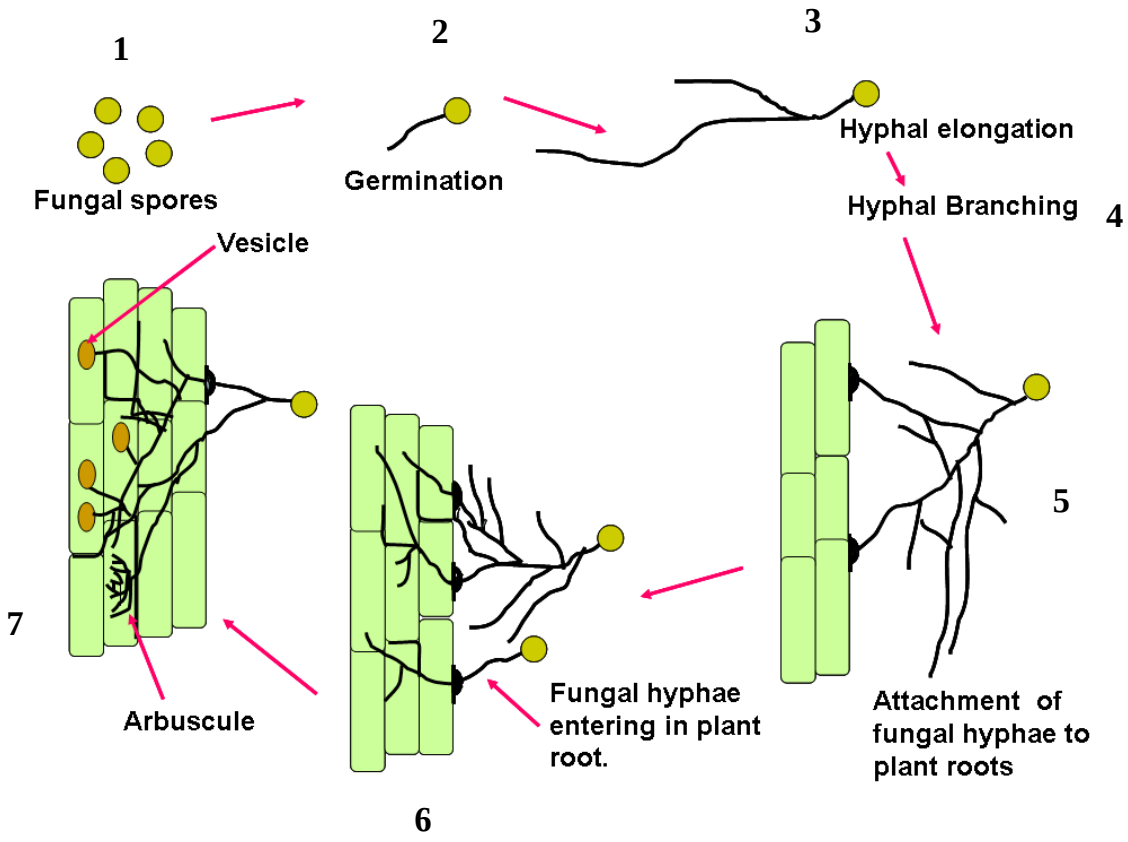


Fig. 1.2: Developmental stages of the arbuscular mycorrhizal association. 1: fungal spores, 2: spore germination, 3: hyphal elongation, 4: hyphal branching, 5: hyphal attachment to plant root, 6: penetration of the plant root by fungal hyphae, 7: Arbuscule and vesicles formation.

1.3 Mechanism of nutrient transport in AM symbiosis

The symbiotic interfaces for the transport of nutrients are developed during the colonization process of host plant root (Smith and Smith, 1990). Two different types of symbiotic interface are found: 1: intercellular in which fungal hyphae occur within the intercellular spaces of root cortex. 2: intracellular in which fungal hyphae penetrate the walls of the root cells (Fig. 1.2). These interfaces alongwith symbiotic structures (arbuscules and hyphae) play important role in nutrient transport (Gianinazzi, 1991).

1.3.1 Nutrient transport through passive and active transport system

The transport of nutrients between the two partners of the mycorrhizal association involves two processes: 1: The solutes flow out from donor organism as passive transport process into the interfacial apoplast. 2- These nutrients are taken up by active uptake by the receiver organism (Smith and Read, 2008). Woolhouse (1975) hypothesized the existence of active mechanisms involved in the transfer of carbon and phosphate. The passive nutrient transport system involves the transport of nutrients to and from the plant along a concentration gradient without the use of metabolic energy. Diffusion of nutrients to the plant occurs, when these nutrients are low in concentration in the plant cells, while being higher concentration in plants, the carbon compounds are transported from the leaves to the fungal cells as fungi need carbohydrates to fulfil their food requirements.

It was proposed that the nutrient transport processes in the AM association are linked to transport proteins which are present in the plant and fungal plasma membranes (Harrison, 1999). Various cytochemical studies have indicated that the periarbuscular membrane shows H^+-ATPase activity, hence a proton gradient is created which facilitates the transport of nutrients to the plant. H^+-ATPases belong to a large family of pumps which are called P type ATPases. All these are energized by ATP and form a phosphorylated aspartyl intermediate during the reaction cycle, hence the name P type. The plasma membrane H^+-ATPase is a single subunit protein of approximately 950-1000 amino acid residues (Geisler and Venema 2010).

The intracellular and extracellular pH is also maintained as a result of activity of H^+-ATPases (Smith and Raven, 1979). H^+-ATPases are widespread in plants, for example they are present in guard cells and function in stomatal opening. H^+ATPase in root hairs function in the transport of nutrients. They are highly concentrated in phloem and help in long distance transport. Their main function is energization of transport of nutrients. H^+-ATPases are integral membrane proteins that move metabolic solutes across the membranes against their concentration gradient. These are called transmembrane ATPases. Several genes encoding H^+-ATPases in different plants have been identified previously. Gianinazzi-Pearson et al. (2000)

demonstrated the induction of two H^+-ATPase genes in arbuscule-containing cells by using promoter β-glucuronidase (GUS) fusions. AM fungi regulate the expression of H^+-ATPase genes in tomato (Rosewarne et al., 2007). Ferrol et al. (2002) also indicated the presence of transcripts of H^+-ATPase genes in wild type while they were absent in the mycorrhiza defective mutants plants of tomato. This indicates that H^+-ATPases may be associated with mycorrhizal symbiosis.

1.3.2 Phosphate transporters

Roots of plants supporting arbuscular mycorrhizal association have extra radical hyphae which increase the area for absorption of phosphorus (Jackobsen, 1999). Phosphate transporters are located in the extraradical hyphae which help to absorb phosphorus from the soil (Harrison and Buuren, 1995; Harrison and Dixon, 1993). This shows that fungal hyphae are initial sites of phosphate uptake.

Two different groups of phosphate transporters have been cloned in recent years. The high affinity transporters as the name suggests show a high affinity for phosphate and operate in the micromolar range while the low affinity transporters work at the concentrations in the millimolar range (Bieleski 1975). The low-affinity phosphate transporters show sequence similarity with the eukaryotic sodium dependent phosphate transporters (Daram et al., 1999). A low affinity phosphate transporter is present in the chloroplast membrane and it also influences the allocation of phosphorus within the plant (Versaw and Harrison, 2002). High affinity phosphate transporters are expressed during phosphorus starvation conditions and have been cloned from the roots of various plant species, for example *Medicago truncatula MtPT1* and *MtPT2* (Chiou et al., 2001; Liu et al., 1998b); tomato *LePT1* and *LePT2* (Daram et al., 1998; Liu et al., 1998a); Arabidopsis (*AtPT1* and *ATPT2*) (Muchhal, 1996; Smith et al., 1997) (Table 1). Expression of the phosphate transporter in tomato root suggested its contribution in phosphate uptake (Daram et al., 1998).

Table 1: Some plant and fungal phosphate transporters

Organism	(a) Name Of Phosphorus transporter	(b) Expression pattern	References
L. esculentum	*LePT3*	AM +	(Nagy et al., 2006)
L. esculentum	*LePT4*	AM S	(Nagy et al., 2006)
L. esculentum	*LePT5*	AM +	(Nagy et al., 2006)
S. tuberosum	*StPT3*	AM +	(Karandashov et al., 2004), (Rausch et al., 2001) (Nagy et al., 2006)
S. tuberosum	*StPT4*	AM S	(Nagy et al., 2006)
S. tuberosum	*StPT5*	AM S	(Nagy et al., 2006)
M. truncatula	*MtPT4*	AM S	(Harrison et al., 2002)
L. japonicas	*LjPT3*	AM+	(Maeda et al., 2006)
O. sativa	*OSPT11*	AM S	(Paszkowski et al., 2002), (Guimil et al., 2005), (Glassop et al., 2007)
O. sativa	*OSPT13*	AM +	(Paszkowski et al., 2002), (Guimil et al., 2005), (Glassop et al., 2007).
H. vulgare	*HvPT8*	AM +	(Rae et al., 2003), (Glassop et al., 2005)
Triticum aestivum		AM S	(Glassop et al., 2005)
Z. mays	*Pht1;6*	AM +	(Nagy et al., 2006)
Glomus versiforme	*GvPT*		(Harrison and Vanbuuren, 1995)
Glomus intraradices	*GiPT*		(Maldonado-Mendoza et al., 2001)
Glomus mosseae	*GmosPT*		(Benedetto et al., 2005)
Neurospora crassa	*PHO5*		(Versaw, 1995)

Table headings are defined as: (a) Name of plant and fungal Pi transporters as indicated in the original references. (b) Expression pattern of plant Pi transporters in response to AM symbiosis: mycorrhiza specific (AMS), up-regulated (AM+). (From Javot et al., 2007 a).

Different phosphate transporter genes, their expression pattern and references are presented in different plants and fungi (Table 1). *StPT3* in potato and *MtPT4* in *M. truncatula were* found to be expressed in the arbuscule-containing cells (Harrison et al., 2002; Rausch et al., 2001). This suggests the role of these genes in symbiotic association for the transport of phosphorus. Similarly a rice phosphate transportor *OsPT11* was identified to be expressed during the arbuscular mycorrhizal symbiosis. Its activation was independent of the soil phosphate and nutrient availability, while it was strictly correlated with the degree of root colonization by *Glomus intraradices* (Paszkowski et al., 2002). Another tomato phosphate transporter had been identified that may be involved in the uptake of phosphorus. This was suggested by obtaining the high transcript levels in arbuscule containing cells (Rosewarne et al., 1999). Chiou et al. (2001) showed that *MtPT1* protein levels decrease in roots during

development of a symbiosis and it was not detected in roots colonized by AM fungi indicating that this transporter may not be involved in symbiotic phosphate transport. The activity of phosphate transporters is linked to H^+-ATPases, for the production of a proton gradient to provide a force for the transport of nutrients between fungi and plant (Schachtman et al., 1998). One member of H^+ ATPase gene family was expressed in roots of *M. truncatula* in arbuscule containing cells (Krajinski et al., 2002) on AM colonization. The association of phosphate transporters with the periarbuscular membranes suggests the uptake of phosphorus through the mycorrhizal pathway.

1.4 Control of symbiosis

The development of mycorrhizal symbiosis as well as nodulation in plants is under the control of a genetic programme (Harrison, 2005; Paszkowski, 2006; Paszkowski et al., 2006; Reinhardt, 2007; Stacey et al., 2006). Plant mutants with defects in both mycorrhiza and nodule development were isolated, hence it was suggested that mycorrhizal symbiosis shares a common signaling pathway with the nodulation pathway (Marsh and Schultze, 2001; Oldroyd and Downie, 2004) (Table 2). Several genes that are induced during nodulation are also expressed during mycorrhization, providing some information about the functional overlap between root symbiosis (Albrecht et al., 1998; Journet et al., 2001).

Two nodulation specific Nod-factor receptor kinases, NFR1 and NFR5 have been known to affect the earliest Nod-factor responses (Radutoiu et al., 2003) , but not the AM symbiosis (Wegel et al., 1998), suggesting that the fungal signaling factor (Kosuta et al., 2003) is different from Nod factor (van Rhijn et al., 1997). Little is known about the mechanism of association between the fungi and the plant, however some plant signaling components that play a role in symbiosis are already known. These include a receptor like kinase (Endre et al., 2002; Stracke et al., 2002), a predicted ion channel (Ane et al., 2004; Imaizumi-Anraku et al., 2005), and calcium and calmodulin dependent protein kinase (Levy et al., 2004; Mitra, 2004), controlling the common bacterial and fungal symbiotic pathway. Genes encoding these proteins are called the common SYM genes. These genes control the process of symbiosis

(Kistner and Parniske, 2002). Seven genetic loci have been identified so far in *Lotus japonicas* for their participation in the common symbiotic pathway. Calcium spiking (oscillations in cytoplasmic calcium level due to certain signaling process of symbiosis) was observed in several legumes (Kosuta et al., 2008; Oldroyd and Downie, 2006; Wais et al., 2000) showing nodulation and mycorrhizal development. Membrane intrinsic protein MtAqp1 was induced during mycorrhizal symbiosis (Krajinski et al., 2000), which was predicted to play it's role during AM association. A lipid transferase protein gene was expressed in epidermal cells and it may be linked with appressorium formation (Blilou et al., 2000), as the transcript level increased when the fungus formed appressoria and penetrated the roots. Pumplin et al. (2010) indicated a gene encoding VAPYRIN protein required for arbuscular mycorrhizal symbiosis.

Saito et al. (2007) found that Nucleoporin 85 is required for mycorrhization and nodulation. *Nup85* encodes a nucleoporin 85 which is a family of proteins forming nuclear pore complex, which together with nucleoporin 133 may help in controlling symbiosis. This gene is required for calcium spiking during symbiotic signalling process. Nuclear pore complex mediate mRNA export and protein import. Nod factor failed to show calcium spiking in Nup85 mutant so symbiosis was affected (Saito et al., 2007). Study of a *M. truncatula* 6k root interaction transcriptome (Mt6k-RIT) revealed the identification of 752 genes up-regulated in mycorrhizal tissues and also involved in nodulation (Küster et al., 2004; Manthey et al., 2004). Seven *Lotus japonicas* genes (*SYMRK, CASTOR, POLLUX, SYM3, SYM6, SYM15,* and *SYM24*) were identified to be required for bacterial and fungal symbiosis (Kistner et al., 2005). Contrary to these common SYM genes, not many genes involved specifically in mycorrhiza formation have been characterized through mutational studies although Zhang et al. (2010) has reported a mycorrhizal specific ABC transporter gene. Another phosphate transporter which is indispensable for mycorrhizal symbiosis has been demonstrated by Javot et al. (2007 b).

AM symbiosis is very ancient as compared to rhizobial symbiosis (Parniske 2008; Remy et al., 1994). This gives an indication that the AM signaling pathway had developed first and rhizobial symbiosis later (Sprent 2007), hence many genes became common between the two symbiotic pathways, performing their functions in

mycorrhizal and in nodulation processes. Predicted functions of these genes include membrane transport, defense and stress responses, primary metabolism and regulation of gene expression. Two genes, *MtC93310* and *MtC50410*, were identified to be induced during arbuscular mycorrhizal symbiosis. *MtC50410* belongs to Gras family of transcription factors and it is a homologue of RGA1 and GAI from *A. thaliana* (Manthey et al., 2004; Peng et al. 1997; Pysh et al. 1999; Truong et al. 1997). Transcription factors perform their function alone or with other proteins in a complex by activating or blocking the recruitment of RNA polymerase (an enzyme that performs the transcription of genetic information from DNA to RNA (Latchman, 1997). Previously two GRAS transcription factors *NSP1* and *NSP2* were identified to play their role during symbiosis (Smit et al., 2005; Kalo et al., 2005; Maillet et al., 2011).

1.5 Mutants defective in AM colonization

A number of plant mutants have been found to be defective in AM colonization, this includes tomato, *Petunia*, maize, pea and *Medicago* mutants. Plant mutants can be defective in different developmental stages of symbiosis. The majority of them have a defect in the earliest stage of arbuscular mycorrhizal formation, like penetration of the roots and appressorium formation. This is evident by the mutants identified in *Lycopersicon esculentum* (Barker et al., 1998); *Pisum sativum* and *vicia faba* populations (Duc et al., 1989); *Medicago truncatula* (Sagan et al., 1995); *Medicago sativa* (Bradbury et al., 1991); *Lotus japonicus* (Senno et al., 2000a); *Pisum vulgaris* (Shirtliffe and Vessey, 1996) (Table 2). Reduced fungal colonization was observed in *cyclops* mutants of *L. japonicus* and *Oryza sativa* (Kistner et al., 2005; Gutjahr et al., 2008; Yano et al., 2008). CYCLOPS form a complex with CCaMK and function during the initial stages of fungal penetration (Yano et al., 2008). Fungal penetration was blocked in *Medicago sativa* (Bradbury et al., 1991) and appressoria became abnormal or complex. The appressoria became large and irregular in shape in a failed attempt at colonization. As a result the spread of the fungal colonization was stopped.

Many AM mutants were isolated from already existing Nod^{-} and Nod^{+} fix^{-} lines (Bradbury et al., 1991; Catoira et al., 2000; Duc et al., 1989b; Kawaguchi et al., 2002; Lum et al., 2002; Sagan et al., 1995; Schauser et al., 1998b; Senoo et al., 2000;

Szczyglowski et al., 1998; Wegel et al., 1998) (Table 2). The mutants isolated were affected in common Sym genes. Besides the isolation of mutants showing defects in early stages of fungal colonization, two mutants were isolated with defects in the later stages of symbiosis (arbuscule formation). Truncated arbuscules were observed in RisNod24 mutant of pea, while the initial stages like appressorium formation and fungal penetration were normal (Gianinazzi-Pearson, 1996). Mutants exhibiting reduced mycorrhizal colonization were also identified in a transposon-tagged population of maize (Paszkowski et al., 2006). A reduced colonized mutant was isolated in a mutagenized population of tomato (David-Schwartz et al., 2001). Similarly by using direct microscopical screening a tomato mutant M20 was identified with defects in the initial stages of symbiosis (David-Schwartz et al., 2003). A large number of mutants were found in *L. japonicus* which showed normal appressoria formation and penetration of the roots, but arrested hyphal development was observed (Bonfante, 2000; Parniske, 2008; Wegel et al., 1998). This abnormal hyphal development may be the result of the mutations at six loci (*Ljsym2, Ljsym3, Ljsym4, Ljsym5, Ljsym23, and Ljsym30*). There are different genetic requirements for colonization of different cell types for *example Ljsym2, Ljsym3, Ljsym4* are required for symbiotic penetration of rhizodermis and the underlying cell layers but are not essential for arbuscule formation in the cortex. A detailed overview of plant myc^{-} mutants is given in Table 2.

Specific mutants of *Medicago truncatula* called the ram (reduced arbuscular mycorrhiza) mutants, form normal root nodules (Marsh et al. unpublished) but very reduced mycorrhizal colonization is exhibited. Therefore, they are affected in a process specific to mycorrhiza formation. The ram mutants appear to be affected at an early stage in the symbiotic interaction. They show a dramatically reduced penetration of the root. A Nod^{+} mutant in *Medicago truncatula* STR (stunted arbuscules) was identified in which AM symbiosis failed to develop due to a defect in arbuscule formation (Zhang et al., 2010). The STR mutant was defective in the AM specific pathway, indicating the role of identified half ABC transporter may be linked to mycorrhizal development.

Table 2: Study of plant Myc⁻ mutants

Host	Locus/allele	Mutant	Myc⁻	Nod/Fix	Reference
Lotus japonicus	Lj sym2-1 Lj sym2-2 Ljsym4-1, Ljsym3-2 Ljsym4-2 Ljsym71-1 Ljsym72 Ljsym4-1, Ljsym4-2 Ljsym15 LjSYMRK	282-287 282-288 282-227 2557-1 EMS1749 Mcbex Mcbep Ljsym4-1 Ljsym4-2 Ljsym15 LjSYMRK	Coi⁻ Coi⁻ Coi⁻ Coi⁻ Coi⁻ Ici ⁻ Pen ⁻ Pen ⁻, Coi⁻ Ici ⁻ Arb, Arb ⁻		Wegel et al. (1998) Schauser et al. (1998) Schauser et al. (1998) Wegel et al. (1998) Bonfante et al. (2000) Senno et al.(2000); Senno (2000b) Senoo et al., (2000a) Noveroet al. (2002) Demchenko et al., (2004)
*Lycopersicon esculentum*cv 76R.	rmc	rmc M161 M20	Pen − Pen − * Pen −		Barket et al. (1998) David-Schwartz et al. (2001, 2003)
Medicago sativa	MN NN-1008 MN IN-3811	MN NN-1008 MN IN-3811	Pen− Arb−	Nod⁻ Nod⁺/Fix⁻	Bradbury et al. (1991)
Medicago trunculata cv Jemalong	Str or ABC dmi2-2 dmi2-3 domi/dmil-1 dmil-2 dmil-3 dmi2-1 dmi3-1	Str TR25 TR26 C71 B129 Y6 P1 TRV25	Ard⁻ Pen⁻ Pen⁻ Pen⁻ * * Pen⁻ ** Pen⁻ * * Pen⁻ * Pen⁻	Nod⁺ Nod ⁻ Nod ⁻ Nod ⁻ Nod ⁻ Nod ⁻ Nod ⁻ Nod ⁻	Zhang et al., (2010) Sagan et al. (1995) Catoira et al. (2000) Penmetsa & Cook (1997) Catoira et al. (2000) Catoira et al. (2000)
Phaseolus vulgaris, cv.OAC Rico	R69	R69	Arb⁻	Nod⁺/Fix⁻	Shirtliffe & Vessey (1996)
Petunia	Pam 1	Pam 1	Pen⁻		Sekhara et al. (2007)
Zea mays		Nope 1, Tac1 , Pram 1	App⁻, Ici⁻, wt		Paszkowski et al. (2006)
Pisum sativum ***cv. Finale*** ***cv. Frisson*** ***cv. Sparkle*** ***cv. Rondo*** ***cv. Sparkle*** ***cv. Sparkle***	S a a a/sym30 c/sym19 c/sym19 a/sym30 p/sym8 b/sym9 p/sym8	RisNod24 P1 P2 F4-1 NMU1 N/A E140 R72 R19	Ard− Pen− Pen− Pen−* Pen−* Pen−* Pen−* Pen− Pen− Pen−	Nod+/Fix− Nod⁻ Nod− Nod− Nod− Nod− Nod− Nod− Nod− Nod−	Gianinazzi-Pearson (1996) Duc et al. (1989) Sagan et al. (1994) Weeden et al. (1990) Weeden et al. (1990) Gianinazzi-Pearson (1996) Gianianazzi-Pearson (1996) Balaji et al. (1994) Albrecht et al. (1998)
Vicia faba	sym1	Indian 778	Pen−	Nod⁺/Fix⁻	Duc et al. (1998a)

The Column headings are defined as follows. Host: plant species and cultivar in which mutant phenotype was identified. Mutant: designation of AM mutant plant lines. Locus/Allele: designation of genetic loci and alleles mutated in AM mutant plant lines. Myc⁻ stage of mycorrhizal colonization affected by mutation; no penetration (Pen⁻), penetration of epidermal cells, but absence of cortex invasion (Coi⁻), exodermis colonized, but absence of inner cortex invasion (Ici⁻), cortex colonized in the absence of arbuscules (Arb⁻) or arbuscule developmentis abnormal (Ard⁻). Resistant to pen. of roots when the inoculum is fungal spores. (Pen-*) Nod⁺/Fix⁺: Nod/nit. fixation phenotype of mutant; does not form nodules (Nod⁻) or forms non nitrogen fixing nodules (Nod⁺/Fix⁻). *Phenotype is inferred from alleles in which the affected stage has been established. **Phenotype is predicted from the biochemical epistasis between alleles revealed by their affect on intracellular calcium spiking (Wais et al., 2000). Table updated from Marsh and Schultze 2001.

1.6 Signal exchange and recognition

A mutual dialogue occurs between the plants and the fungi due to exchange of signals as a result of which the plant and AM fungal interaction is initiated (Harrison, 2005). Plant roots release signal molecules which are called the branching factors (BFs), AM fungi develop branching as a result of these signal molecules. Akiyama (2005) isolated a branching factor from the root exudates of a model legume *Lotus japonicus*, and it was identified as 5, deoxystrigol (strigolactone). These were previously isolated as seed germination stimulants, from the parasitic plants *Striga* and *Orobanche* (Bouwmeester et al., 2003). Fungal spores can germinate in the soil in the absence of the plant signals, however extensive growth of the fungal hyphae occurs due to the root exudates of the plants especially the strigolactones.

Strigolactones from the host roots can trigger the molecular and cellular events in the AM fungi, which help in stimulating the fungal growth (Akiyama et al., 2005; Besserer et al., 2006). These root exudates also induce expression of mitochondrial related genes and hence fungal respiratory activity is activated (Tamasloukht et al., 2003). A few examples of natural strigolactones are: 5, deoxystrigol, strigol, strigyl acetate, sorgolactone, orobanchol, alectrol. GR24 and GR7 are the examples of synthetic analogs (Fig. 1.3). Strigolactones have been isolated from root exudates of many monocots like maize, millet, sorghum and dicots including cotton, cowpea, red clover, *Menispermum dauricum* and *Lotus japonicus* (Akiyama et al., 2005, 2007; Cook et al., 1966, 1972; Hauck et al., 1992; Muller et al., 1992; Siame et al., 1993; Yasuda et al., 2003; Yokota et al., 1998). Their characterization is difficult due to their instability and very low concentration.

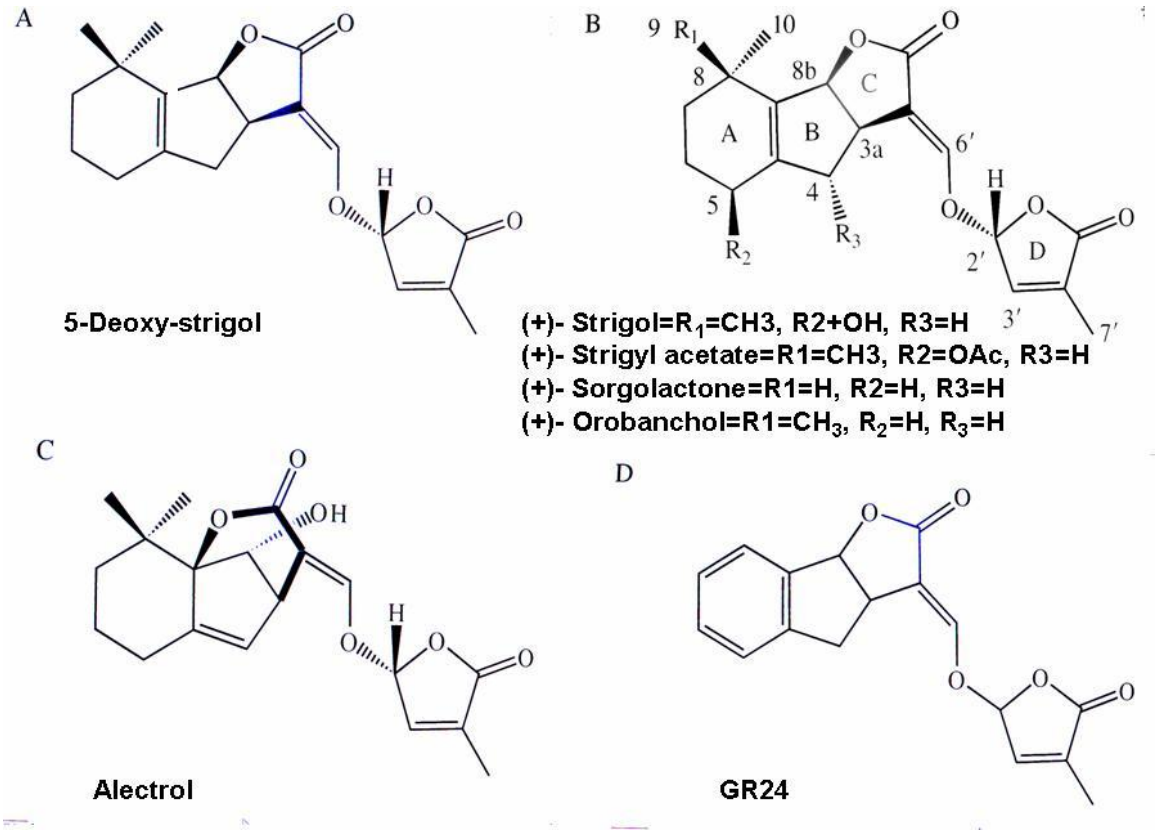


Fig. 1.3: General chemical structure and numbering scheme of strigolactones. A) 5-Deoxy strigol. B) Four natural strigolactones. C) Alectrol (tentative structure). D) Synthetic analogue GR24. (Structures from Akiyama and Hayashi, 2006).

Flavonoids are also present in plant root exudates (Fig. 1.4), and are important in the symbiotic rhizobium legume interaction. They are inducers of rhizobial nodulation genes, involved in the synthesis of lipochitooligosacchride signals called Nod factor (Perret et al., 2000). Flavonoids (Fig. 1.5) also stimulate the growth and branching offungi (Bécard et al., 1992; Gianinazzi-pearson et al., 1989; Tsai and Phillips,1991).

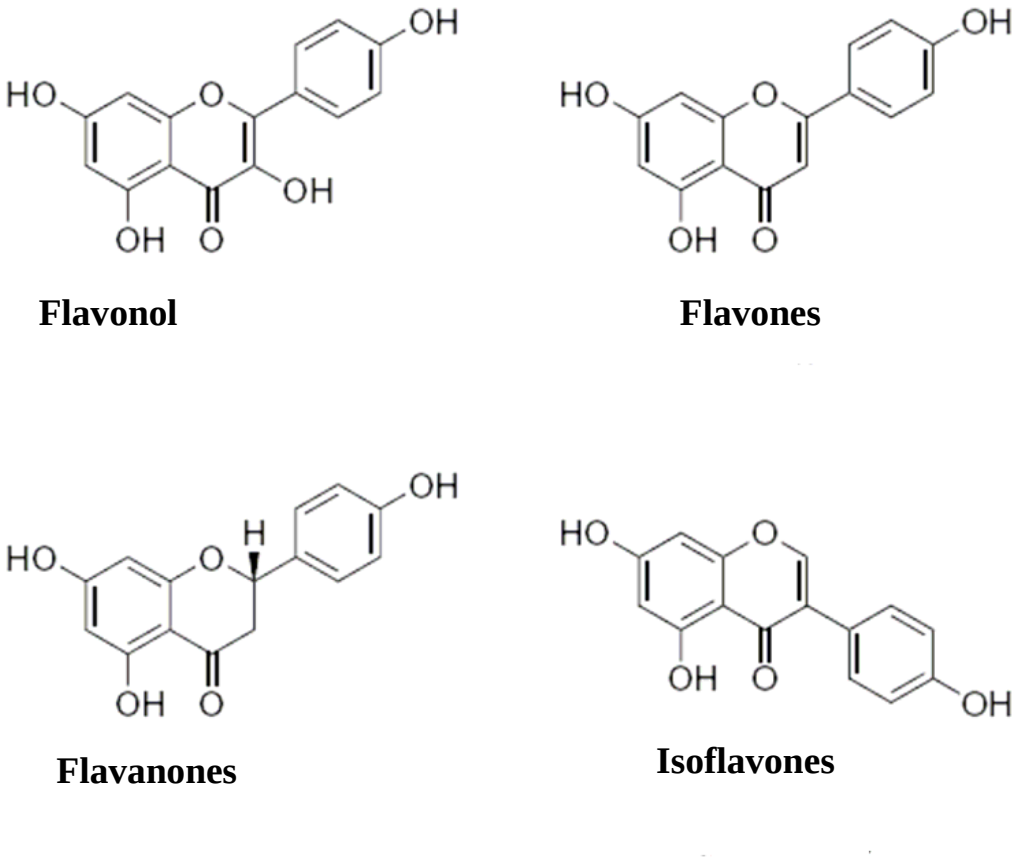


Fig. 1.4: Chemical structures of flavonoid compound (Steinkellner et al., 2007).

The stimulatory effect of flavonoids on AMF hyphal growth depends on the chemical structure of the compound (Becard et al., 1992; Chabot et al., 1992; Scervino et al., 2006). The flavonoid pattern was altered in mycorrhizal roots and it may be due to the developmental stage of the AM symbiosis as demonstrated by Harrison and Dixon (1993) and Larose et al. (2002).

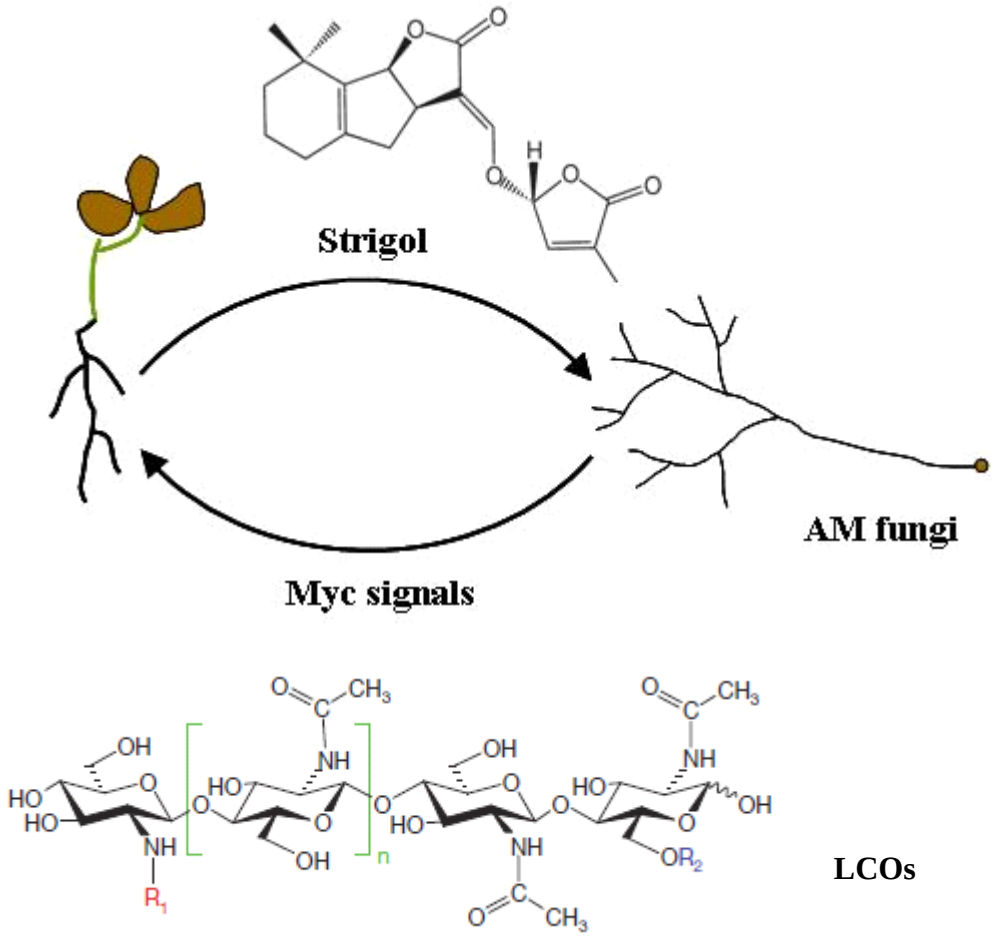


Figure 1.5: Strigol released by plant roots is perceived by AM fungi. Myc signals are released by fungi which cause the expression of some genes in plant roots for AM association. (Figure of plant and fungi from Michael Schultze). LCOs (Maillet et al. 2011).

Fungi release diffusible symbiotic signals which are called Myc factors (Maillet et al., 2011). These myc factors are similar to Nod factors in their structure and are a mixture of sulphated and non-sulphated simple lipochitooligosaccharides (LCOs) (Fig. 1.5). This diffusible factor is recognized by the plants and certain genes are activated in the plant roots which may help in the development of AM symbiosis. A mycorrhiza specific factor (Myc) induced the expression of *MtENOD11* in the roots of *Medicago truncatula* (Kosuta et al., 2003). Chabaud et al., (2002) also reported the activation of *MtENOD11* gene in epidermal and cortical cells in response to inoculation by *Gigaspora rosea*. Mycorrhizal colonization was also reported to be increased as a result of Nod factor (Oláh, 2005). About hundred genes expressed in mycorrhizal roots have been isolated. Inoculation with different fungi *Glomus mosseae* and *Glomus intraradices* resulted in overlap of genetic programe (Hohnjec et al., 2005). One member of an AM-induced gene encoding blue copper binding

proteins (*MtBcpl*) was expressed in arbuscule containing cells, indicating some role of this gene in mycorrhizal symbiosis (Hohnjec et al., 2005). Recently two mycorrhiza specific blue copper binding genes were identified in *M. truncatula* Jemalong 5 (Parádi et al., 2010). It is possible that these copper binding genes play some role in mycorrhizal symbiosis. To understand the mechanism of association of functional AM symbiosis, detailed analysis of the promotor can also help in the identification of upstream regulatory mechanism of AM. By using the promotor reporter gene fusion the expression pattern driven by mycorrhiza specific promoters of *M. truncatula* was identified as described by Krajinski and Frenzel (2007). Table 3 indicates some genes expressed in arbuscules containing cells and adjacent cells and their predicted functions.

Table 3: Expression of some genes in arbuscule-containing cells

Name	Current Annotation	Assumed function	Reference
Arbuscule containing cells specific expression			
MtPt4	Phosphate transporter	Plant phosphate uptake	Harrison et al. (2002)
MtScp1	Serine-carboxypeptidase)	Signaling	(Liu et al., 2003)
MtTi1	Protease-inhibitor	Signaling	(Grunwald et al., 2004)
MtLec5	Lectin	Storage	(Frenzel et al., 2005; 2006)
MtLec7	Lectin	Storage	(Frenzel et al., 2005)
MtChit3-3	Chitinase	Signaling	(Elfstrand et al., 2005)
Expression in arbuscule-containing and adjacent cells			
MtGst1	Glutathione-S-transferase	-	(Wulf et al., 2003)
MtCel1	Endo-1,4-b-D-glucanase	Cell-wall modifications	(Liu et al., 2003)
MtBcp1	Blue copper binding protein	Electron transfer	(Hohnjec et al., 2005)

Table from Krajinski et al. (2007).

1.7 *Medicago truncatula* as a model legume to study AM symbiosis

Medicago truncatula is used to study the AM symbiosis as its genome sequencing is under way; as well it is suitable for genetics studies in legumes (Fig. 1.6). It is commonly known as barrel medic because of the shape of the seed pod (Fig. 1.6 B). The plant is self-fertile and its genome, unlike the complex ones of other legume species, is diploid (with just eight pairs of homologous chromosomes) and is relatively small (Frugoli and Harris, 2001; May, 2004). It is used as a forage legume and is excellent quality forage due to its digestibility, nutritional value, and silage properties.

It can also be regenerated from cell culture and there are systems of genetic transformation (Barker et al., 1990; Cook, 1999) in *M. truncatula*. A series of forward and reverse genetics methodologies and populations have also been established (*M.truncatula* handbook version 2006).

Fig. 1.6: *Medicago truncatula,* 'A' leaves and branches. 'B' Developing seeds are indicated by red arrows on branches.

1.8 Mutagenesis as an effective tool to study symbiosis

The basic approach to understand gene function is to a create genetic mutation and then identify the mutated gene. Different types of mutagenesis have been used in the model legume *Medicago truncatula* in the past. These include ethyl methane-sulphonate (EMS) causing point mutations (Penmesta and Cook, 2000; Catoira et al., 2000), retrotransposon or T-DNA tagging causing gene disruption by insertions (d′ Erfurth et al., 2003; Tadege et al., 2008), and fast neutron mutagenesis causing deletions (Rogers et al., 2009). RNA induced gene silencing (Ivashuta et al., 2005) has been used as well.

EMS is a chemical mutagen and is characterized by certain transitions which are caused by methylation of G residues. These mutations may lead to a partial or complete loss of a gene function. For the identification of mutated alleles in EMS mutation, generally map based cloning is used. In *M. truncatula* EMS mutated seeds were used previously to identify several mutants altered in the symbiotic interactions or changed morphologically or physiologically (Catoira et al., 2000).

Deletions and other chromosomal rearrangements are usually caused by fast neutron mutagenesis. Deletions from few base-pairs to more than 30 kb deletions can be obtained (Li and Zhang, 2002). A part of a chromosome can be missing or any number of nucleotides can be deleted from a single base to an entire piece of chromosome. Since fast neutron is a highly efficient mutagen that produces deletion mutations easily detectable by PCR, this method has the potential to enable reverse genetic screens in most plant species. The *ram1* and *ram2* (reduced arbuscular mycorrhiza) mutants were obtained by fast neutron mutagenesis of wild-type line A17. Mutants *ram1* and *ram2* are Nod^{+} myc^{-}, these form root nodules but they are defective in mycorrhiza formation. *dmi3* (does not make infections) was obtained by EMS mutagenesis and it is a known mutant showing reduced mycorrhizal colonization (Catoira et al 2000).

Insertional mutagenesis is mutagenesis of DNA by the insertion of one or more bases of DNA. It can occur naturally mediated by viruses or transposons or can be artificially created for research purposes in the laboratory. Insertional mutagenesis has been used successfully in legumes for the production of a large number of mutant lines and to study the symbiotic relationships. T-DNA, transposons or retrotransposons can be used as insertional mutagens. In T-DNA tagging a DNA sequence is used to mutate and tag the genome. The tag can then be used as an identifier for the mutated site.

RNA induced gene silencing based on small double-stranded RNAs, causes the degradation of sequence-specific mRNA (Hannon, 2002; Helliwell and Waterhouse, 2005; Waterhouse and Helliwell, 2003). It is also called post transcriptional gene silencing. Genes have been effectively silenced in *M. truncatula* and other plant species by using this technique (Limpens et al., 2003; 2004; Wesley et al., 2001). Mutations caused due to this technique result in a range of variable phenotypes, hence for each gene being silenced, there is a need to analyse a large number of transformants.

1.9 Insertional mutagenesis

Retrotransposons are important in insertional mutagenesis, transpose through RNA intermediate, which is then reverse transcribed into linear double stranded DNA. Retrotransposons avoid repetitive sequences, do not transpose in the vicinity of their original location, but are dispersed in the genome so their transposition is entirely random and there are no integration hotspots (Courtial et al., 2001; d'Erfurth et al., 2003; Lucas et al., 1995). Other category is DNA transposon, they transpose through a DNA intermediate. Two subclasses of retrotransposons are: LTR retrotransposons which carry long-terminal repeats (two direct repeats at both ends), non terminal retrotransposons are without these repeats. Some LTR retrotransposons are highly specific for their insertion site. A few examples of retrotransposons include *Tos17* of rice, *Tnt1* and *Ttol* of tobacco. The use of tobacco retrotransposons *Tnt1* and *Ttol* for gene tagging in rice and *Arabidopsis thaliana* indicated that these transposons do not exhibit any obvious site specificity for their integration (Courtial et al., 2001 ; Okamoto and Hirochika, 2000 ; Yamazaki, 2001). Most of the insertions were observed in the coding region in *Arabidopsis* and it was indicated that insertions are unlinked and distributed all over 5 chromosomes (Courtial et al., 2001). *Tnt1* was first isolated after its transposition into the nitrate reductase (NR) gene of tobacco and it's transposition was detected in NR deficient mutant lines (Grandbastien et al., 1989). It is 5334 nucleotide long LTR element and produces 5 base pair duplication upon insertion. It contains two 610 base pair long terminal repeats and a single open reading frame of 3,984 nucleotides. The *Tnt1* transposon was used as a successful mutagen in *Medicago truncatula* due to its highly efficient transposition during the regeneration process resulting in multiple inserts (average 25) in *Medicago* lines (d'Erfurth et al., 2003; Tadege et al., 2008). The *Tnt1* gene-tagging strategy is more efficient as compared to T-DNA tagging (Krysan et al., 2002; Scholte et al., 2002). This was also explained by Courtial et al., (2001) and d'Erfurth et al., (2003) by observing more inserts in genes in *M. truncatula* and *A. thaliana.* Transposition of retrotransposons (eg, *Tnt1*, *Tos17*) is activated by biotic and abiotic stresses (Mhiri et al., 1997; Moreau et al., 1996) and in tissue culture. Another benefit of using *Tnt1* mutagenesis is that a large number of mutant individuals are produced. About 7600 mutant lines carrying 190 000 insertions were generated using *Tnt1* (Tadege et al., 2008). These characteristics make *Tnt1* best candidate for the insertional mutagenesis

and it could be used in other plants as well. In the present research transposon mutant population was produced by using tissue culture regeneration method (Tadege et al., 2008).

1.10 Aims and hypothesis of the Project

The aim of this PhD project was to carry out a detailed phenotypic characterisation of low colonized mutant lines of *Medicago truncatula* and to isolate and characterize the mutated gene from at least one of the mutants. Plant mutants affected in mycorrhizal development are an important tool to define roles for the genes affected. A number of plant genes involved in symbiosis have been characterized previously; however the majority of these genes have been involved in controlling the common pathway of nodulation and mycorrhiza formation. Genes that are specifically involved in mycorrhiza formation needed to be discovered. The hypothesis of this project was that low colonized mutants are defective in the arbuscular mycorrhizal signaling pathway or symbiotic functions such as nutrient transfer might be affected. Alternatively the mutants might be impaired in the production and secretion of substances stimulating fungal growth. Primary mutant screening of fast-neutron mutant lines and transposon mutagenized lines was carried out at the Noble foundation by Dr. Michael Schultze. M_3 plants were retested to isolate reduced colonized mutants. Then a PCR based approach was used for the isolation of sequences flanking the transposon insertion. Two genes were identified which are suggested to be involved in the mycorrhiza development or its function.

Chapter 2

Materials and Methods

2.1 Plant Lines

Table 4: All plant lines used in this thesis are detailed.

Plant lines	Description	Source
cv. Jemalong A17	Wild-type *M. truncatula*	Schultze lab stock
ram 1, ram2	Reduced arbuscular mycorrhiza, Fast neutron mutants of A17	John Innes Centre
FNB2, FNB4	Reduced AM colonization, impaired nodulation (Murray et al., 2011). Fast Neutron lines	Noble foundation
A2909, A2925, A2926, A2935, A2942, A2962 A2990, A2995, A3000, A3012, A3031, A3032	Fast neutron mutants	Noble foundation
DMI3	EMS mutant, $Nod^{-}Myc^{-}$, (Ané et al., 2002)	John Innes Centre
cv. tricycla TNK	Starter line for *Tnt1* mutagenesis (Tadege et al., 2008)	Pascal Ratet
R108	Wild-type, (Hoffmann et al., 1997)	Schultze lab stock
NF131, NF249, NF383, NF400, NF413, NF423, NF425, NF426, NF428, NF447, NF472, NF473, NF577, NF629, NF689, NF762, NF788, NF807, NF819, NF841, NF850, NF871, NF905, NF919, NF940, NF984, NF1248	Transposon mutants (Tadege et al., 2008)	Noble foundation

2.2 Fungal inoculum

The inoculum used for screening was Endorize mix (manufactured by Agrauxine, Saint Evarzec, France). It is a mixture of different *Glomus* species along with dried soil substrate. For nurse pot experiments, six-week old leek plants colonized with *G. intraradices* were used. To produce colonized leek plants, leek seedlings were planted in 9 parts of a mixture of autoclaved sand: Terragreen (equal volumes) and 1 part (by volume) of leek root pieces colonized by *G. intraradices* spores. The plants were allowed to grow for about 6 weeks and then individual colonized leek plants were used as nurse plants.

2.3 Physiological techniques

2.3.1 Seed sterilization and germination

Medicago truncatula seeds were surface sterilized by soaking in 3 % Chlorox for 2 minutes washed with distilled water 2 times and dried using autoclaved filter papers. The seeds were scarified by gently abrading them with sand paper and then germinated on moist filter paper, in Petri-plates, in the dark at room temperature. Normally the seeds took two days to germinate. Newer seeds (less than three months) need a vernalisation treatment prior to germination. The Petri-plates of new seeds were kept at 4˚ C for one to two weeks and then germinated as stated.

2.3.2 Plant growth conditions for testing mycorrhizal colonization

After germination, the seedlings were planted in 9 parts of sand and Terragreen (1:1 v/v) to one part of Endorize mix (by volume). Substrate and inoculum was evenly mixed by hand. Plants were grown in P40 trays (50 ml volume of plant pots) and these were kept in the glass house for four or seven weeks before testing. Plants were watered with tap water by horticultural staff. The light duration was maintained at 16 hr day and 8 hr night and temperature was kept within the range of 17─22 ºC. Plants in P40 trays (50 ml pots) were fed once a week with 5 ml per plant of feeding solution containing reduced phosphate, while 10 ml feeding solution was used for

plants in P15 pots (250 ml). Feeding solution was nitrate based Long Ashton nutrient solution (Hewitt and Smith, 1975).

Long Ashton nutrient stock solutions:

100X stock KNO_3: 40.4 g/l (400 mM)

100X stock $NaH_2PO_4\,2H_2O$ ($1/10^{th}$): 2.08 g/l (13.3 mM)

100X stock $Ca(NO_3)_2.\,4H_2O$: 94.4 g/l (400 mM)

100X stock $MgSO_4$: 36.8 g/l (150 mM)

]

Micronutrients

Micronutrients 100X stock: 2.45 g/l $FeC_6H_5O_7$ (10 mM) heated into solution, 0.233 g/l, $MnSO_4.4H_2O$ (1 mM), 0.029 g/l $ZnSO_4.7H_2O$ (0.1 mM), 0.025 g/l $CuSO_4.5H_2O$ (0.1 mM), 0.31 g/l H_3BO_3 (5 mM). 0.012 g/l, $NaMoO_4.2H_2O$ (0.05 mM), 0.58 g/l NaCl (10 mM), 0.0056 g/l $CoSO_4.7\,H_2O$ (0.02 mM), filter sterilised.

Reduced phosphate feed was prepared by using 10 ml/l of each of the 5 stocks in 950 ml d H_2O. All solutions were filter sterilized and stored at 4°C.

2.3.3 Nurse pot experiment

A six-week old colonized leek plant was planted in the middle of a round pot P6 (1000 ml volume) and 4-5 *Medicago* mutant seedlings were grown surrounding the leek plant. Four pots for each plant line were tested hence there were almost 16 plants in each case. The plants were allowed to grow for about four weeks and tested for colonization using the magnified intersection method. (McGonigle et al., 1990).

2.3.4 Root harvesting and staining

For harvesting of roots, plants were removed from pots and the soil washed from the roots. 4.5 cm section of roots was collected from 1.5 cm below the hypocotyl (Fig. 2.1). After removal of the root samples, the plants were repotted in *Medicago* mix (2:1:1 Levingtons: vermiculite: perlite). The root pieces were cleared in 10% KOH solution for four days, then washed with tap water and stained with Pelikan ink for

10 minutes as described by Vierheilig et al. (1998). The staining solution was prepared by mixing 10 ml of Pelikan ink in 25 ml of acetic acid and distilled water was added to make 100 ml volume. The stain was then washed off with tap water and the root samples were left in 25 % acetic acid for 10 minutes. The acid was washed off and the root samples were kept in tap water (sufficient quantity to cover the root pieces) at 4ºC until examination by microscopy.

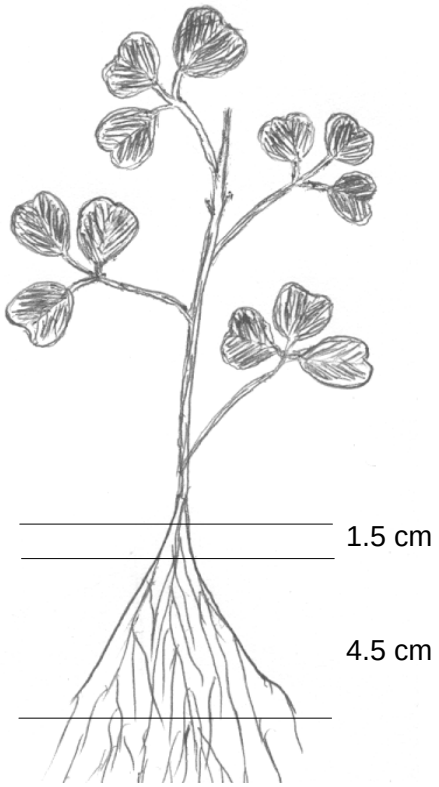


Fig. 2.1: 4.5 cm section of *Medicago truncatula* roots was sampled for staining.

2.3.5 Microscopy and grid line intersection method

For microscopy, the root sections were cut into 1 cm pieces and placed in a Petri plate situated over a grid of 1×1 cm squares. The root pieces were separated apart with forceps where needed. Fungal colonization was counted under the microscope (Nikon SMZ800) using the grid line intersection method (Giovannetti and Mosse 1980) (Fig. 2.2), 50–80 intersects were observed. Percentage root length colonization was measured by dividing the number of intersects showing colonization by the total

number of intersects, multiplied by 100. This method scored only total colonization. The number of arbuscules, vesicles and hyphal structures were not counted separately.

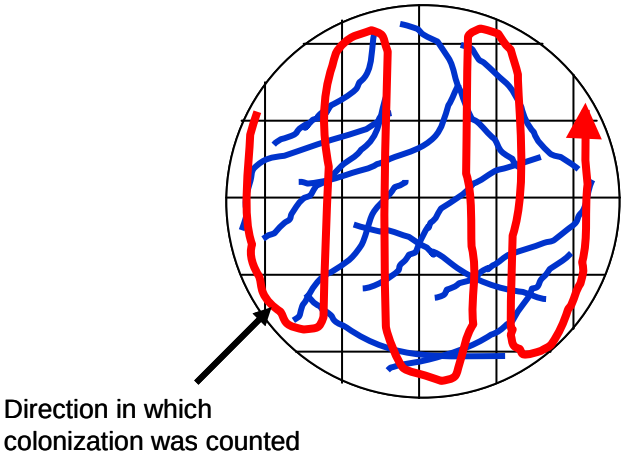


Fig. 2.2: Grid line intersection method. Arrow line showing the direction in which colonization was counted.

2.3.6 Magnified intersection method for counting the colonization

To count the proportion of roots colonized by specific fungal structures, a magnified intersection method was used (McGonigle et al., 1990) (Fig. 2.3). The intersections between microscope eye piece cross hair and roots were counted under 200X magnification. If the vertical eye piece crosshair crossed one or several arbuscules, it was called arbuscular colonization, while if the cross hair crossed one or more vesicles, then it was considered vesicular colonization. Fungal arbuscules, vesicles and internal hyphae were counted in this way. Intersections were also counted for no fungal material in roots. The count for each specific category was increased by one in each case regardless of how many structures corresponding to that category were cut by the crosshair. The percentage colonization for each category was calculated by dividing the number of intersects for that category by the total number of intersects multiplied by 100.

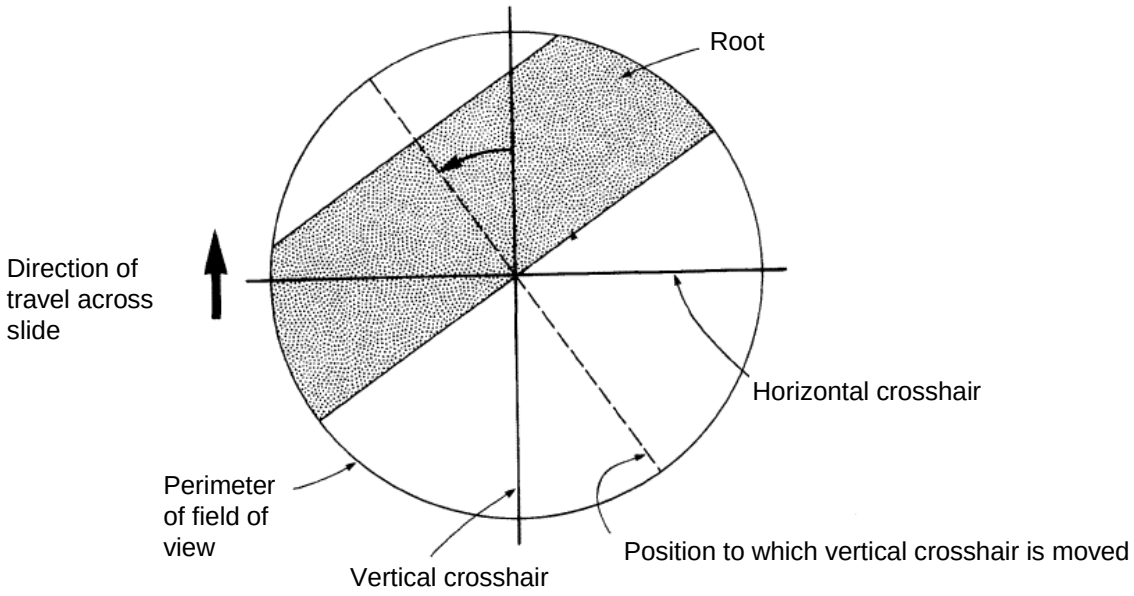


Fig. 2.3: Diagrametic representation of magnified intersection method in which a root is aligned to the vertical crosshair by moving the stage of microscope. The vertical crosshair is moved to make the perpendicular intersection. Picture from McGonigle et al. (1990).

2.4 Crossings

Plants were grown in the glass house for 4-7 weeks. Flowers were inspected to see whether the pollen was ripe. If under the microscope the pollen grains were seen bursting out of their pollen sac (Fig. 2.4 A), then it was considered to be at the right stage to use. Five-week old plants were ready for crossing in most cases.

With the help of very fine forceps, sepals, petals and anthers were removed from the pollen recipient and a thin strip of coloured tape was placed around the peduncle to mark the stripped pistil. A flower was cut from the pollen donor and forceps were used to remove the sepals, petals and pistil leaving the stamens. The pistils of mutant lines were pollinated using wild type pollen. To avoid desiccation the flower was inserted into falcon tube (15 ml) containing 2 ml water. The tube was closed with a cotton plug and it was kept vertically in the plant pot (Fig. 2.4 B). After 5-7 days the

partially developed seed pod was removed from the tube and was netted in order to keep it separate from other seed pods (Fig. 2.4). The pollination was repeated every day for 5-7 days. Usually the mutant plant was used as a female parent. Some reciprocal crosses were also made in which the mutant line was used as male parent. 10-11 crosses were made in each line and 7-8 crosses were successful in each case.

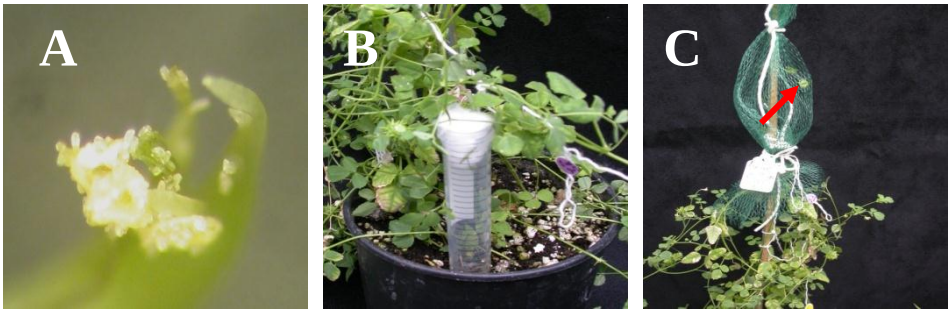


Fig 2.4: *M. truncatula* crossings. A: exhibiting stigma and stamens with pollens. B: crossed flower inserted in a falcon tube. C: Netting, Arrow pointing seed pod.

2.5 Molecular biology techniques

2.5.1 DNA isolation from plants

DNA was extracted using cetyltriethyl ammonium bromide (CTAB) method.

Following solutions were prepared.

2xCTAB buffer: 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2 % CTAB.

10 % CTAB: 10 % CTAB, 0.7 M NaCl.

CTAB precipitation buffer: 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 % CTAB.

2x CTAB buffer was kept in a boiling water bath for five minutes. 1g of freshly opened leaves were ground to a fine powder using a pestle and mortar filled with liquid nitrogen. The powder was transferred to a Falcon tube (50 ml). 1-2 ml of 2x CTAB was added, the mixture was vortexed and kept in a water bath at 56°C for 45 minutes. The mixture was cooled to room temperature and an equal amount of chloroform/isoamylalcohol (24:1) was added and shaken gently. It was centrifuged at room temperature in a Jouan CR312 centrifuge for 10 minutes at maximum speed (4000-5000 rpm at 6000 G). The upper aqueous phase was removed to a new tube and 1/10th volume of 10% CTAB was added, mixed thoroughly, and the chloroform

isoamylalcohol extraction repeated. The new aqueous phase was transferred to a new tube and an equal volume of CTAB precipitation buffer was added, mixed thoroughly, and the tube was kept at room temperature for 20 minutes. The tube was centrifuged at a speed of 13000 rpm (15000 G) in a Jouan A14 centrifuge, the supernatant was discarded and the pellet resuspended in 1 M NaCl (1 ml per gram of starting material). After addition of 2 volumes of 100% ethanol, the tube was centrifuged for 10 minutes and the supernatant was removed. The pellet was washed twice with 70 % ethanol. The ethanol was removed and pellet was dried at room temperature. Finally the pellet was dissolved in 100 µl of autoclaved distilled water. The concentration of the DNA was determined using a nanodrop spectrophotometer (ND-1000, V3.0.1). Usually from 1 g of leaves, about 2–3 µg/µl (200–300 µg) of DNA was obtained. Then the DNA was stored at –20°C.

For some experiments (genotyping) DNA was isolated on a small scale in which 100 mg of freshly opened leaves were ground and transferred to a microcentrifuge tube. 200 µl of 2 x CTAB was used and after chloroform isoamylalcohol extraction all centrifuge steps were carried out at a speed of 13000 rpm in a Jouan A14 centrifuge. The step of adding 10% CTAB was the same as was used for large scale preps of DNA. 100 µl of NaCl was added (100 µl/100 mg leaf material). DNA washing steps were also same as mentioned above. In the final step DNA pellet was dissolved in 25 µl of autoclaved distilled water. Roughly about 100-800 ng/µl DNA was obtained.

The DNA was treated with RNase A (Fermentas, 1 mg/ml) by adding 0.5 µl to 2 µg/µl of DNA in a volume of 50 µl (half volume of the DNA extracted was used for RNase treatment), and incubated for 1 h at room temperature. The concentration of DNA was also determined after RNA removal. It was about 1.4 µg/µl.

2.5.2 Agarose gel electrophoresis

DNA was separated by gel electrophoresis on 1% agarose gel with SYBR Safe. For smaller PCR fragments a 1.5 % agarose was used. The samples were run with 1 kb DNA ladder (New England Biolabs or Fermentas or Promega) and DNA bands were visualised by illuminating with blue or ultraviolet light. 0.5 X TBE buffer and agarose gels were prepared as described by Sambrook and Russel (2001).

2.5.3 PCR

PCR-primers were designed by using the Primer3 plus programme. (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), or used as published previously (Ratet et al. 2006; 2009). Table 2.3 lists all primers and oligonucleotides used in this project. Primers were purchased from Sigma-Aldrich (UK) and re-suspended in autoclaved distilled water to give a stock solution of 100 µM, which was further diluted to give a 10 µM working solution, and stored at –20ºC.

Standard PCR:

2 µl of 10 x buffer

1.2 µl of 25 mM $MgCl_2$

0.4 µl of 10 mM dNTP

0.5 µl of 10 µM forward primer

0.5 µl of 10 µM reverse primer

0.5 µl Taq DNA polymerase (Fermentas, 5 units/µl)

13.9 µl of autoclaved distilled water.

1 µl of 100 ng/µl template DNA

Total reaction volume was 20 µl.

The standard PCR programme was as follows:

Step 1.	Initial denaturation	94 ºC	5 minutes
Step 2.	Denaturation	94 ºC	30 seconds
Step 3.	Primer annealing	55 ºC	30 seconds.
Step 4.	Extension	72 ºC	1-2 minute
Step 5.	Final extension	72 ºC	10 minutes.

Steps 2-4 were repeated 35 times for standard PCR. Depending on primer Tm temperature was changed in some cases.

Table 5: Sequences of Oligonucleotides used during research project

Oligonucleotides	Sequence 5'-3'
MtPT4 F1 MtPT4 R1	GACTCGATCCACAACAAAGATT TTGGCCAGAAAGTGTTCC
MtPT4 F2 MtPT4 R2	TTTCAACCGTTTCGAAGCTC GTGTAACGACCGGTTTCAGG
MtPT4 F3 MtPT4 R3	TTCCAGCTGCGATGACTTAC CATCAATTTTGGCCCCATTA
Gras F1 Gras R1	ACCGAGTCAATCTTCATCGATCT TGGACTTCCACATCACCATCT
Gras F2 Gras R2	TGTGGAAGCTCAAATTCCCTCAA TGCCACTGCTTCAGCACAT
Gras F3 Gras R3	AAGGATTACCAGCGCAGCTT GCCACCCAAGCAACAAACAA
Gras F4 Gras R4	GGTGGCAAGACAGAGCCATT AAACACCGACGGTTCAAGGA
Gras F5 Gras R5	GCAGGGTTGAGTTGCCCTAC GGGGCTGTGAACCGTCATAA
Gras F6 Gras F7	TCCTTGGACGCCACTTTTCC CCGGGAATCATCTCGGGAAC
NF 807-1 F NF 807-1 R	GGCTCAACGAGGCGATGATA GTGCAACCAAGTGACCAAACA
NF788-1 F NF788-1 R	CTTGCTGCAAAGGGTGCTTC TGAGAAACAAAGGGTTTTCCAACA
NF788-3 F NF788-3 R	AGCACACTCTTAGGTCTCAAGTC GGGCAAGGTTGGTGAATGGT
NF905-1 F NF905-1 R	ACAGAACAGCATCGGATGGGGGAA TTCAACGTGGCGGCGCTCTT
NF905-3 F NF905-3 R	GCCATTGCGGATGCAAATGT CGGACACGCCGACATCATTA
Ram2 F1 Ram2 R1	TGAACCGTGTTTTCCCAAGTCA AGCCATGAAGGGAGCATCAGT
Ram2 F2 Ram2 R2	CACCGCGAATCCAAGGATCA GGCATGACCAACACAATACAAACC
LTR51* LTR6*	CAAAGC TTCACC CTCTAAAGC C GCTACCAACCAAACCAAGTCAA
LTR31* LTR4*	GCTCCTCTCGGGGTCGTGG TACCGTATCTCGGTGCTACA
Mtha1 F Mtha1 R	TGACGGTTTTGCTGGTGTCT CTGGAAAATTGCTCGACTGGT
Adaptor-1* Eco Adaptor-2*	CCCCTCGTAGACTGCGTACC AATTGGTACGCAGTCTACG
Ase adaptor-2* NdeII-adaptor 2*	TAGGTACGCAGTCTACGA GATCGGTACGCAGTCTACGA
Eco1* Eco2*	CTCGTAGACTGCGTACCAA CGTAGACTGCGTACCAATT
Ase1* Ase2*	CTCGTAGACTGCGTACCTA CGTAGACTGCGTACCTAAT
NdeII-1* NdeII-2*	CTCGTAGACTGCGTACCGA CGTAGACTGCGTACCGATC

The oligonucleotides with stars (*) in the table are as reported by Ratet et al. (2006).

2.5.4 Isolation and analysis of flanking sequences

Genomic DNA flanking the transposon insertion sites were isolated as described by Ratet et al. (2006). See Fig. 2.5 for an overview.

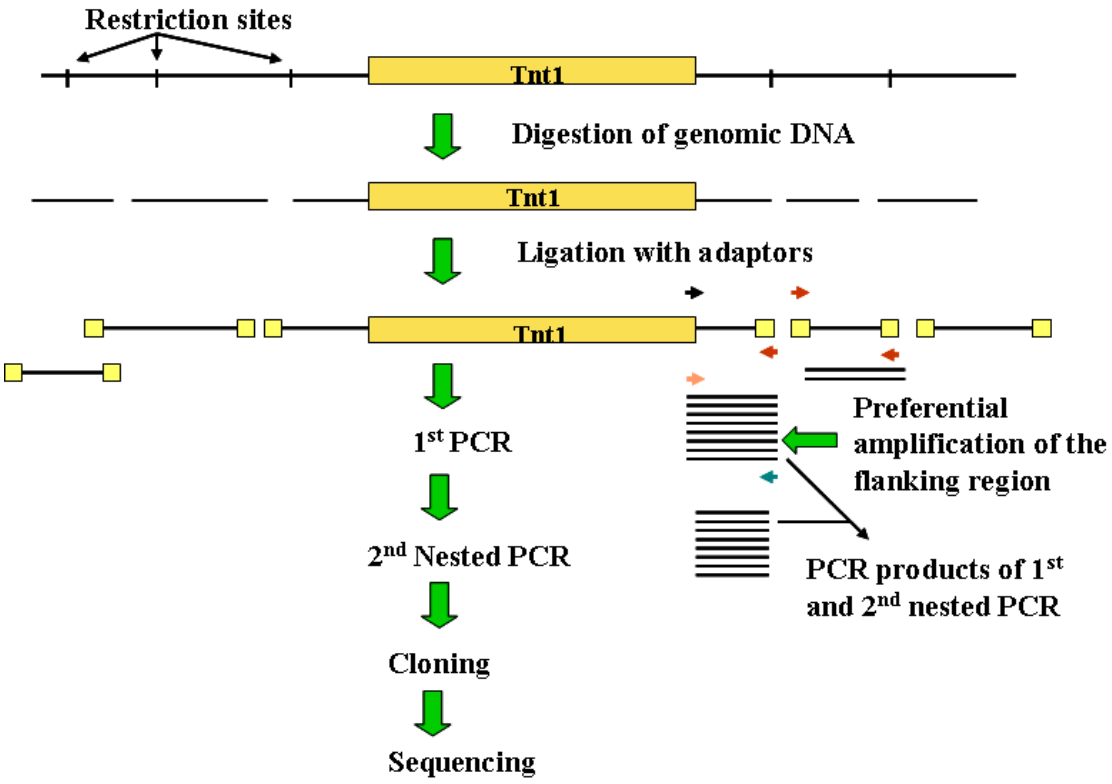


Fig. 2.5: Strategy of identification of insertions sites.

2.5.4.1 Solutions for genetic analysis

Standard solutions if not stated otherwise were prepared as described by Sambrook and Russel (2001).

2.5.4.2 Amplification of flanking sequences

Amplification of flanking sequences (Fig. 2.5) was as follows. 2 µg/µl of DNA was digested with restriction enzymes (Fermentas) in a total volume of 50 µl following the manufacturer's protocol. The restriction enzymes EcoRI, MfeI, AseI and NdeII were used. To verify that the digest was complete an aliquot of the digest was tested by agarose gel electrophoresis. The enzymes were inactivated by incubation at 65 ºC for 20 min.

To produce the double stranded adaptor, equal volumes of 100 µM adaptor 1 and adaptor 2 oligonucleotide solutions were mixed, heated at 95 ºC for 10 minutes and allowed to cool to room temperature. For ligation 5 µl of digested DNA, 1 µl double stranded adaptor (5µM), 2 µl 10X ligation buffer, 0.1 µl T4 DNA ligase (New England Biolabs ref: M02025, 400U/µl), were mixed in a total volume of 20 µl and incubated overnight at room temperature.

PCR1 was set up as follows:
2 µl 10X Taq buffer
1.6 µl 2.5 mM dNTP mix
0.5 µl 10 µM primer 1
0.5 µl 10 µM primer 2
0.08 µl 5 units/µl Takara ExTaq (Lonza)
Water, 13.32 µl.
2 µl ligated DNA

PCR1 conditions:
94°C 2mn 1X
94°C 20s, 60°C 20s, 72°C 2mn 5X
94°C 20s, 58°C 20s, 72°C 2mn 5X
94°C 20s, 56°C 20s, 72°C 2mn 20X

PCR1 product was diluted 20 times with autoclaved distilled H_2O and 2 µl was used in PCR2. The concentrations of all chemicals used during PCR2 were the same as in PCR1.

PCR2 conditions:

94°C 2mn 1X

94°C 20s, 55°C 20s, 72°C 2mn 10X

94°C 20s, 52°C 20s, 72°C 2mn 25X.

2.5.4.3 Cloning of PCR products

PCR products were analysed on agarose gels. In some cases, bands were extracted using a gel extraction kit (Sigma, NA1111) and cloned into the pGEM-T easy vector (Promega) according the manufacturer's procedure. In other cases, the entire PCR2 mixture was purified using a PCR purification kit (QIAquick PCR purification kit, 28104) and the mixture of products was cloned. PGEM-T easy vector was used for cloning the PCR product.

Chemically competent JM109 (Stratagene) were transformed according to the manufacturer's protocol and selected on LB plates containing X-gal/IPTG and carbenicillin. To identify clones containing different insert sizes colony PCR (conditions) was used. This was accomplished by touching a colony with a micropipette tip and dipping the tip into a standard PCR reaction mix. The conditions of colony PCR were same as standard one (see 2.5.3). Suitable colonies were then used to inoculate 3 ml of LB containing carbenicillin. Plasmid DNA was isolated using the Qiagen plasmid purification kit (QIAprep Spin Miniprep).

2.5.4.4 Sequencing and sequences analysis

Plasmids were sequenced at the technology facility, University of York by using either T7 or SP6 primers. Sequence data were inspected and analysed using Sequence Scanner (version 1.0, Applied Biosystems) and lasergene. After trimming away vector and *Tnt1* regions, the sequences were analysed by using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). EST and genomic data bases was searched for identity or similarity to the flanking sequences. Sequences with a perfect (or near perfect) match to a data base sequence were analysed further using a longer region left and right to the isolated sequence and performing blastn, blastx and tblastn. Protein sequences were obtained by using Expasy translate tool (used to translate

nucleotide sequences) and then blastp was performed with these protein sequences. Phylogenetic trees were made by using protein sequences from blastp and ClustalX programme was used.

2.6 Data Analysis

Data was analysed statistically by using software “R” (version 2.7.2). A Kruskal-Wallis test with multiple comparison correction was applied to the colonization data.

Chapter 3

Screening of *M. truncatula* mutants

3.1 Screening of *Medicago* mutants for reduced AM colonization

3.1.1 Preliminary experiments to test the experimental conditions

Initial experiments were performed to test suitable conditions for mutant screening. It was decided to use some wild-type and known mutant lines to see which conditions were best to discriminate mutants from wild type. Conditions that were varied were the type of growth substrate, inoculum strength and length of cultivation before testing. We aimed at finding conditions under which wild type was reasonably well colonized, e.g. 50% root length colonization. We also reasoned that colonization should not reach saturation, as this would increase the risk of not detecting mutants with a weak phenotype. Ideally, conditions should be such that there is little variation between individual plants. All wild-type plants should be colonised and every individual mutant plant should be distinguishable from wild type.

Although 10% inoculum was used in primary screening of mutants, we tested whether 5 % inoculum would be sufficient to observe a difference of colonization between wild-type and mutants. In the primary mutant screening a sand/Perlite mix (50% v/v) was used. However, since a mixture of sand and Terragreen was used earlier in the lab, this substrate was also tested here. To find the right time for testing colonization, plants were analysed after four and seven weeks of sowing. Previously some mutants were identified by using a time point of 6 weeks (David-Schwartz et al., 2001; Barker et al., 1998) and 8 weeks (Paszkowski, 2006). For wild-type plants the expectation of colonization was about 40-90 % and normally this range of colonization is observed in wild types of different plant species, e.g. tomato, maize and *Petunia* (Barker, 1998; David-Schwartz et al. 2003; Paszkowski et al., 2006; Reddy et al., 2007). In *Medicago truncatula* wild-type A17 a colonization range of 60-75 % at different time points was observed (Zhang et al. 2010).

Figure 3.1 shows the average percentage of root length colonization for an overall view of differences of fungal colonization between wild-type and mutant lines. To exhibit the plant to plant variation of colonization, individual graphs have been displayed in Fig. 3.2. The average percentage colonizations were higher after seven

weeks as compared to four weeks (Fig. 3.1). All three wild-type lines were highly colonized as compared to the mutant lines after four and seven weeks and it was not easy to decide which condition was best although at 5% with perlite the colonization was generally quite low and many wild-type plants were not colonised (Fig. 3.2). This suggested that the use of P + 5 % inoculum was not suitable to distinguish the mutant lines from wild types. There was a danger of selecting many false mutants. Two plant samples of *ram1* were exhibiting surprisingly higher colonization after seven weeks as compared to wild-types, which seems an artifact. With Terragreen + 5 % inoculum and Perlite + 10 % inoculum, colonization of many individual wild-type plants was variable and a few plants were not colonised. It was not convincing to choose such a condition with too many wild-type samples exhibiting reduced fungal colonization, as it was likely that many false positives would be found among the mutant lines.

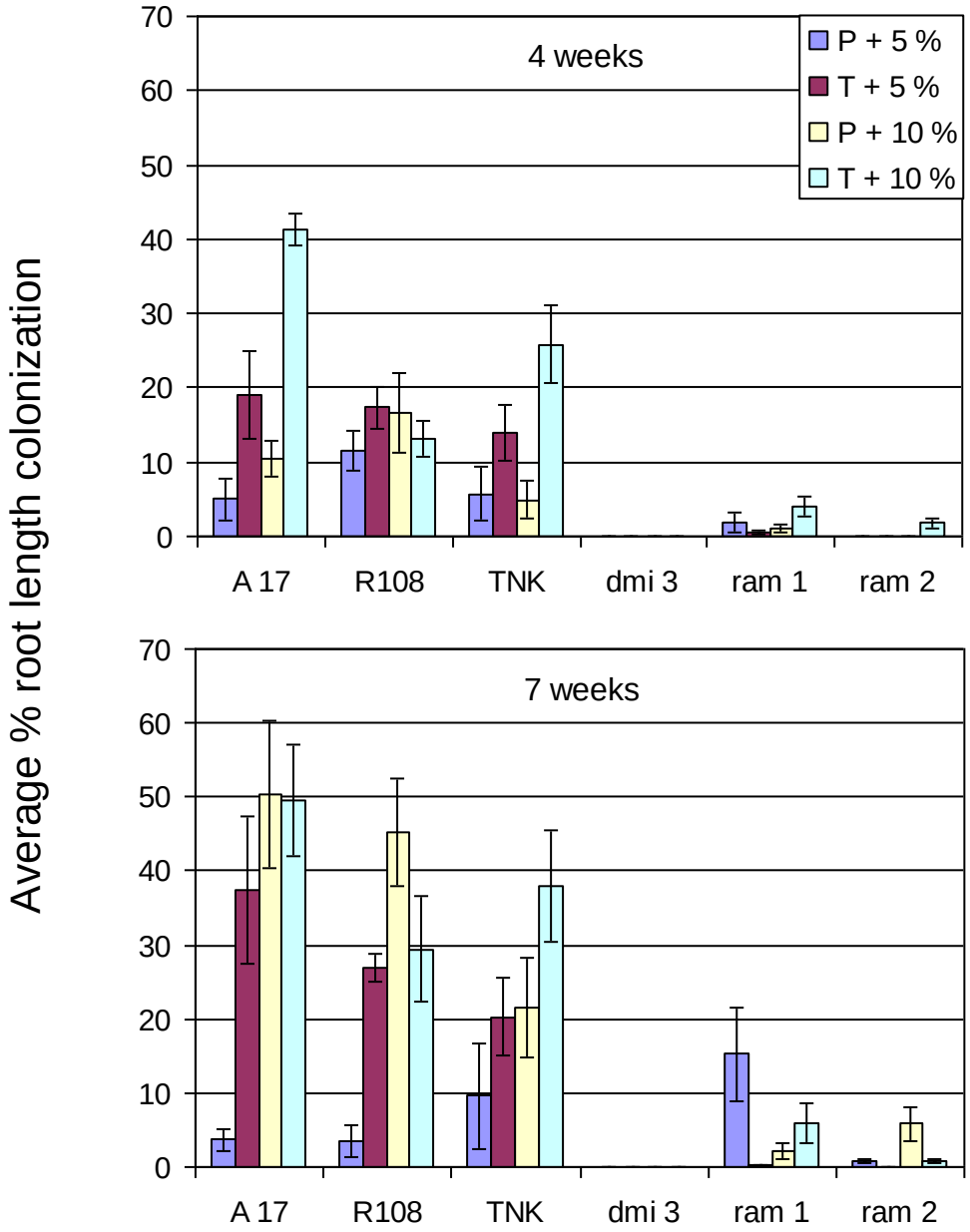


Figure 3.1: Internal fungal colonization percentages in roots of *M. truncatula* lines observed after four and seven weeks. 6-7 plants of each line were tested. Error bars indicate standard errors of mean values. Perlite = P; Terragreen = T. 5 and 10 % of endorize mix was used. A17, R108 acted as control lines. TNK is a starter line. Dmi3 is $nod^{-}myc^{-}$ line obtained by EMS mutagenesis. The reduced mycorrhizal colonization mutants ram1 and ram2 mutants are $nod^{+}myc^{-}$ obtained by fast neutron mutagenesis method.

With Terragreen + 10 % inoculum wild-type samples were highly colonized except uniformally low colonization of R108 after four weeks and absence of fungal colonization after seven weeks in one sample of R108, in which case the low colonization could be explained because the plant looked unhealthy.

Terragreen + 10 % inoculum was chosen as a suitable condition for mutant screening because good and uniform colonization was observed as compared to mutants in most of the individual wild-type plants and there was a clear difference between wild-type and mutant colonization after four and seven weeks. 10 % inoculum was better than 5 % because with 10 % inoculum colonization on wild-type plants was more robust while for known mutants it was consistently low. The four and seven week time points seemed both suitable for mutant screening. Therefore, four weeks were chosen to save time. Fig. 3.3 illustrated a clear difference in colonization between wild-type and mutant lines (absence or reduced). Many arbuscules, internal fungal hyphae and some vesicles are visible in wild-types R108, A17 and starter line TNK. In wild-type fungal penetration and further development of arbuscules, vesicles and internal hyphae was normal. In *ram1* and *ram2* although hyphal penetration was observed at some places in roots, it seemed to be arrested after entry. No fungal structures in mutants *dmi3* and severely reduced colonization in *ram1* and *ram2* was observed, hence explaining the suitability of substrate (Terragreen) and inoculum percentage (10 %) for mutant identification from wild-types. This indicated that mutants were easily discriminated from wild-types by using 10 % inoculum with Terragreen.

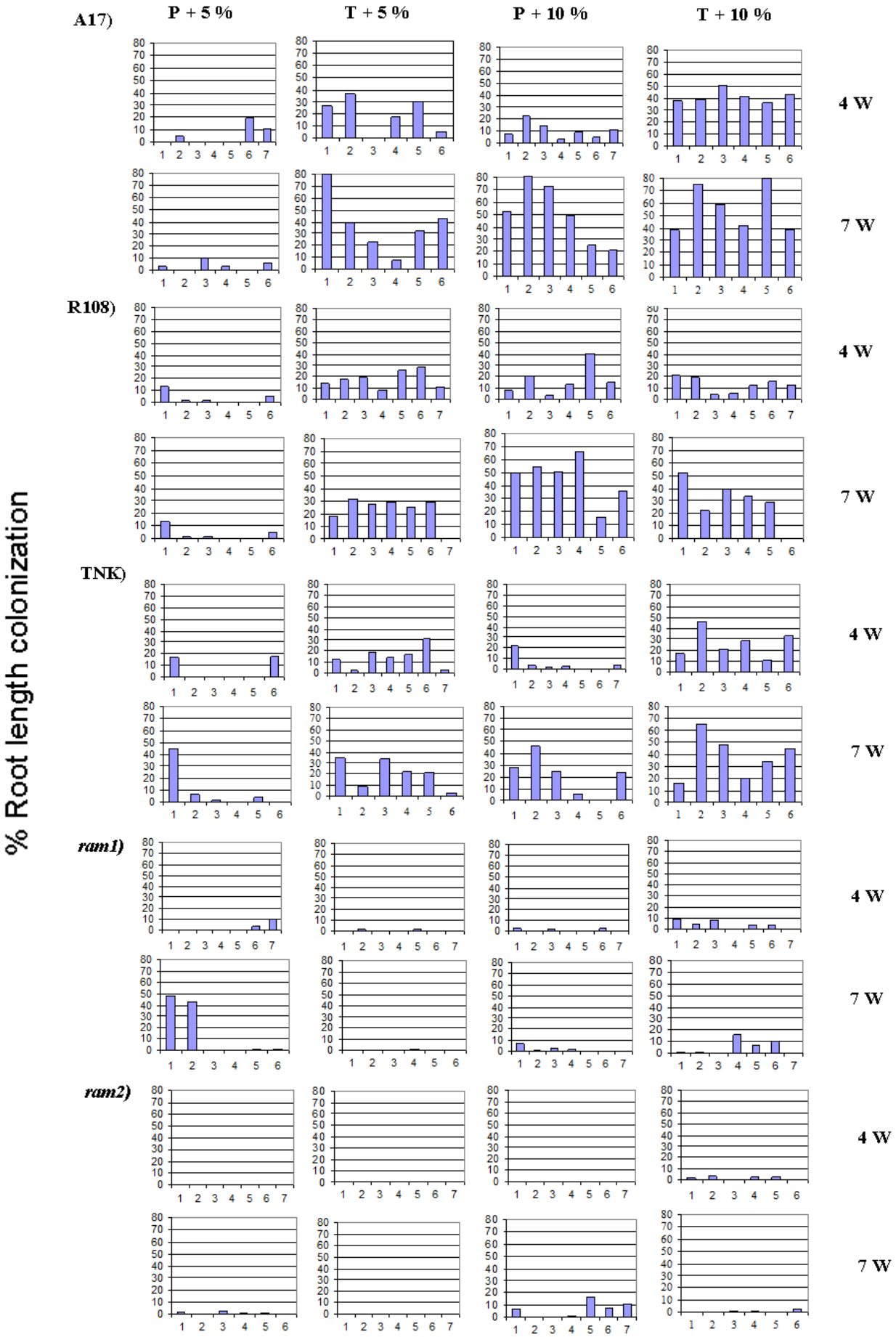


Figure 3.2: Percent root length colonization in individual plants. Fungal colonization was observed after four and seven weeks.

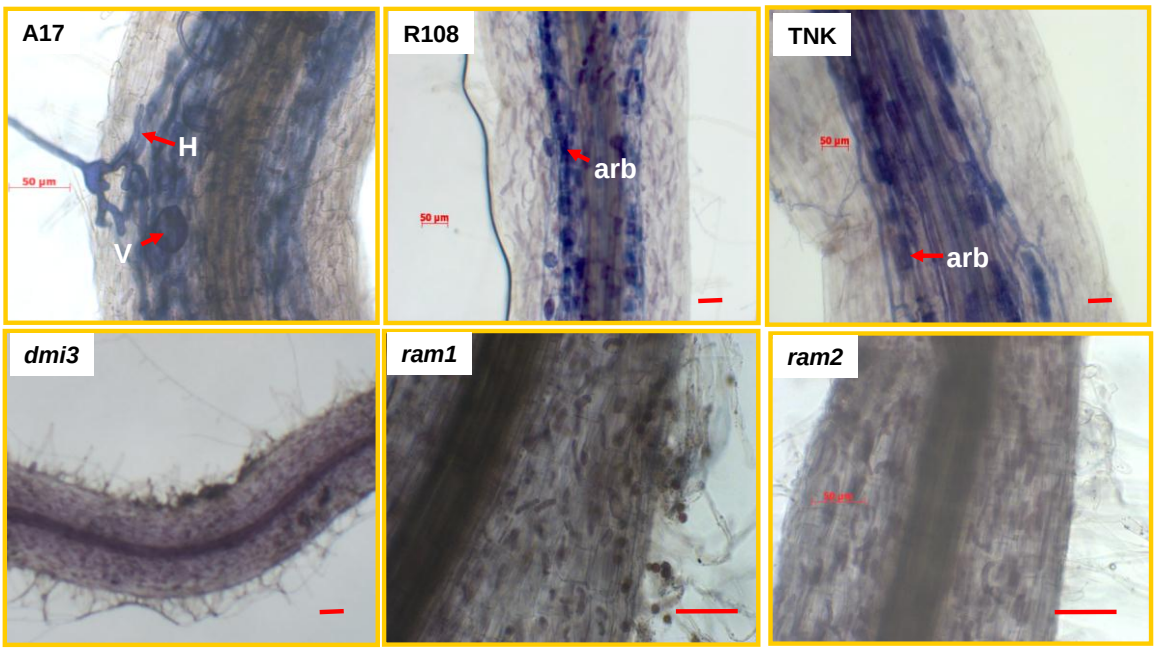


Fig.3.3: Stained roots of *M. truncatula* lines for phenotypic study of initial experiments. H: Hyphae, V: Vesicle, arb: arbuscule. Representative pictures when Terragreen + 10 % Endorize mix was used. Scale bar: 50 µm

3.1.2 Testing colonization of fast neutron mutagenized lines

In a primary mutant screen at the Noble foundation 12 fast neutron mutagenized *M. truncatula* plants were selected as candidates showing reduced AM colonization. Here, the progeny of these plants were retested to confirm the mutant phenotype (Fig. 3.4). Wild-type A17 and three reduced colonization lines *ram1*, *FNB2* and *FNB4* were used as controls. The plants were analyzed four weeks after planting. Colonization of A17 was surprisingly low compared to the preliminary experiment (Fig.3.4 compare with 3.1). It was also lower than the colonization of many mutant lines. The reason for this reduced fungal colonization is unknown.

In the control mutants, *FNB2*, *FNB4*, and *ram1* colonization was extremely reduced, with a maximum of 1 %. Lines *FNB2* and *FNB4* are defective in fungal penetration and arbuscule development and are also deficient in nodulation (Murray et al., 2011). Colonization of the mutant candidate lines varied between 15 % and 44 % (Fig. 3.4). Since the tested plants of each line were progeny of single plants selected in the primary screen, the average root length colonization should have been significantly below that of wild type. This was not the case. Therefore, all the lines appeared to be false positives and no clear mutant line was identified from this fast neutron population.

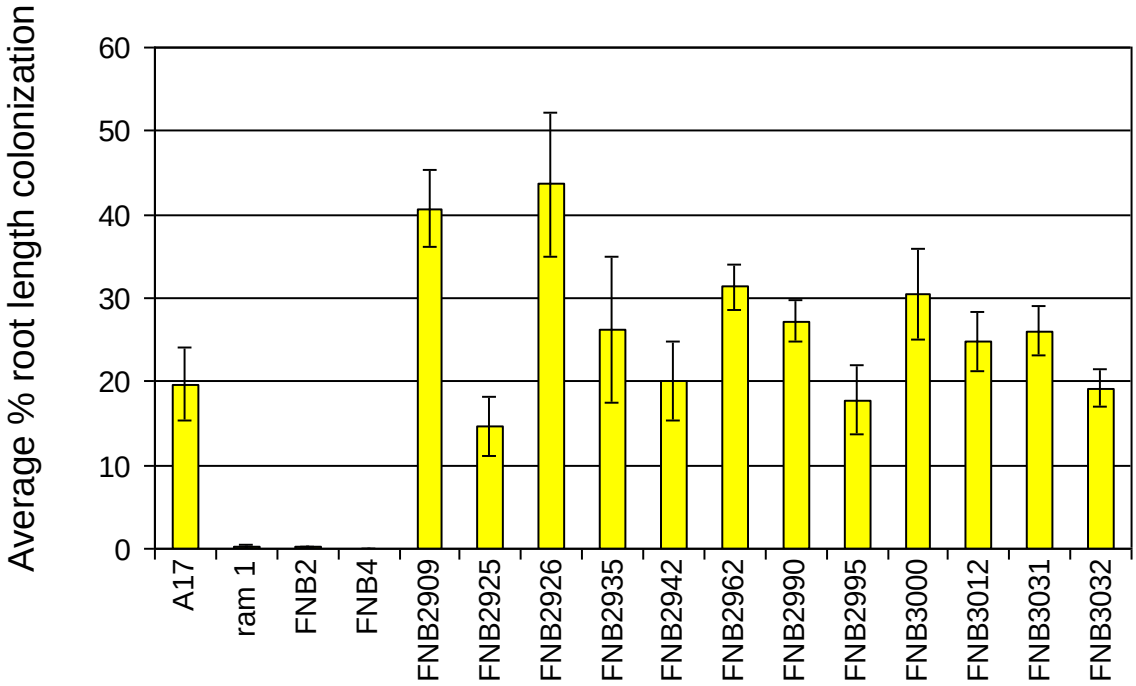


Figure 3.4: Internal fungal colonization in the roots of fast neutron mutagenized lines. A17: wt control, FNB2, FNB4 and *ram1*: known mutant controls. Error bars displaying standard errors of the mean of 5-9 plants.

3.1.3 Selection of mycorrhizal mutants from transposon mutagenized lines

M. truncatula lines mutagenized with the transposon *tnt1* had been screened at the Noble foundation for a number of different phenotypes, including mycorrhizal colonization (Tadege et al., 2008). Up to 12 individual M_1 plants grown from seeds of plants regenerated from tissue culture explants (M_0) had been tested. AM colonization was analysed between four and six weeks after sowing by inspecting the root system for the presence of external fungal hyphae. Plants exhibiting no or reduced external fungal hyphae were considered as having mutant phenotypes (Table 3.1, column 3), while plants with external hyphae were scored as wild type. From the mutant candidates root samples were removed and stained. These were then inspected for the presence of internal root colonization (Table 6, column 5). Lines, for which the staining results confirmed the reduced mycorrhizal colonization, were retested in this project to verify the mutant phenotype (Fig. 3.5).

Mutant candidates from a single line were combined and planted in a single pot after the noninvasive testing for external hyphae, but before the result for the stained root samples were obtained. Seeds were harvested from these pooled plants. Plants that did not show a phenotype of interest were also pooled and grown in a separate pot to maintain and multiply the given insertion line. Therefore, it was possible that descendants of the mutant candidate pools were a mixture of wild-type (false positives) and mycorrhizal mutants. Conversely, the "wild-type" seed stocks would contain a mixture of mycorrhizal mutants (descendants from heterozygous plants or from false negatives) and wild types.

At the onset of this project, for some lines seed stocks were available that were derived from the pooled mutant candidates (NF0000* number labelled with asterisk). For the other lines only the seeds descending from the "wild-type" pools were available (NF0000 number). Depending on the seed stock used and the number of plants in each pool, it was difficult to predict the number of plants displaying a mutant phenotype. It was expected that a homogeneous result could be obtained from some of the mutant pools, whereas a variable number of mycorrhizal mutants would be obtained in all other cases.

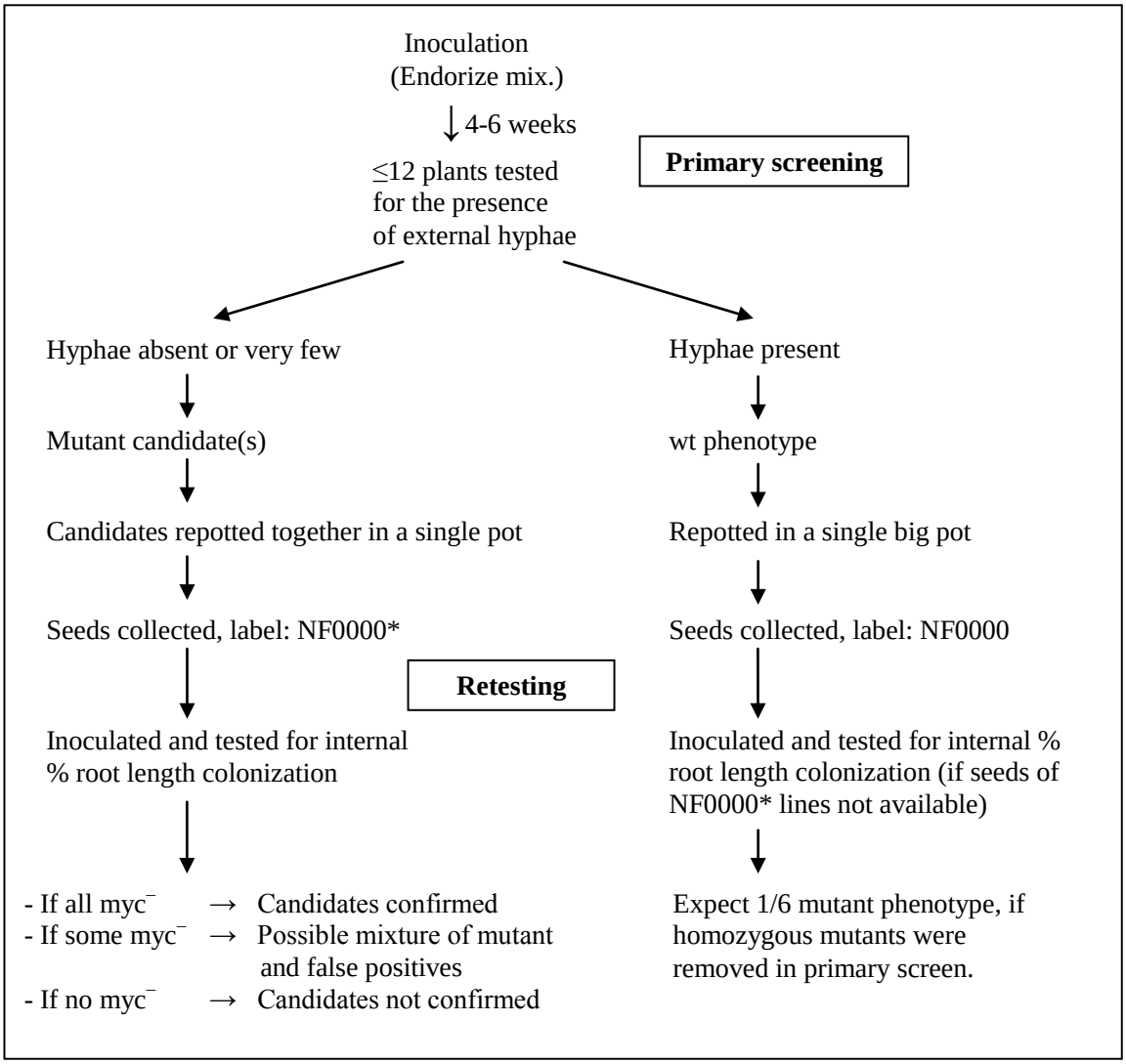


Fig.3.5: Overview of primary mutant screen and expected results of retesting.

Table 6 Comparison of primary and secondary mutant screening

Primary Screen (at Noble foundation)					Secondary Screen (this work)			
Line #	Number of plants tested	Plants with reduced external hyphae	Other phenotype	Myc phenotype upon ink staining	Number of plants tested	Myc phenotype upon ink staining	Confirmed mutants	Comments
NF0131	12	1		myc$^-$	6	0	No	
NF0249	9	1		red.	9*	2 myc$^-$	No	Expected 9/9 (single plant progeny)
NF0383	12	1		myc$^-$	6	0	No	
NF0400	1	1		red.	5	0	No	
NF0413	6	2		red.	5	1 myc$^-$	Possible	
NF0423	12	3		2 red., 1 wt	5*	2 red.	Possible	
NF0425	12	2		1 myc$^-$, 1 wt	6*	2 myc$^-$	Possible	
NF0426	12	3		1 red. 2 wt	6*	3 red.	Possible	
NF0428	10	2		red.	6*	0	No	
NF0447	12	3		2 red, 1wt	8	5 myc$^-$	Possible	Myc$^-$ plants were small
NF0472	12	4		1 myc$^-$, 1 red., 2 wt	5	2 myc$^-$	Possible	
NF0473	5	3		2 myc$^-$, 1 wt	8	0	Uncertain	Need to retest plants from original seed stock
NF0518	12	3		1 myc$^-$, 1 red., 1 wt	8	4 myc$^-$	Possible	Numbers higher than expected
NF0577	7	1	Nod$^-$	red.	10*	0	No	
NF0629	12	3		1 myc$^-$, 2 red.	6	0	No	
NF0689	12	2		red.	8*	6 red.	Possible	
NF0762	12	2		red.	9*	0	No	
NF0788	12	3		2 myc$^-$, 1 red.	9*	4 myc$^-$,5 red.	Yes	Retested, out of 5:1 myc$^-$, 4 strong reduced
NF0807	12	2		1 myc$^-$, 1 red.	10*	5 myc$^-$,5 red.	Yes	Retested, out of 5:2 myc$^-$, 3 strong reduced
NF0819	12	3		1 myc$^-$, 2 red.	6*	0	No	
NF0841	12	1		Pen$^-$	5*	2 red.	Possible	Pen$^-$ phenotype not confirmed
NF0850	12	1		Myc$^-$	5*	0	No	
NF0871	7	2		1 reduced, 1 wt	10	2 red.	Possible	
NF905	8	3		1 myc$^-$, 2 red.	9*	7 red.	Likely	
					9	2 myc$^-$,3 red.	Likely	
NF0919	12	3		1 myc$^-$, 1 red., 1 wt	6*	0	No	
NF0940	10	1	shootless	Pen$^-$	6	1	Possible	Pen$^-$ phenotype not confirmed
NF0984	6	3 Myc^{++}	Nod^{++}	2 Myc^{++}, 1 wt	6	0	No	Myc^{++} not confirmed
NF1248	12	2	small plants	myc$^-$/red.	6	0	No	

Primary and secondary screening results. * Retesting of progeny of "pooled mutants candidates" (see Fig. 3.5). Myc$^-$. no fungal structures observed. Red: reduced % colonization. Pen$^-$. Appressoria present, but no penetration. wt: colonization as in wild-type.

28 candidate lines were retested for mycorrhizal phenotypes. This was done in four batches, whereby R108 and TNK were included as wild-type controls in each batch. Plants were harvested after four weeks of sowing, and their roots were tested for internal fungal colonization (see chapter 2.3). Four lines, NF788, NF807, NF905 and NF447, showed a strong average reduction in colonization compared to the starter line TNK (Figs. 3.6 & 3.8). However, plants of line NF447 were relatively small compared to others. Therefore, the impaired colonization might be an indirect effect of reduced growth. This defect of colonization in NF447 is obvious from Fig. 3.10. NF447 was ignored from further analysis due to reduced plant growth in all plants. NF518, NF689, NF841, NF919 and NF940 also showed an average reduction of colonization, which was however, less severe. A Kruskall-Wallis test with multiple comparison correction indicated that the reduction in colonization was significant ($P < 0.05$) in these lines. Fungal colonization pattern in NF919 and NF940 can be observed in Fig. 3.10. Reduced number of colonization structures as compared to wild-type was observed. Line NF905 was tested in batches 1 and 3 (Figs. 3.6 & 3.8). In both cases similar results were obtained for average root length colonization, although in batch 1 seeds from "wild-type" stock were used (Fig. 3.5). Therefore, I expected a mixture of wild-type and mutant phenotypes, while mutant phenotype was obtained in all plantlets within the line. It is possible that seeds of NF905 might have been mislabelled as wild-types.

R108 and TNK were always colonised in the secondary screening, hence the chance of getting false positive mutants was low. In line 425, two of the six plants were not colonised (Fig. 3.6). This correlates well with the results of the primary screen, where upon staining one of the two plants appeared normal. Some possible mutant candidates were also ruled out during secondary mutant screening. The examples of which are NF413, NF423, N425, NF426, NF518, NF472 and NF689, where the results of primary and secondary mutant screening were correlating well (Table 6).

In line NF689, primary and secondary screening results exhibited a strong correlation. This was depicted by six reduced colonization plants in which colonization was reduced out of eight in secondary screening, while both plants were exhibiting low colonization in primary screen (Fig. 3.7, Table 6).

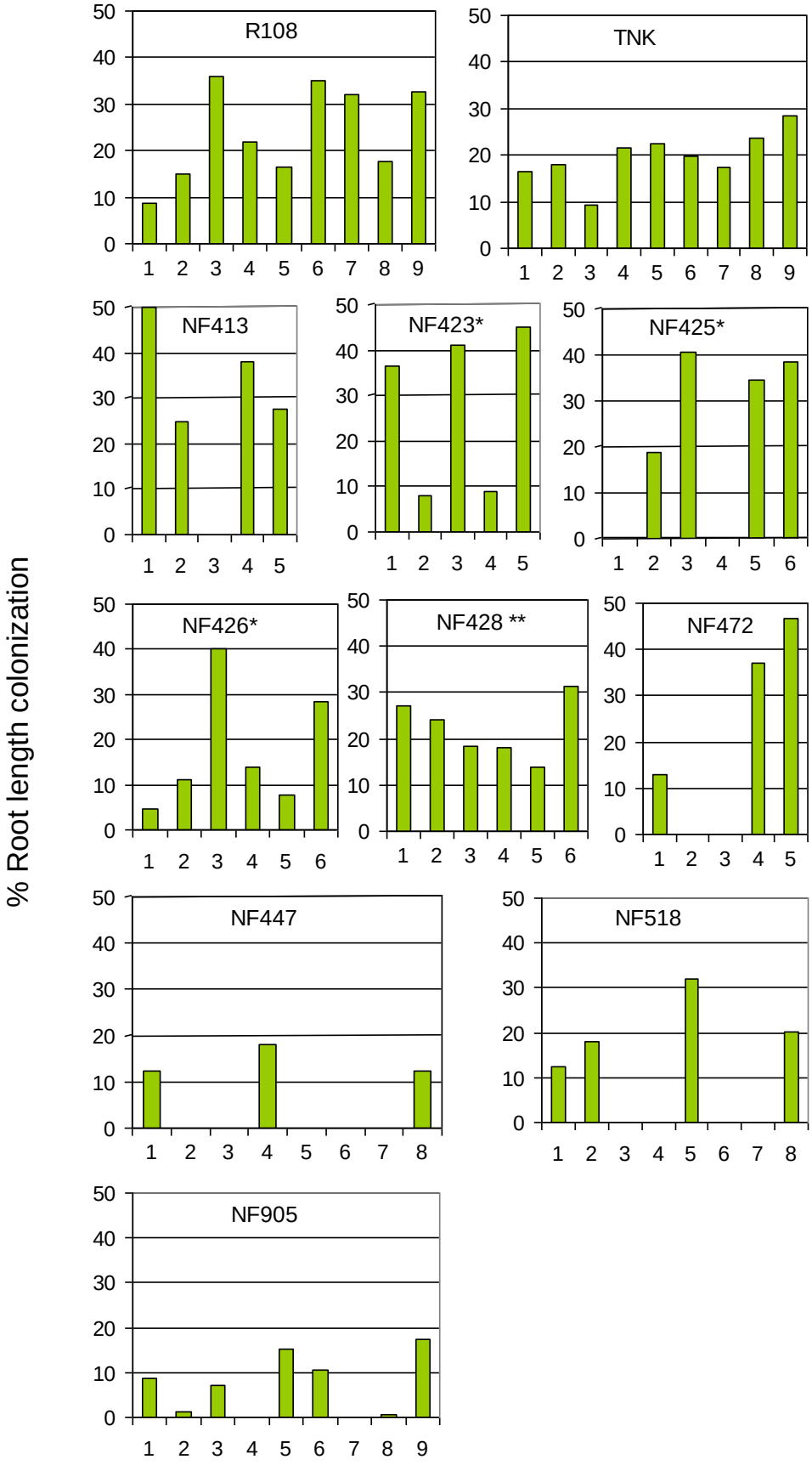


Fig. 3.6: Percentage root length colonizations of individual plants of first batch. Some of the seeds failed to germinate out of total 10, as a result of which the number of plants in different lines was varying, so 5-9 plants of each line were tested. Plant lines R108 and TNK SL were acting as controls.

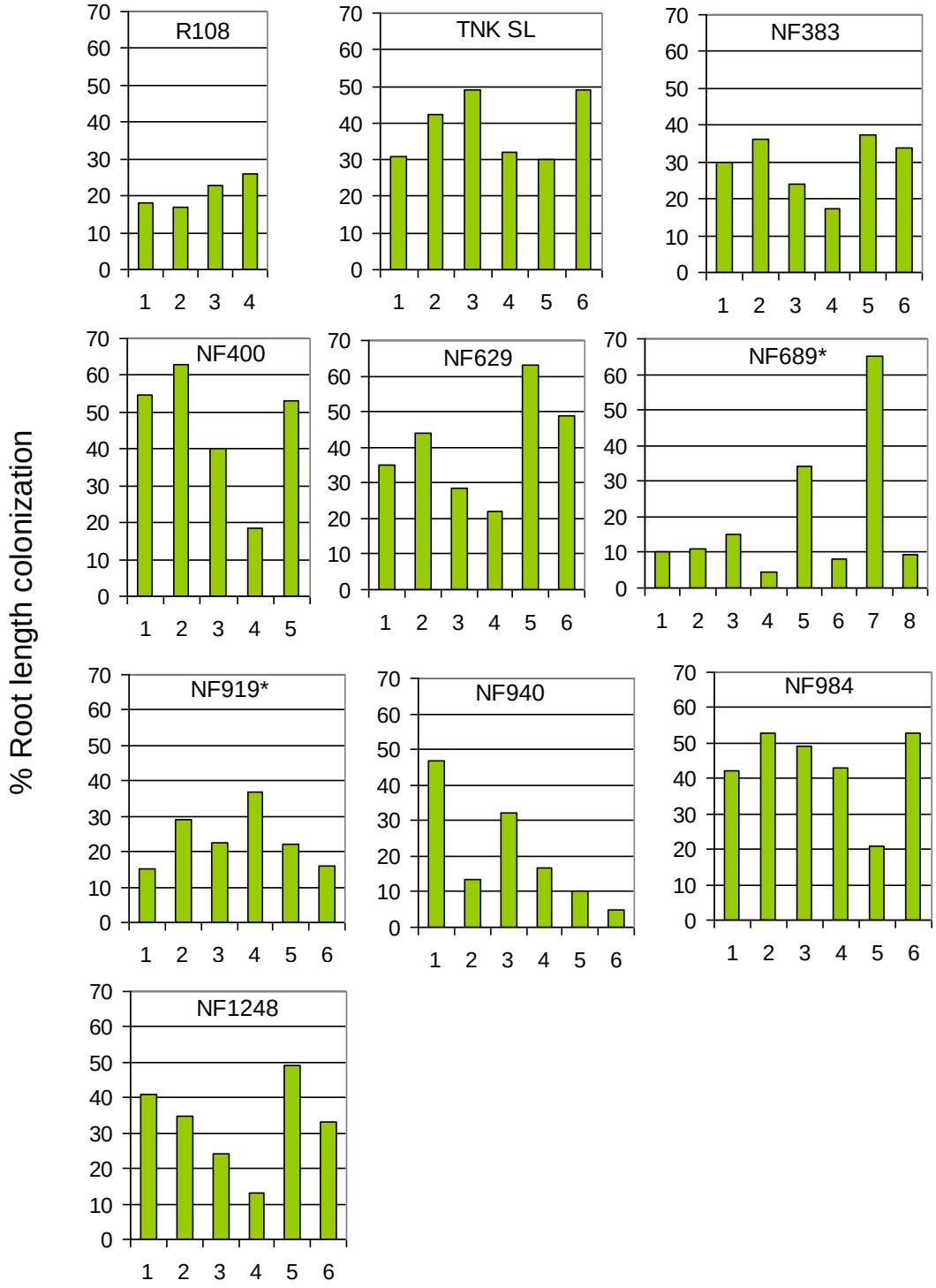


Fig. 3.7: Root length colonization percentages of second batch. 4-8 plants of each line were tested depending on the number of seeds germinated out of total 10. Fungal colonization was analysed on the basis of counting of overall fungal structures. Arbuscules, vesicles and internal hyphae were not counted separately.

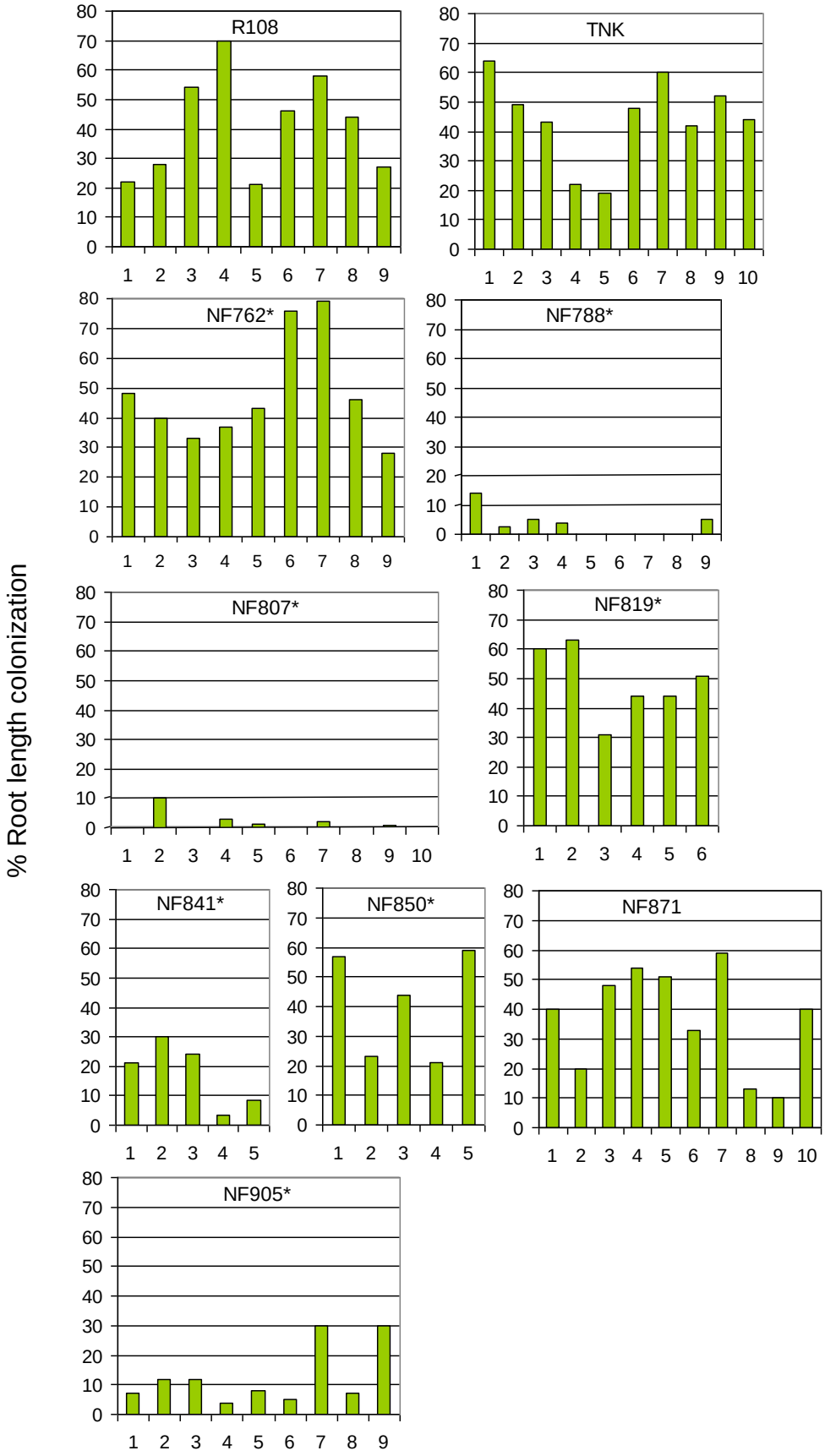


Fig. 3.8: Percentage root length colonizations in individual plants of third batch. 5-10 plants of each line were tested.

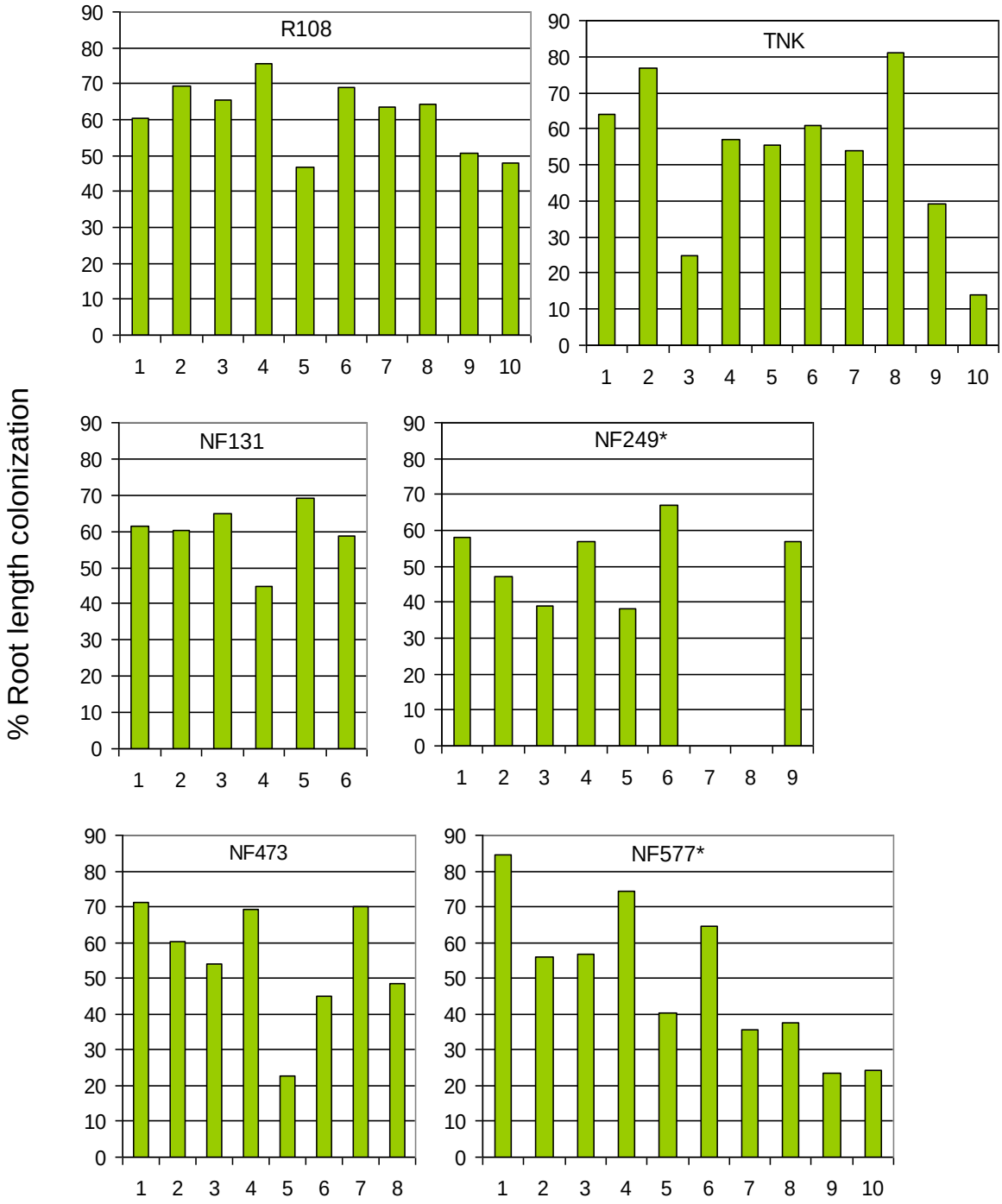


Fig. 3.9: Percent root length colonization in individual plants of fourth batch. 6-10 plants of each line were tested depending on the number of seeds germinated. The time point of testing of all batches was four weeks. The substrate was sand + Terragreen + 10 % Endorize mix for all the batches.

Reduced average percentage colonization displayed by NF841 in secondary screen and penetration defective phenotype in primary screen, indicated the possibility of this line being a mutant (Figs 3.5 & 3.8). Some lines did not exhibit reduced fungal colonization after secondary screening for example NF131, NF473, NF577 etc (Table 6) (Fig. 3.9), and so these were excluded from future analysis. Plants of mutant line NF249 were the progeny of single plant, therefore our expectation was to get reduced fungal colonization in all the plants. This was not the case because two out of nine plants displayed myc$^-$ phenotype (Fig. 3.9). There might be an artifact in primary or secondary mutant screening result in the case of NF249.

After secondary mutant screening the following lines were retained as promising and possible candidates: NF413, NF423, NF425, NF426, NF472, NF518, NF689, NF788, NF807, NF841, NF871, NF905, NF919, and NF940. Three lines with a very strong mutant phenotype (NF788, NF807, and NF905) were further characterised in this project.

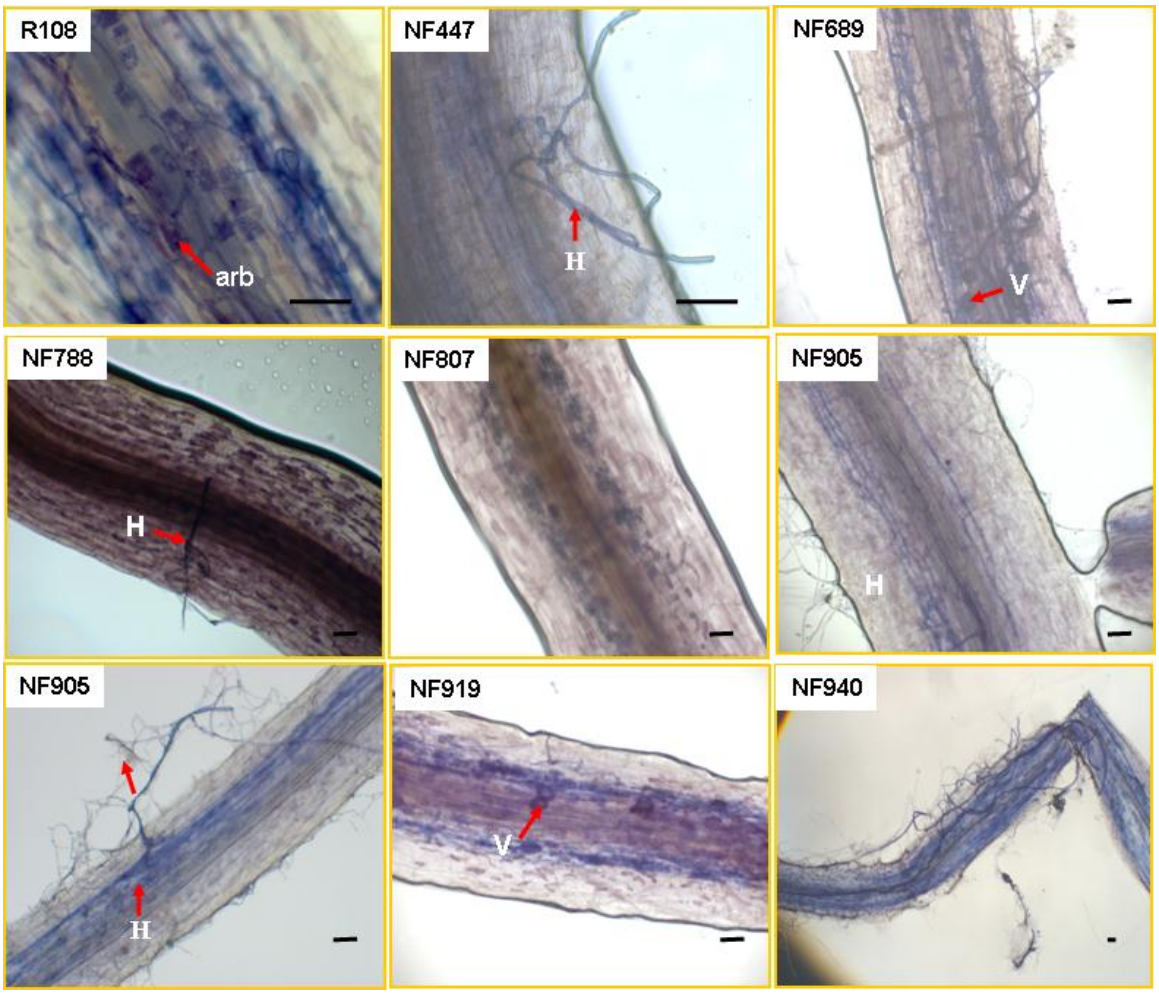


Fig. 3.10: Stained roots of some representative samples of different lines of *M. truncatula.* arb: arbuscule, H: Hyphae, V: Vesicle. Scale bar: 50 µm.

3.2 Defect in arbuscule-development function in mutant lines NF788, NF807 and NF905

When we tested the colonization in M_3 plants of mutant lines NF788, NF807 and NF905 with Endorize inoculum some external hyphae and reduced internal colonization as compared to wild-type R108 was found (Fig. 3.10). Fungal growth was retarded after penetration of hyphae in roots of NF788, NF807 and NF905, suggesting the initial signalling events between plant and fungi are normal. It seems that the defect in these mutants lies at later stages of mycorrhizal development i.e. arbuscule formation. The growth of all three lines appeared normal. Differences of fungal colonization between

wild-type and mutants were obvious seen in Fig. 3.10. Wild-type line R108 exhibited an expected result of a reasonable level colonization of arbuscules, vesicles and internal hyphae. It was concluded here that mycorrhizal fungi were not able to form a successful symbiosis due to the lack of fully developed arbuscules in mutant lines NF788, NF807 and NF905.

Mutants could show higher fungal colonization in the presence of colonized nurse plants. This was reported, for example, by David-Schwartz et al. (2001). Tomato mutants offered less resistance to fungal colonization with inoculated nurse plants, as compared to whole soil inoculum (fungal spores or whole soil inoculum). The improvement of total fungal colonization may be due to hyphal bridges between mutant and colonized nurse plants because there is continuous infectious pressure on mutants due to wild-type colonization (Bethlenfalvay et al., 1991). Fungal hyphae from the inoculated nurse plants may have a continuous supply of nutrients and energy for plants. We carried out an experiment to test the possibility of overcomming the reduced colonization and impaired arbucule formation in mutants NF788, NF807 and NF905 by use of inoculated leek plants. Based on the results of David-Schwartz et al., (2001) our expectation was that in a nurse pot experiment, mutants should show higher colonization of arbuscules, vesicles and internal hyphae. This was not the case because the mutant phenotype was retained in the form of incompletely developed arbuscules, although the total colonization was increased compared to the screening experiment where Endorize mix was used. This result was similar to what was reported by Reddy et al. (2007) and Murray et al., (2011) for *Petunia* pam1 mutants and *M. truncatula* mutant FNB4, where overall level of colonizations of mutants was reported to be similar to wild-type except for the defect in arbuscule formation.

It was assumed that absence of arbuscules may result in defective symbiotic interaction. Figures 3.11 and 3.12 show the formation of two types of arbuscules, fully developed and incomplete in NF788, NF807 and NF905. A fewer number of fully developed arbuscules were observed in mutant lines NF788, NF807 and NF905 as compared to the wild-type. This indicated that the arbuscule development was not ruled out completely even by inoculated nurse plants. Wild-type R108 exhibited a reduced number of incompletely developed arbuscules compared to mutant lines (Fig. 3.11). The defect in the development of arbuscules in mutants in the presence of inoculated leek plants

suggested a fault or malfunction in mycorrhizal pathway. Further analysis is needed to test whether the mutants are also impaired in phosphorus transport.

Fig. 3.12 shows the differences in structures of incomplete arbuscules in mutant NF807 as compared to complete arbuscules observed in R108, indicating the lack of complex arbuscule structure in incompletely developed arbuscules. NF788 was completely devoid of arbuscules, indicating the impairment of post penetration steps in mycorrhizal symbiosis. Fewer numbers of hyphae and vesicles as compared to wild-type R108 and absence of arbuscules in NF905 was obvious as can be seen in figure 3.12. Hence the mutation could not be rescued by the use of inoculated leek plants in a nurse pot experiment.

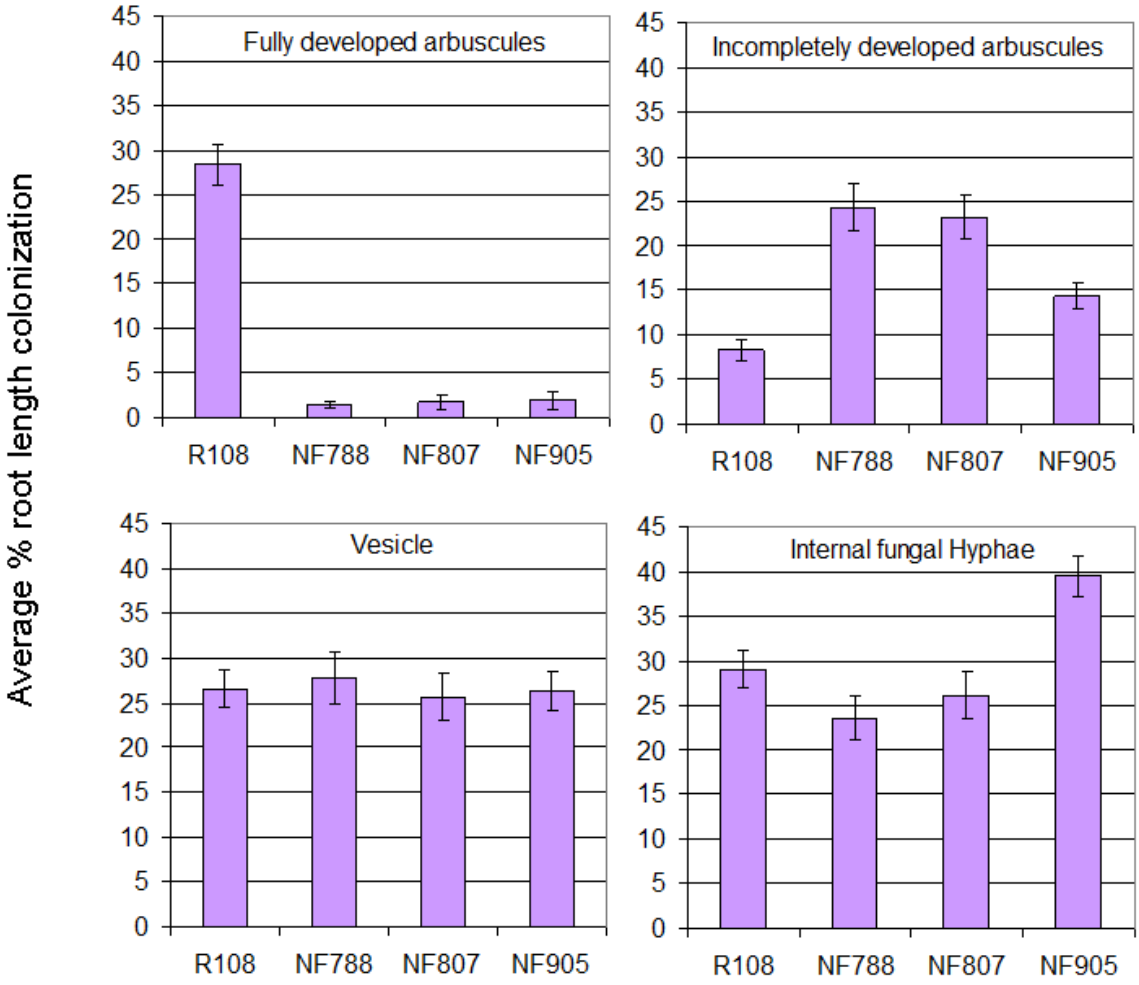


Fig. 3.11: Internal % root length colonization of mutants in the presence of leek nurse plants. Wild-type line R108 was used as a control. Graphs are representative of three experiments. Error bars show standard errors of mean values of 19−21 plants.

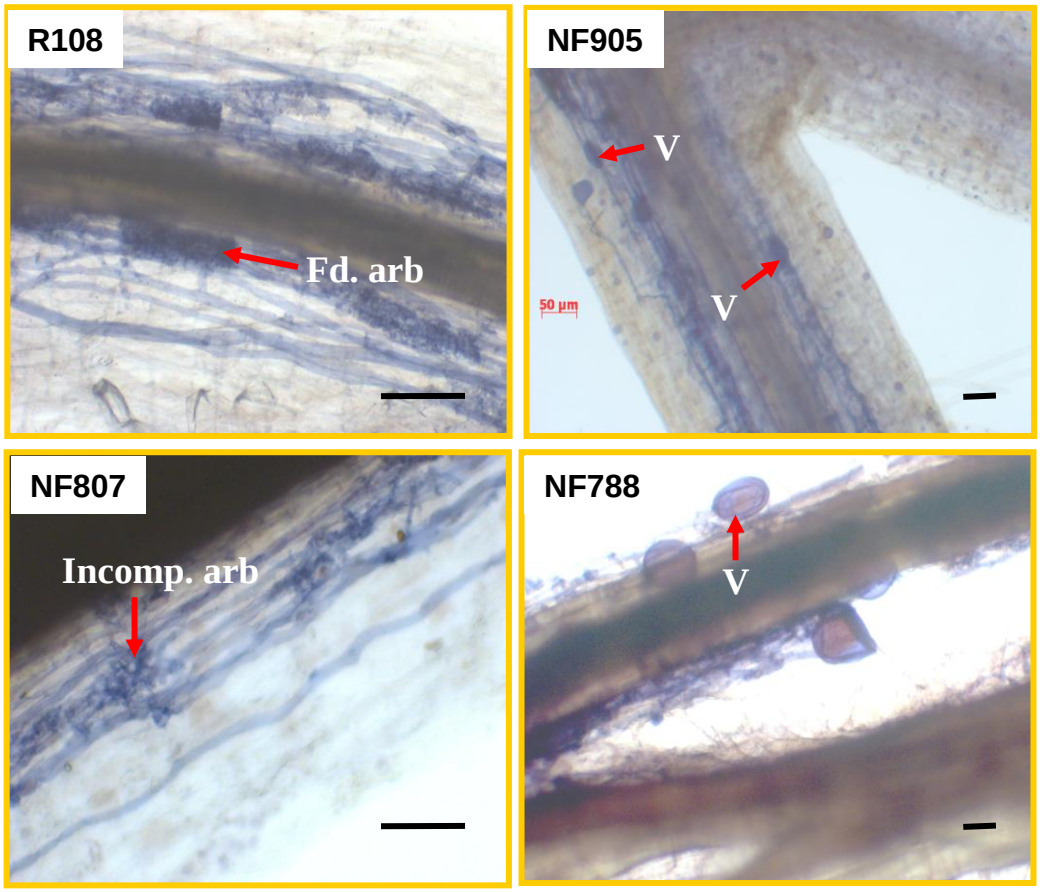


Fig. 3.12: Light microscopical view of stained roots of wild-type (R108) and mutants for internal fungal colonization. Fd. arb: Fully developed arbuscules, Incomp. arb: Incompletely developed arbuscules, V: Vesicle, H: Hyphae. Scale bar: 50 µm.

Chapter 4

Isolation of flanking sequences and segregation analysis

4.1 Testing mutant lines for insertions in some known genes

The phenotype of the mutants NF788, 807 and 905 is reminiscent of that found upon mutation of the mycorrhiza-specific phosphate transporter *MtPT4* (Javot et al., 2007 b). This gene is required to sustain AM symbiosis and arbuscule life and morphology. To test whether insertion of *Tnt1* in this gene could be the cause of the mutant phenotype, we analysed the mutants for the presence of wt alleles. Using three sets of primers, a region of 2733 bp, covering the entire coding region of *MtPT4* and 943 bp upstream of the start codon, was amplified from DNA isolated from M3 plants (Fig. 4.1). Products of expected sizes were observed for all three mutants, suggesting that mutation of *MtPT4* is not related to their mycorrhizal phenotype.

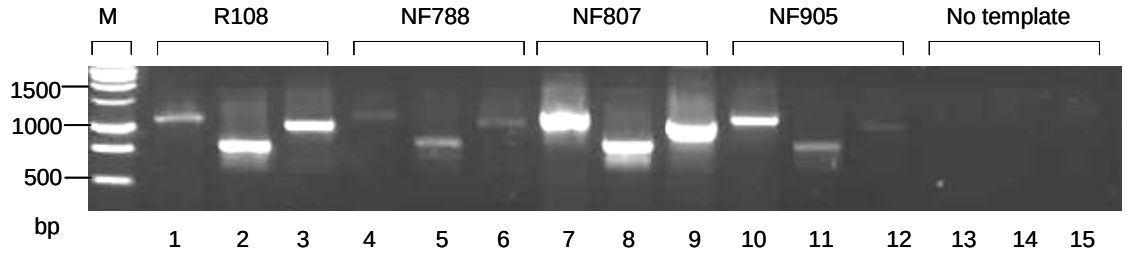
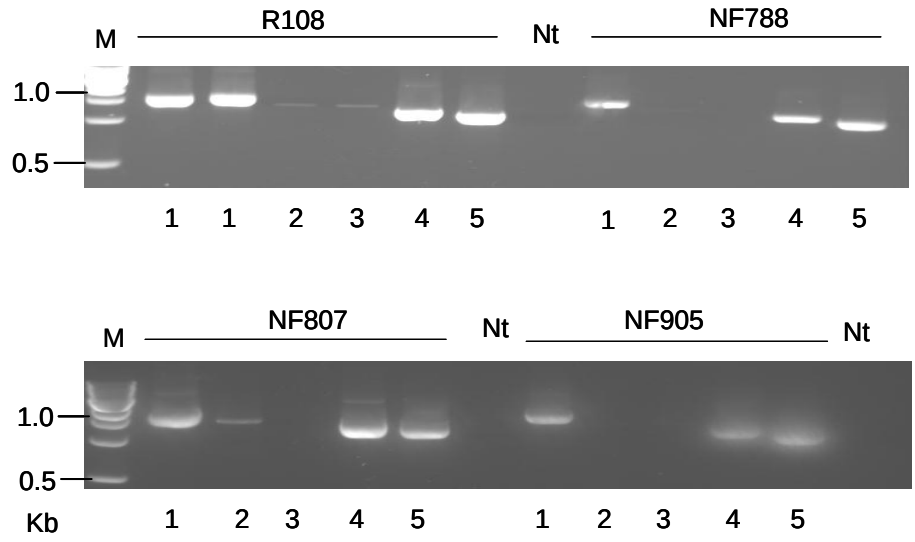


Figure 4.1: PCR amplification of intact *MtPT4* gene in mutant lines. Three primer pairs were used. Expected sizes: *MtPT4* F1/R1: 1111 bp, F2/R2: 799 bp, F3/R3: 1012 bp. 1 kb DNA ladder was used from Promega.

I also analysed the gene that was isolated at the John Innes Centre from mycorrhizal mutant *ram1*. Different PCRs gave some conflicting results. Wild-type bands were amplified (Fig. 4.2 A) with 1st, 4th and 5th primer pairs. In a different PCR reaction wild-type amplification was also observed with 2nd and 3rd primer pairs (Fig. 4.2 B). Therefore, it was concluded at this point, that there was no *Tnt1* insertion on *ram1* gene in lines NF788, NF807 and NF905. However, sequencing of insertion sites in line NF807 and subsequent retesting revealed a *Tnt1* insertion in the *ram1* gene between the region of 4th primer pair. Hence later it was concluded that results shown in Fig 4.1 were PCR contaminants. Moreover, NF807 and NF788 turned out to be siblings lines (see next section).

A)



B)

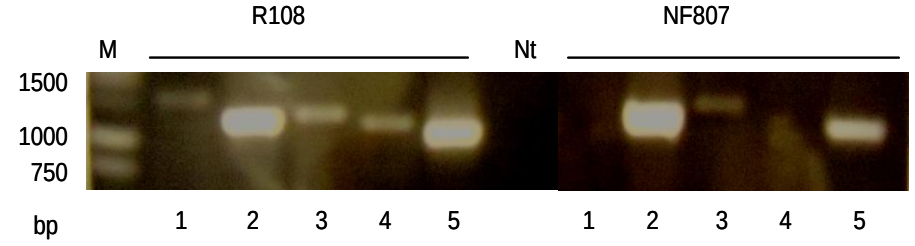


Fig. 4.2: A) PCR amplification of *ram1* candidate gene in mutant lines for the presence of the wild-type copy of that gene. Wild type R108 acted as a positive control. Nt: Non template control. Five primer pairs were used with expected sizes: F1/R1; 1187 bp, F2/R2; 1300 bp, F3/R3; 1288 bp, F4/R4; 1215 bp, F5/R5; 1165 bp. 1 kb DNA ladder was used (New England Biolabs). B) Repeated PCR in which mutant line NF807 showed wild-type bands with 2nd, 3rd and 5th primer pairs.

4.2 Isolation of insertion sites

The DNA sequences of mutants NF788, NF807 and NF905 flanking the transposon insertions were isolated by PCR of adaptor-ligated genomic DNA (Ratet et al. 2006). Genomic DNA was isolated from M3 plants and digested with restriction enzymes EcoRI and MfeI in combination, AseI or NdeII (Fig. 4.3).

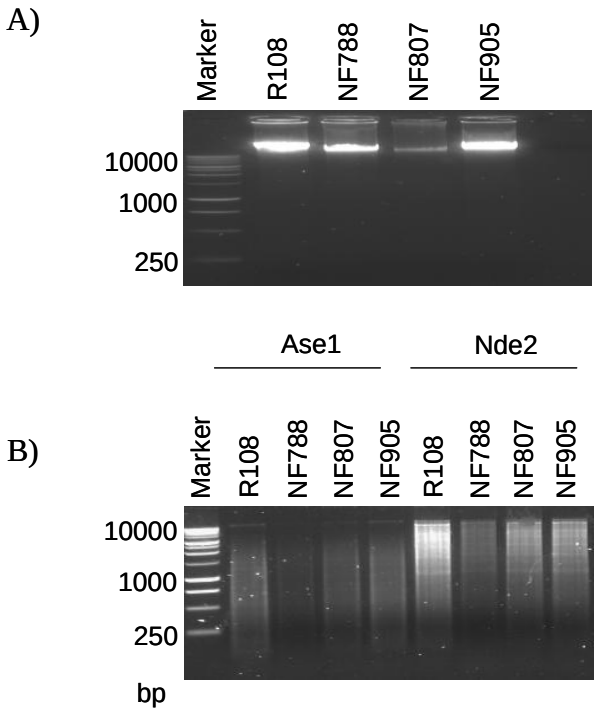


Fig. 4.3: Agarose gel (1 %) electrophoresis of DNA: A) before, B) after digestion. The DNA was digested with AseI and NdeII. 100 bp 1 kb Promega ladder were used. (An example of DNA digestion done during the project).

Given the average GC % of 38 % of *M. truncatula* genomic DNA (Blondon et al., 1994), the expected average size of restriction fragments was as follows. EcoRI/MfeI (GAATTC and CAATTG): 1500 bp; AseI (ATTAAT): 1126 bp; NdeII (GATC): 288 bp. Few thousand bp of average sized fragments were recovered for EcoRI/MfeI, AseI and NdeII. Fig. 4.3 is an example exhibiting DNA samples before and after digestion. Completely digested DNA should appear as smear around the expected average size. Digestion with NdeII was not complete. However, given the frequency of occurrence of the four-base recognition site, it was thought that a partial digest was acceptable and would allow recovery of longer stretches of flanking sequences.

Genomic DNA flanking the transposon on the 3' and 5' side was amplified after ligation of restriction fragments to synthetic adaptor oligonucleotides. PCR1 was performed for preferential amplification of the flanking region. Some non specific products are also amplified during PCR1, so PCR2 is carried out to remove these products. PCR2 results (Fig 4.4) gave fragments of different sizes corresponding to various flanking sequences. Bands of the same sizes in NF788 and NF807 produced a suspicion that these lines could be siblings or these bands may correspond to the donor line sequences. PCR2 products were cloned and sequenced. Nine sequences were recovered from line NF788, seven from NF807 and eleven from NF905. Three sequences corresponded to three of the five insertions sites in the starter line. Four sequences were shared between NF788 and NF807 that were not derived from donor lines. This confirmed the suspicion that these two lines are siblings. Later, crossing results also confirmed their allelic nature. In total, nine new insertion sites were isolated from each NF788/807 and NF905, respectively (see annexe and Tables 7 and 8).

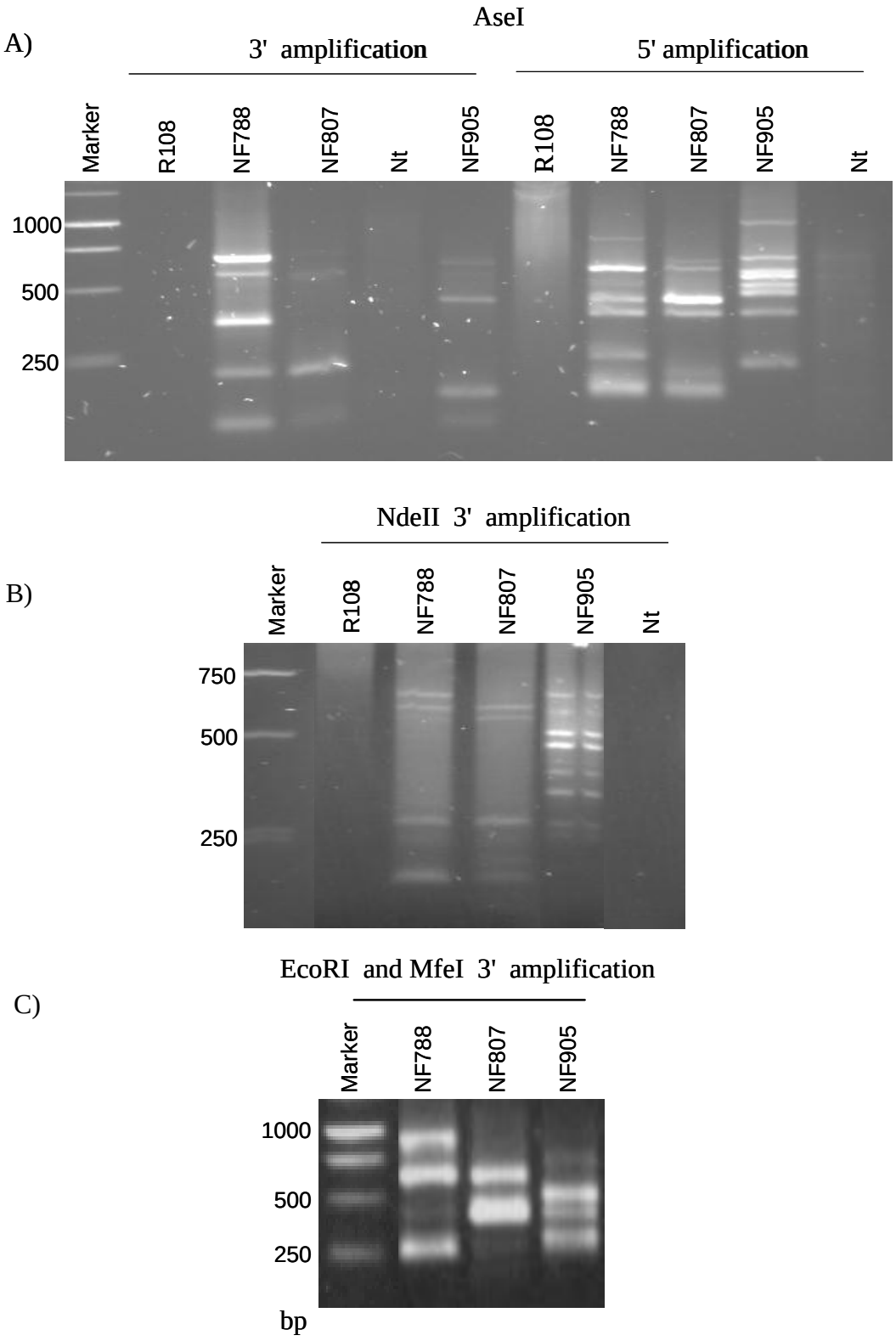


Fig. 4.4: Some examples of PCR2 products. Insertion sites were amplified either 3' or 5' sides of *Tnt1*. EcoRI/MfeI, AseI and NdeII were used for the digestion of genomic DNA, PCR1 and PCR2 were performed after ligation reaction. M: marker (1 kb from Promega). Nt: Non template control.

NF788 was crossed with NF807 by using NF788 as a male parent. Very reduced fungal colonization was observed in the progeny of crossed plant (Fig. 4.5). This confirmed that NF788 and NF807 are allelic.

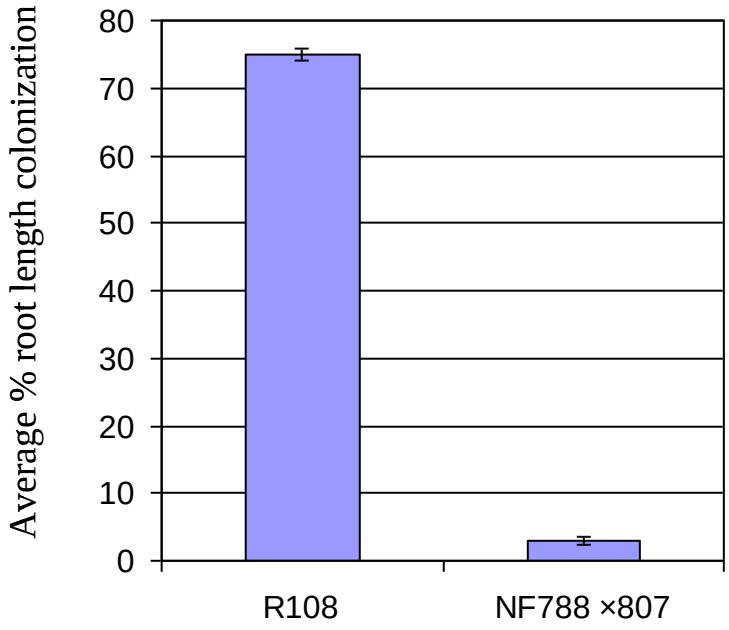


Fig. 4.5: Internal average percentage root length colonizations in the roots of R108 and progeny of cross NF788 x NF807. Error bars are representing standard errors of mean values of 4-5 plants.

Table 7: Flanking sequences isolated from NF788 and NF807

Insertion site number[(a)]	Size of PCR Product [(b)] (bp)	Enzyme used[(c)]	3′ or 5′of *Tnt*	Trimmed length[(d)] (bp)	Hit in nucleotide data base[(e)]	Match in EST data base[(f)]	Predicted protein[(g)]
NF788-1	610	Ase1	5′	488	—	*M. truncatula* developing flower, stem and leaf, *M. truncatula/Meloidogyne incognita* mixed EST, drought *M. truncatula*	Isoflavonoid glycosyl transferase
NF788-2	390	Ase1	3′	293	—	—	Non coding
NF788-3	180	Ase1	5′	64	mth2-94j16	—	Unknown *Medicago truncatula*
NF788-4	230	NdeII	3′	137	mth2-97e21,Chromosome 8 mth2-17m16	Aphid infected shoot *M. truncatula*, Developing flower and leaf, Insect herbivory. *M. truncatula/Glomus versiformae* mixed EST, MtBA *Medicago truncatula* cDNA clone	Auxin induced in roots
NF788-5	270	NdeII	3′	145	Mth2-94j16	*M. truncatula* cDNA clone; MtTA02P14S6, HOGA-2G1, Glandular trichomes *Medicago sativa* cDNA, Germinating Seed *M. truncatula*	Non coding
NF788-6	390	Ase1	5′	271	Mth2-81g19 (Donor line)		
NF788-7	260	EcoR1, Mfe1	3′	163	mth2-24f21	—	Non coding
NF788-8	600	Ase1	3′	499	Mth2-3h12	—	
NF788-9	160	Ase1	5′	53	Mth2-24o19	—	
NF807-1	560	Ase1	3′	458	Mte1-33n12	—	Mitogen-activated protein kinase kinase kinase
NF807-2	570	NdeII	3′	472	Mth2-31h5	—	GRAS transcription factor
NF807-3	660	EcoR1 , Mfe1	3′	537	mth2-17c13	—	Unnamed protein product

Table headings are defined as follows: (a): Numbers of flanking sequence isolated in mutant line NF788. (b): Sizes of PCR2 fragments obtained after gel electrophoresis. (c): Different restriction enzymes were used to digest the DNA. (d): Length of sequence after excluding adaptor and *Tnt1* sequence. (e & f): A near perfect match in nucleotide and EST data base. (g):Identity or similarity to protein found in data base. For 3′ insertion sites the PCR product included 77 bp of *Tnt1* 15 bp of the adaptor, so the expected difference in length between product and trimmed sequence was 92. For 5′ insertion sites, the difference was 114 bp (99 bp from *Tnt1*). The four shared sequences in NF807 were deleted because NF788 and NF807 is the same line. No match in nucleotide and EST data bases has been indicated by dashed line (—). Non coding is exhibiting no predicted coding region.

Table 8: Flanking sequences isolated from NF905

Flanking seq. insertion site number	Size of PCR fragment (bp)	Enzyme used	3′ or 5′ side of *Tnt1*	Trimmed length of flanking sequence (bp)	Hit in nucleotide data base	EST data base	Similarity to any protein
	510	EcoR1 , Mfe1	3′	410	mth2-116e22	*M. truncatula* cDNA clone HOGA-26L6	Unknown protein
NF905-2	670	NdeII	5′	550	mth2-103j6	—	Non coding
NF905-3	330	EcoR1 , Mfe1	3′	200	mth2-24f21	—	Nodulin like protein
NF905-4	870	Ase1	3′	764	mth2-69e15? [(a)]	—	Non coding
NF905-5	500	Ase1	3′	401	—	—	No significant. similarity to protein in data base
NF905-6	170	Ase1	3′	75	—	—	No significant. similarity to protein in data base
NF905-7	130	Ase1	3′	27	mth2-81g19	—	
NF905-8	640	Ase1	3′	541	*Mtha1* (AJ132891)	*M. truncatula* cDNA clone mtgmacc120007e03,mtgmacc120006g06, mtgmacc120017d05, MtBC43B08, Mycorrhizal roots	H^+-ATPase[(b)]
NF905-9	450	Ase1	3′	345	mth2-20e4	—	Non coding
NF905-10	690	NdeII	5′	566	No	Developing leaf, Elicited cell culture *M. truncatula, Nodulated* root *M. truncatula* cDNA clone	MYBR domain class transcription factor
NF905-11	230	NdeII	3′	130	Mth2-3h12 (Donor line)	—	

(a): NF905 exhibited a discontinuous hit in the nucleotide data base. (b): NF905-8 dispalyed identity with mycorrhiza specific gene *Mtha1*. The dashed lines (—) are showing no match in nucleotide and EST data bases. Non coding is exhibiting no predicted coding region.

Most of the sequences were obtained from the 3′ side of *Tnt1*. Any sequences from the 5′ side were inspected for the presence of the 5 bp target site duplication to see whether there was a match among the sequences from the 3′ side. This was the case for two of the five 5′ flanking sequences. It is likely that additional sequences could have been found. However, as soon as insertion sites were discovered that were highly likely to be responsible for the mutant phenotype, sequencing of insertion sites was not continued. Since NF788 and NF807 lines are siblings, the name NF807 or *ram1*-2 is used in further experiments to represent both lines. Before the identification of promising genes in NF807 and NF905 as candidates required for mycorrhizal symbiosis, some other flanking sequences were tested for the presence of wild-type alleles. Flanking sequence NF807-1 corresponded to insertion in coding region of a gene encoding mitogen-activated protein kinase kinase kinase. There was no previous report of indication of this gene to be involved in mycorrhizal symbiosis, however this sequence was analysed to take an initial step for testing of sequences for the presence of wild-type sequences. Gene specific primers were used on both sides of *Tnt1*. Wild-type copy of NF807-1 sequence was amplified (Fig. 4.6 A), hence indicating that *Tnt1* insertion on mitogen-activated protein kinase kinase kinase gene cannot be responsible for the phenotype of reduced mycorrhizal colonization in mutant line NF807.

Flavonoids are also important in mycorrhizal interaction and their accumulation in *M. sativa* root was reported during AM symbiosis (Larose et al., 2002). Fungal growth was also observed to be increased by flavonols (Bécard et al., 1991). Flanking sequence NF788-1 corresponded to *Tnt1* insertion in coding region of a gene encoding isoflavonoid glycosyltransferase. This sequence was also tested for the presence of wild-type alleles by using gene specific primers on both sides of *Tnt1*. PCR results showed the absence of wild-type copy of isoflavonoid glycosyltransferase encoding gene, which indicated that insertion is homozygous (Fig. 4.6 B). *Tnt1* insertion on isoflavonoid glycosyl transferase gene may be responsible for reduced colonization phenotype in NF807.

Similar to NF788-1 and NF807-1, some other *Tnt1* insertions were also observed in the coding region. So it was decided to test these sequences as well to rule out that these insertions are interrupting the function of genes related to mycorrhizal association (Fig. 4.6). Flanking sequences NF788-3, NF905-1 and NF905-3 were tested. Gene expression

studies indicated the expression of nodulin like proteins such as ENOD11 in cortical cells containing arbuscules (Journet et al., 2001). Similarly some other nodulins like *MtN21* and *MtN26* were observed to be induced during symbiosis including nodulation and mycorrhizal development (Manthey et al., 2004). Insertion in the coding region of nodulin like protein (MtN21) in mutant line NF905-3 may be associated with reduced fungal colonization in NF905. A wild-type band was not amplified for a nodulin like gene in a PCR reaction (Fig. 4.6 D). This indicated that NF905-3 insertion is homozygous for a nodulin like gene. Two other sequences, NF788-3 and NF905-1, were also tested for the presence of wild-type copy of a gene encoding unknown protein product (Figs 4.6 B & C). Insertion on flanking sequence NF905-1 cannot be responsible for mutant phenotype in getting wild-type copy of that gene (Fig. 4.6 B). Flanking sequence NF788-3 corresponded to a homozygous insertion site. The sequences which are homozygous for *Tnt1* insertion cannot be excluded from further analysis, but not all the sequences that show homozygous insertion site can be responsible for mycorrhizal phenotype. Cosegregation analysis is needed to test whether the phenotype is cosegregating with the insertion site or wild-type copy of those genes can be introduced in mutant for the restoration of wild-type colonization and this can also explain the correlation between phenotype and genotype.

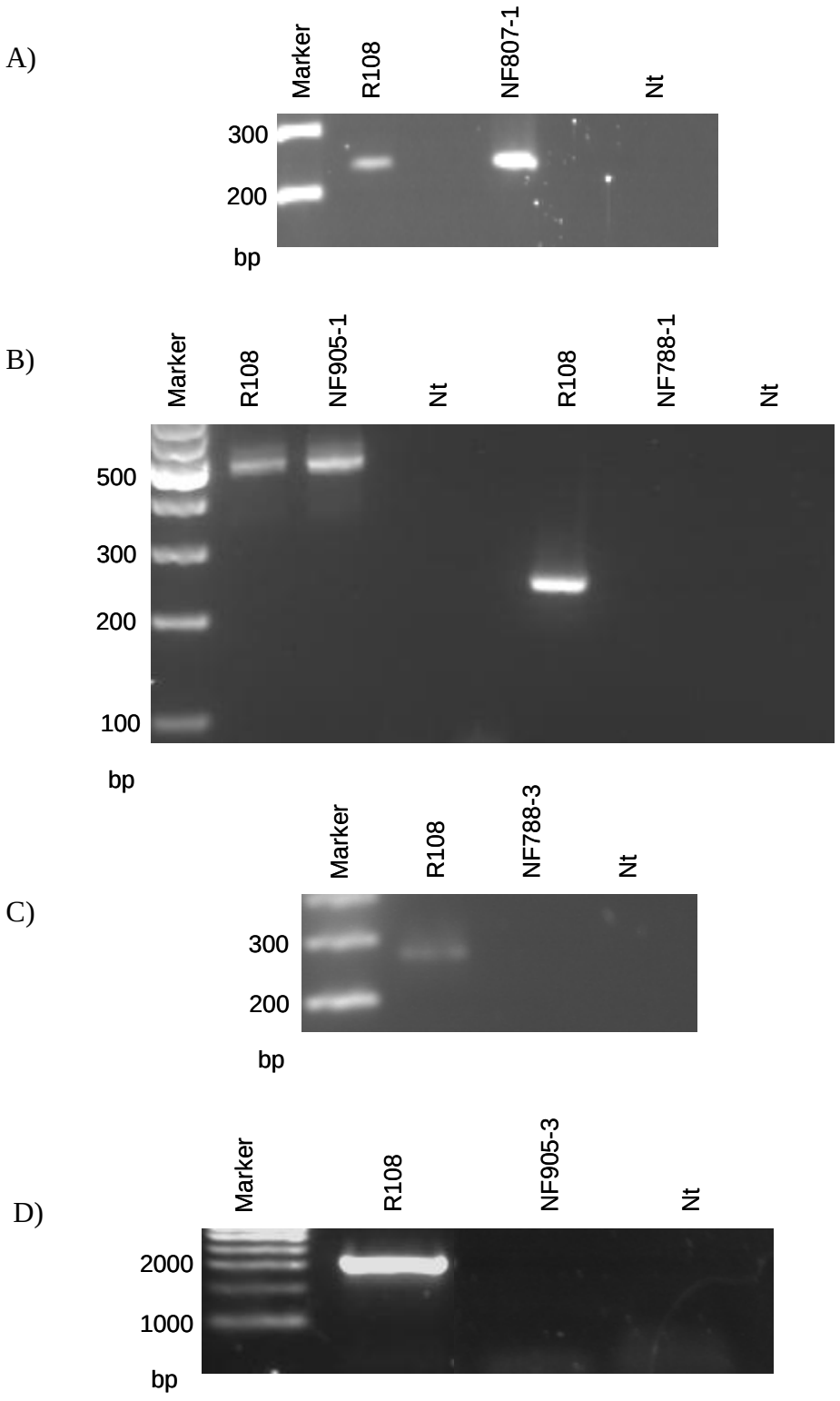
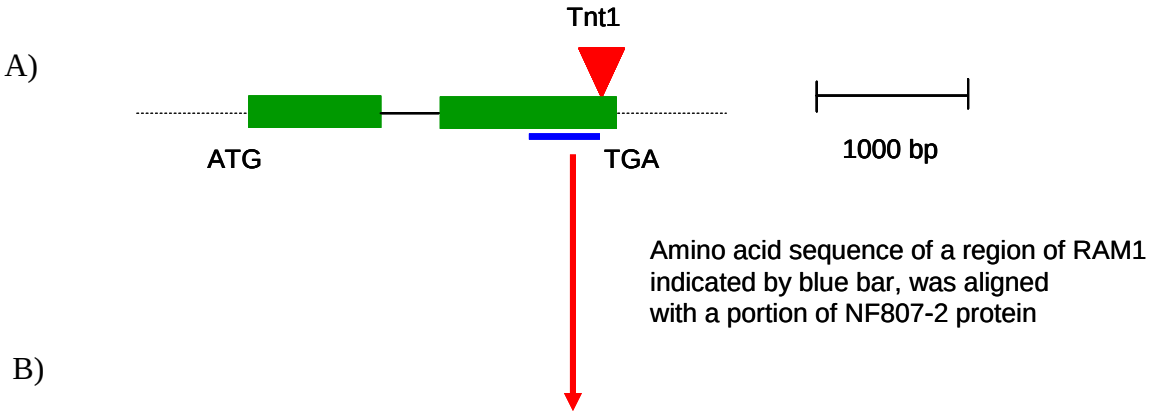


Fig. 4.6: A and B) Amplification of wild-type copies of NF807-1 and NF905-1 sequences, B, C and D) Absence of wild-type copies of NF788-1, NF788-3 and NF905-3 sequences. Nt: Non template. R108: wild-type control. Expected product sizes for: NF807-1: 265 bp, NF905-1: 466 bp, NF788-1: 224 bp, NF788-3: 271bp, NF905-3: 1930 bp. 100 bp DNA ladder (Promaga) was used in A, B and C. 1 kb DNA ladder (Promaga) was used in section D. Primers used are given as under: A) NF807-1 F/NF807-1 R, B) NF905-1 F/NF905-1 R, NF788-1 F/NF788-1 R, C) NF788-3 F/NF788-3 R, D) NF905-3 F/NF905-3 R.

Insertion site NF807-2 corresponded to a predicted GRAS-type transcription factor. The same gene was deleted in the fast-neutron mutant *ram1* (E. Gobbato, G. Oldroyd, personal communication). Therefore, with the NF807-2 insertion a second mutant allele of the *ram1* gene, hereafter called *ram1*-2, was discovered. It is highly likely that the mutation of this gene is responsible for reduced mycorrhizal colonization in the line NF788/807. The transposon was inserted near the 3′ end of the coding region (Fig. 4.7 A), within the second exon, such that the last 25 amino acids of the 674 amino acid protein are lost. The region of the *Tnt1* insertion in *ram1-2* (NF807) is within a SAW motif, which is conserved among different GRAS domain transcription factors (Fig. 4.7 C). Therefore it can be assumed the insertion of *Tnt1* at this position prevents production of a functional protein. Insertion in SAW motif is known to play an important part during nodulation (Smit et al., 2005). It was assumed that this SAW motif might also play some role in mycorrhizal symbiosis.

Fig. 4.7 B shows an alignment of a region of the RAM1 protein with a region of NF807-2 protein displaying 100 % identity. This shows that mutant line NF807-2 and a deletion mutant *ram1* are affected in the same gene.



B)

ram1 DLKPHMFNRRVGEALAVNTVNRLHRVPGNHLGNLLSMIRDQAPNIVTLVEQEASHNGPYF
NF807-2 DLKPHMFNRRVGEALAVNTVNRLHRVPGNHLGNLLSMIRDQAPNIVTLVEQEASHNGPYF

ram1 LGRFLEALHYYSAIFDSLDATFPVESAPRAKVEQYIFAPEIRNIVACEGEERIERHERLE
NF807-2 LGRFLEALHYYSAIFDSLDATFPVESAPRAKVEQYIFAPEIRNIVACEGEERIERHERLE

ram1 KWRKIMEGKGFKGVPLSPNAVTQSRILLGLYSCDGYR
NF807-2 KWRKIMEGKGFKGVPLSPNAVTQSRILLGLYSCDGYR

C)

Fig. 4.7: A) Indication of the presence of *Tnt1* insertion on the *ram1* gene in mutant line NF807 (*ram1*-2). Exons are represented by green blocks and line in between exons is exhibiting intron. Triangle is indicating *Tnt1* insertion. Blue line is indicating the amino acids alignment of a region of RAM1 with NF807-2 protein. B) Alignment of amino acids of a portion of RAM1 with NF807-2 protein exhibiting 100 % identity. C) Highly conserved domain of GRAS protein (C terminus) with five motifs.

Although the number of insertion sites shared between NF788 and NF807 could only be explained by these lines being siblings, the *ram1* allele was not among the sequenced flanking regions in NF788. However, using one Tnt1 and one *ram1*-2 allele specific primer the presence of the insertion in NF788 was demonstrated (Figure 4.8 A). Using

primers binding on both sides of the transposon insertion, a wild-type band was not amplified when DNA from NF807 was tested (Figure 4.8 B). This confirmed that NF807 is homozygous for allele *ram1*-2.

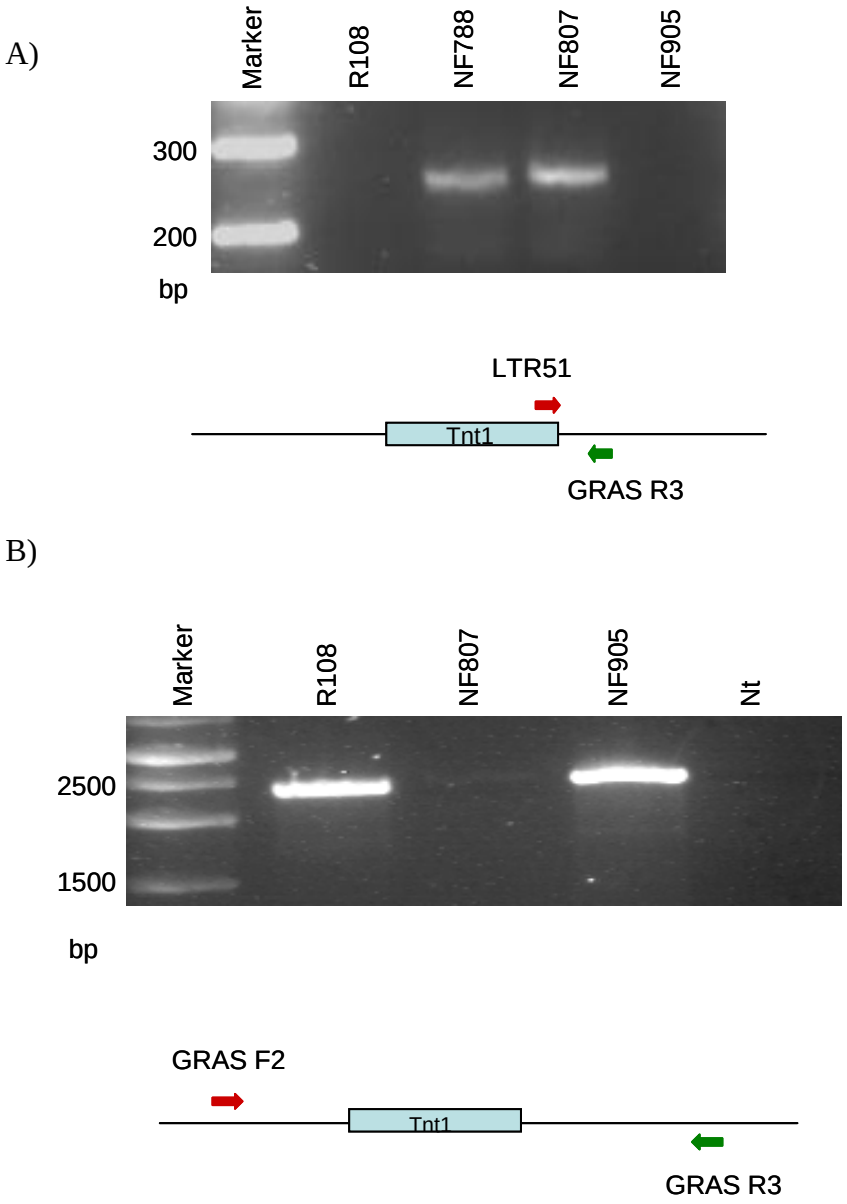


Fig. 4.8: PCR amplification of *ram1* alleles. A) *ram1*-2. Expected product size: 242 bp. B) wt allele. Expected size: 2357 bp. Nt: No template. 1 kb Promega ladder was used for the size of PCR product.

Line NF905 did not carry the *ram1-2* insertion (Figure 4.8). Furthermore, while the isolation of flanking sequences was underway, a candidate gene for the fast-neutron mutant *ram2* was identified at the John Innes Centre (E. Wang, G. Oldroyd, personal

communication). To rule out that this gene could be affected in the transposon mutants, it was tested in NF905. Wild-type alleles were amplified in two overlapping PCR products covering the entire coding region and 1416 bases upstream of the start codon (Figure 4.9). Therefore it was concluded that *ram2* and NF905 were affected in different genes.

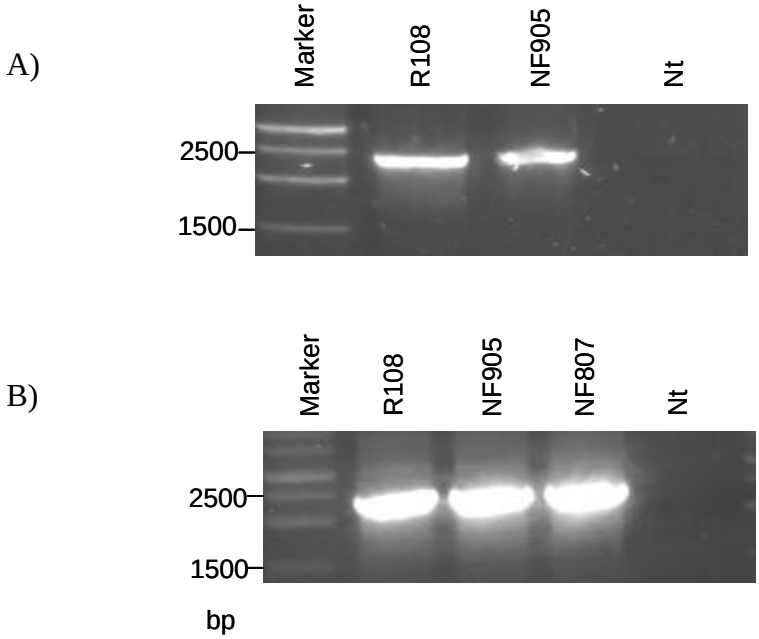
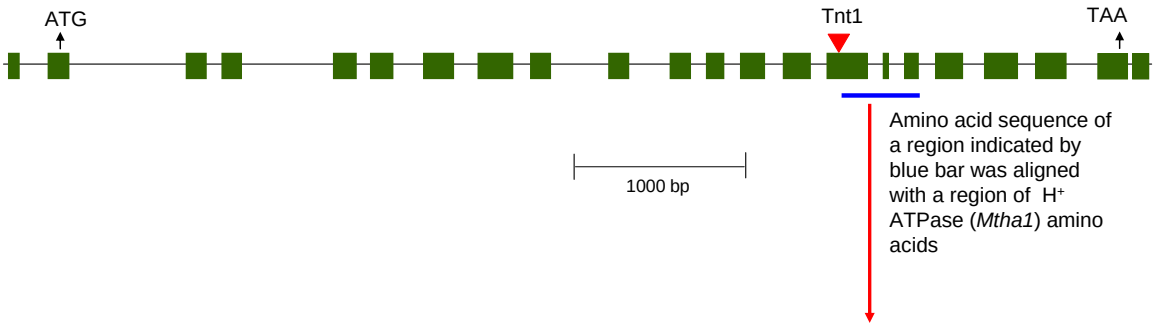


Fig. 4.9: NF905 carries a wild-type copy of RAM2. Amplification of two overlapping PCR products. Expected sizes: A) 2497 bp; B) 2018 bp. Nt: No template. 1 kb Promega ladder was used. Gene specific primers were used *ram2* gene (*ram2* F1/*ram2* R1, *ram2* F2/*ram2* R2).

Among the flanking sequences in line NF905, an insertion in exon 15 of the gene *Mtha1* encoding a plasma membrane H^+-ATPase was found (Fig. 4.10). This gene had been reported to be specifically expressed in arbuscule-containing cells in *Medicago* roots (Krajinski et al., 2002). The insertion of *Tnt1* leads to the loss of last 364 amino acids of the 966 amino acids on mycorrhiza related gene. The insertion on *Mtha1* in NF905 is an indication of requirement of this gene in causing reduced mycorrhizal colonization because this gene was already known to be expressed in arbuscule containing cells by Krajinski et al. (2002). Using primers flanking the insertion site, no wild-type band was amplified from NF905. This confirmed that the line is homozygous for the insertion in *Mthal* (Figure 4.11). Alignment of an amino acids sequence region of *Mtha1* with NF905-8 amino acids showed 100 % identity as indicated in Fig. 4.10 B.

A)



B)

Mtha1	APALKIADIGIAVADSTDAARSASDIVLTEPGLSVIISAVLTSRAIFQRMKNYTIYAVSI
NF905-8	APALKIADIGIAVADSTDAARSASDIVLTEPGLSVIISAVLTSRAIFQRMKNYTIYAVSI

Mtha1	TIRIVLGFMLLNSFWSFDSPPFMVLIIAILND
NF905-8	TIRIVLGFMLLNSFWSFDSPPFMVLIIAILND

Fig. 4.10: A) Structure of *Mtha1* gene with *Tnt1* insertion on 15th exon. Blocks displaying exons (22) while lines in between are showing introns. Triangle: *Tnt1* insertion site. B: Alignment of amino acids of a region of *Mtha1* with NF905-8 amino acids.

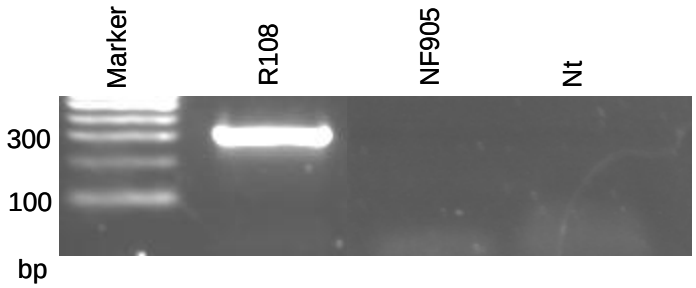


Fig. 4.11: Wild-type copy of *Mtha1* allele is absent in NF905. R108 acting as a positive control exhibited expected sized band of 336 bp. 100 bp Promega ladder was used.

Bidirectional nutrient transport is an important feature of mycorrhizal symbiosis because for proper association fungi and plant both should be benefitted. It is believed that phosphate transporters function in association with H^+-ATPases to develop a proton motive force which helps in the transport of nutrient to plants (Rausch et al., 2001; Schachtman et al. 1998). There are many studies which indicate the strong activity of H^+-ATPases in the plasma membrane surrounding the arbuscules (Benabdellah et al. 1999; Gianinazzi-Pearson et al. 2000). *Tnt1* insertion on *Mtha1* gene resulted in interrupting the function of this gene, the H^+-ATPase may be defective or not produced.

4.3 Analysis of cosegregation of the *ram1-2* allele and mycorrhizal mutant phenotype

To test whether the *ram1-2* allele cosegregates with the mycorrhizal mutant phenotype, line NF788 was backcrossed with R108. Twenty plants of the F_2 generation (seeds from 5 crosses were mixed) were grown in the presence of Endorize inoculum. After four weeks the genotype and degree of colonization were examined. Although repeated PCRs gave conflicting results for some plants, the *ram1-2* insertion allele appeared to segregate in the following ratio: 7 wild-types: 7 heterozygous: 6 mutants (Fig. 4.12 A). The predicted ratio was 5 wild-types: 10 heterozygous: 5 mutant. So the result of segregation of *ram1-2* allele was nearly equal to the expected ratio.

Unfortunately, the level of colonization of plants that appeared to be homozygous for *ram1-2* was unexpectedly high compared to the mutants tested before crossing (Figure 4.12 B, 4.13, compare Fig. 3.8) In hindsight it would have been useful if the original mutant seed stock had been included in this experiment. There was significant plant-to plant variation in total root length colonization as well as in the percentage of vesicles and arbuscules (Fig.4.12 B). Therefore, a clear mutant phenotype was difficult to score for individual plants. That is why the data were averaged for wild-type, heterozygous and mutant plants (Figure 4.13). The average colonization of the 6 mutant plants was lower than that of wild-type and heterozygous plants. The difference was statistically significant when a Kruskal-Wallis test with multiple comparison correction was applied ($P < 0.05$). Similarly, the number of fully developed arbuscules was lower in mutants. In particular the ratio of fully developed to incomplete/degenerated arbuscules was low in the mutants (Fig. 4.13).

Fig. 4.14 exhibits clearly the differences in wild-type and mutant phenotypes. Wild-type samples were highly colonized by arbuscules, vesicles as well as internal fungal hyphae. Many incompletely developed arbuscules were observed in mutant samples (see sample 20). These incomplete arbuscules seemed smaller and simple in structure as compared to fully developed arbuscules. Some parts of roots were not colonized at all in mutant samples. The heterozygous samples exhibited wild-type like colonization. Overall, the data are consistent with the insertion allele *ram1-2* co-segregating with the mutant phenotype. However, it is possible that the relatively weak mutant phenotype observed

in the backcrossed F_2 plants has been caused by segregation of mutations in other genes that could have affected the overall growth and vitality of the mutant lines. The phenotype of homozygous mutants of F2 generation was not supporting the idea of mutation of two or more genes being responsible for reduced mycorrhizal colonization because fungal colonization in homozygous mutants was not showing a dramatic reduction, except the defect in arbuscule formation. Crossing of NF788 with the *ram1-1* deletion mutant has been carried out at the John Innes Centre, and F_1 plants showed a level of colonization nearly as low as *ram1-1* plants (Enrico Gobbato, Giles Oldroyd, personal communication). This indicated that NF788 (*ram1-2*) and *ram1-1* are allelic and mutation of *ram1* gene was responsible for mycorrhizal mutant phenotype in line *ram1-2*.

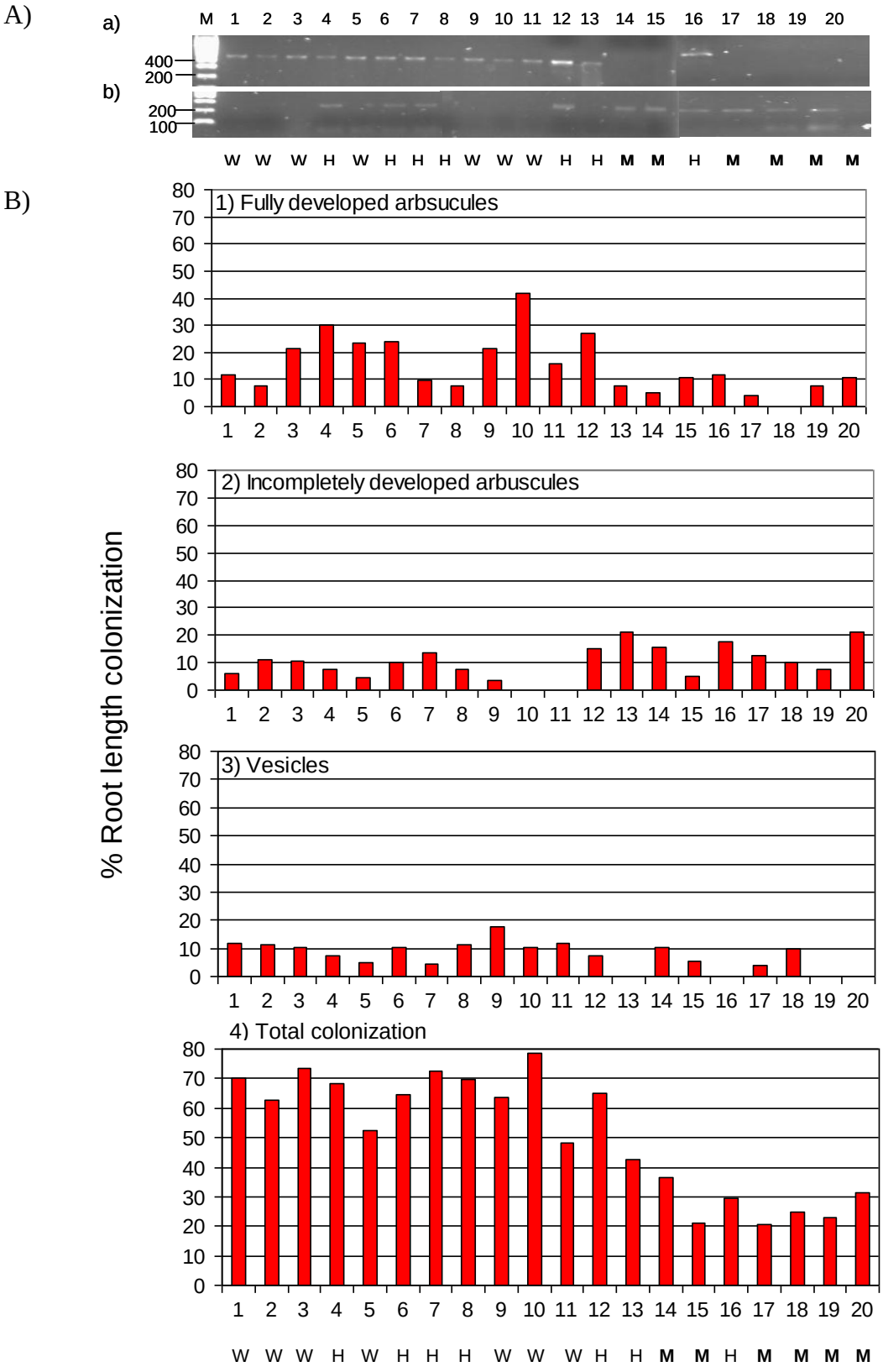


Fig. 4.12: A) PCR amplification of *ram1*-2 alleles. , a): Wild type alleles. Expected product size: 429 bp. Gene specific primers: GRAS F7/GRAS R3, b). mutants. Expected product size: 238 bp. Primers: LTR51 F/GRAS R3. 100 bp DNA marker was used from Bioline. (samples 8, 13 and 20 showed heterozygous bands in a different PCR reaction. B) Percentage root length colonization in offsprings of backcross in *ram1*-2 (NF807) for: 1) fully developed arbuscules, 2) incompletely developed arbuscules, 3) Vesicles, 4) total fungal colonization.

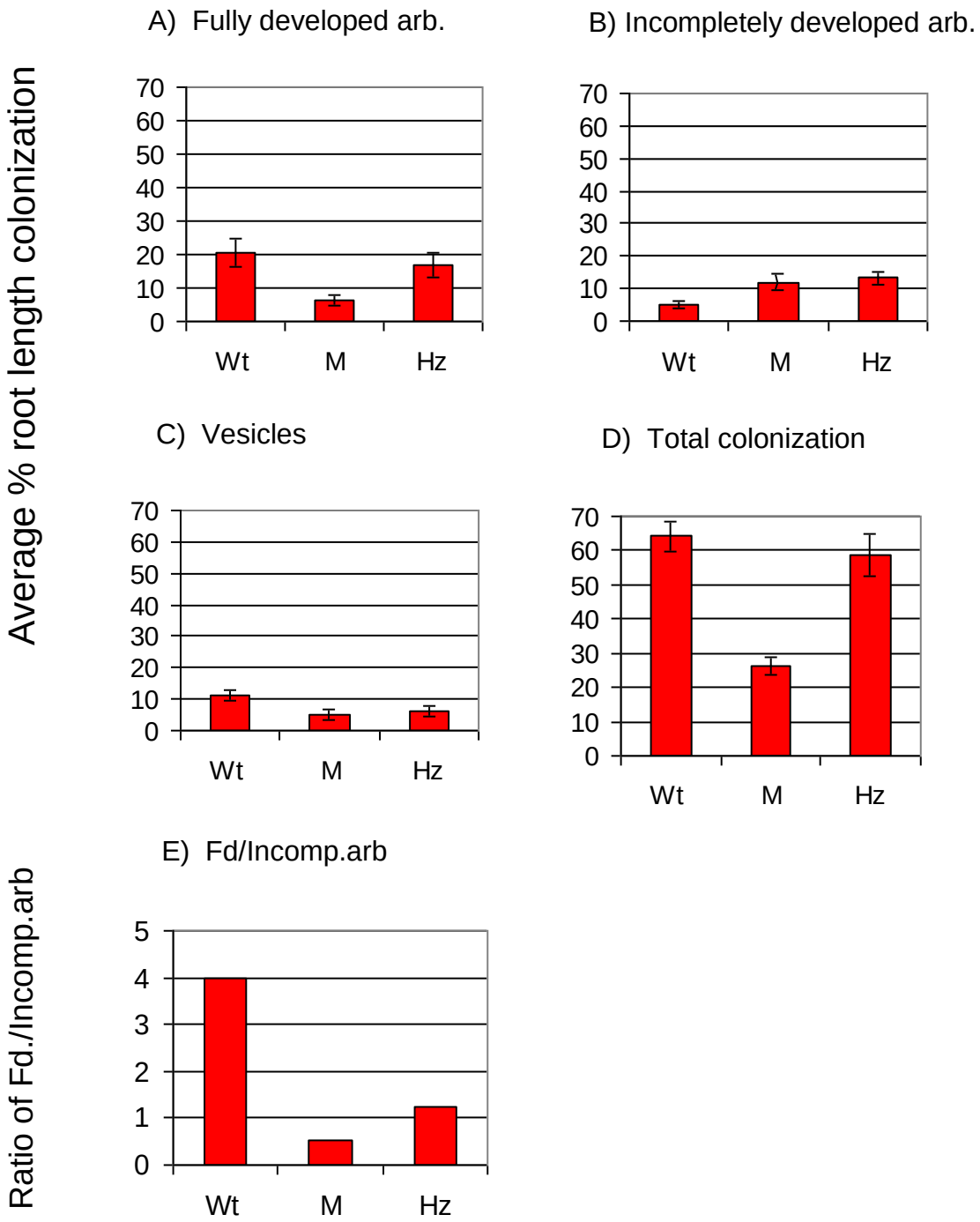


Fig. 4.13: Mycorhizal phenotypic analysis for the average percentage colonizations in offsprings of backcross in *ram1*-2 (NF807) for A) fully developed arbuscules, B) incompletely developed arbuscules, C) vesicles, D) total fungal colonization, E) Ratio of the fully developed to incompletely developed arbuscules. Error bars are showing the standard errors of mean values. Fd/Inc arb= Fully developed/Incompletely developed. There were 7 wild type samples, 7 heterozygous and 6 mutant plant samples in offsprings of backcross in *ram1*-2 (NF807).

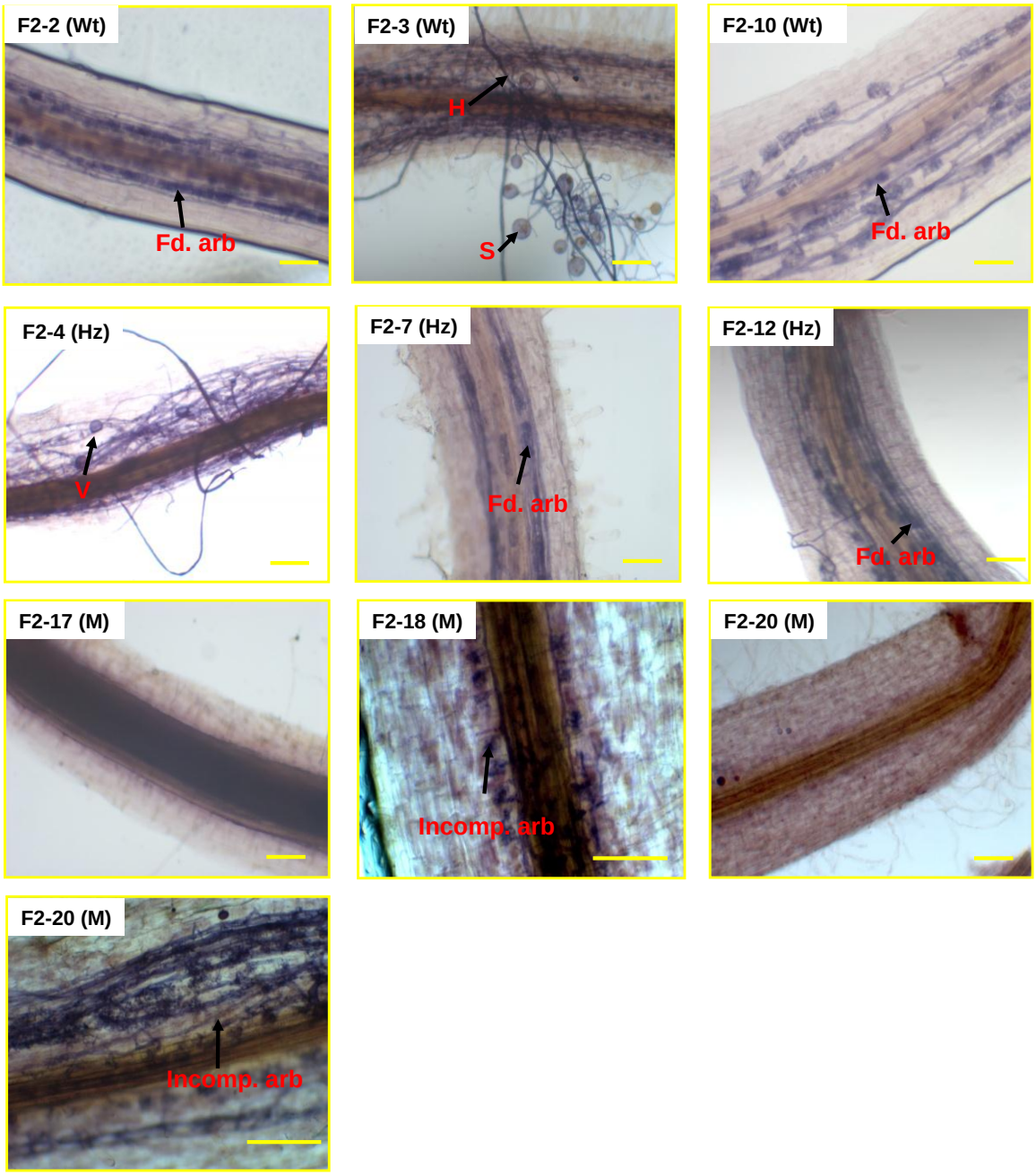


Fig. 4.14: Stained roots exhibiting fungal colonization in offsprings of backcross in *ram1*-2 (NF807). 20 root samples were analysed. Fd. arb: Fully developed arbuscules, Incomp.arb: Incompletely developed arbuscules, M: mutant. V: vesicle. Wt: Wild type: Hz: Heterozygous. S: Spore. Scale bar: 100 µm.

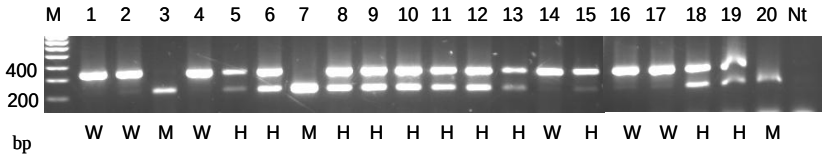
4.4 Cosegregation of the *Mtha1* allele and mycorrhizal mutant phenotype

To determine the cosegregation of the mutant *Mtha1* allele NF905 with the mycorrhizal mutant phenotype, it was crossed with wild type (R108). Twenty seeds of the progeny of backcross were planted in the presence of Endorize mix and the plants were analysed for genotype as well as the phenotype of low fungal colonization after four weeks. For the confirmation of wild-type, mutant and heterozygous samples, three primers were used in combination in a PCR reaction. *Mtha1* allele appeared to segregate in the following ratio: 6 wild-types, 11 heterozygous, 3 mutants (Fig. 4.15 A), while the expectation was 5 wild-types, 10 heterozygous, 5 mutants.

For phenotypic study different fungal colonization structures were counted separately. Similar to *ram1*-2, the plants that scored to be homozygous for *Mtha1* allele were exhibiting higher colonization as compared to the earlier mutant screening experiment (Fig. 4.15 B, 4.16 compare Fig. 3.8). Like mutant line *ram1-2* the data of fungal colonization for individual plants was displaying much variability and a clear phenotype was difficult to score (Fig. 4.12 B & 4.15 B). Therefore the data was averaged for the indication of wild-type, mutants and heterozygous samples (Fig.4.16). Applying Kruskal-Wallis test with multiple comparison correction ($P < 0.05$), it was clear that the average fungal colonization values of fully developed arbuscules of the 3 mutants were significantly smaller as compared to that of wild-types and heterozygous samples (Fig. 4.16). The ratios of fully developed/Incomplete arbuscules for mutants were also significantly smaller as compared to wild-type and heterozygous plants. Total colonization of mutants is not very different from wild-types and heterozygous plants. Fewer fully developed and more incompletely developed arbuscules in mutants were a discriminative point between mutant and wild-type plants. Many arbuscules, vesicles and internal hyphae were observed in all wild-type samples. The 3 mutants were less colonized on the average as compared to wild-type but the differences were not statistically significant. Some internal hyphae can be observed in the mutant sample 3 of a representative picture of mutant of progeny of backcross (Fig. 4.17).

Overall, phenotypic and genotypic results for cosegregation analysis of NF905 suggested that reduced fungal colonization phenotype cosegregated with the insertion on *Mtha1* gene. The *Tnt1* insertion on *Mtha1* is the most likely mutation responsible for reduced fungal colonization in NF905.

A)



B)

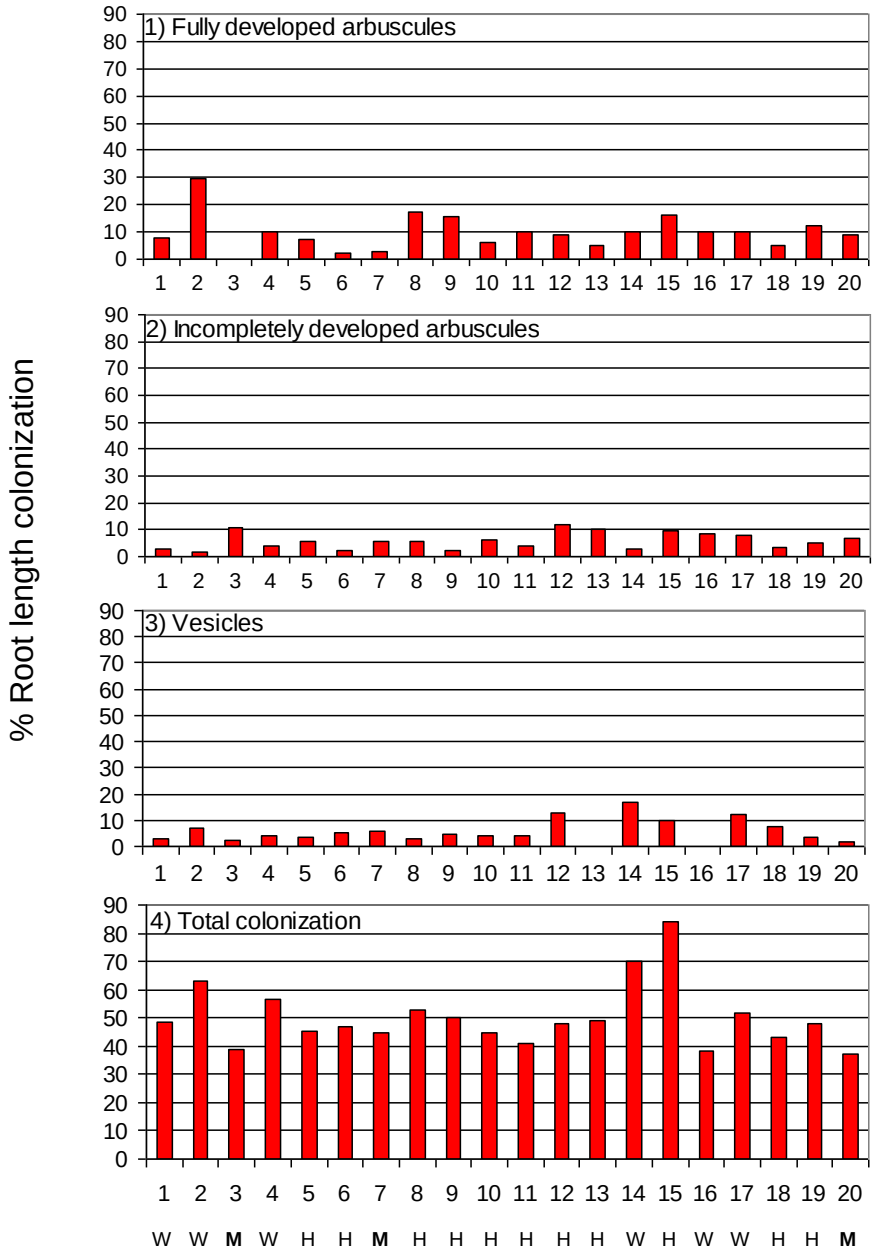


Fig. 4.15: A) PCR amplification of *Mtha1* alleles. Wild type alleles. Expected product size: 336 bp. Primers: *Mtha1* F, LTR4-F/*Mtha1* R (three primers used in a PCR) 2. mutants. Expected product size: 221 bp. 100 bp DNA marker was used from Bioline. B) Percentage root length colonization in the progeny of backcross in NF905 for: 1) fully developed arbuscules, 2) incompletely developed arbuscules, 3) Vesicles, 4) total fungal colonization.

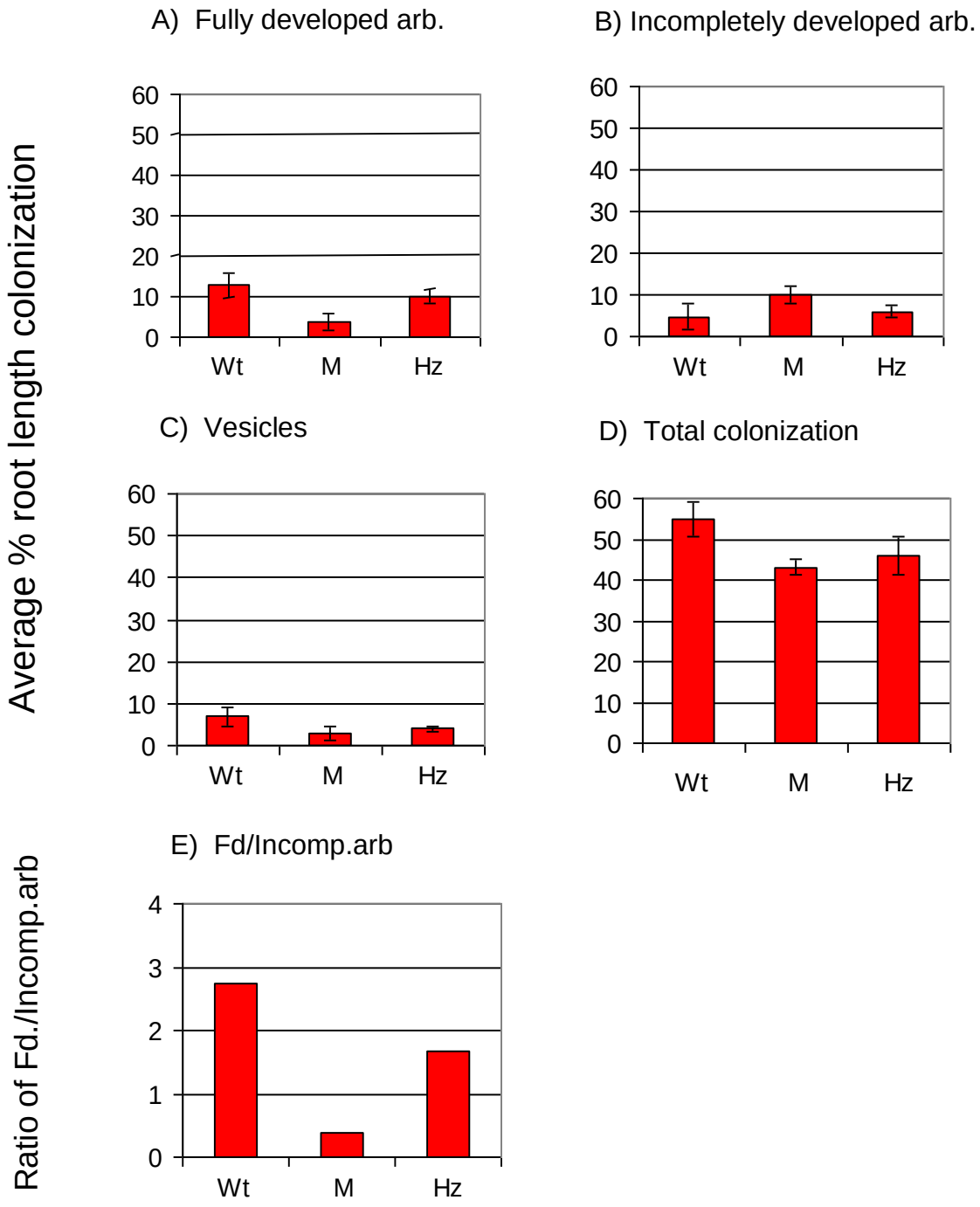


Fig. 4.16: Phenotypic study of fungal colonization for segregation analysis displaying the average percentage colonizations in the progeny of backcross in NF905: A) fully developed arbuscules, B) incompletely developed arbuscules, C) vesicles, D) total colonization, E) ratio of the fully developed to incompletely developed arbuscules. Error bars are showing the standard errors of mean values. Fd/incomp.arb= Fully developed/Incompletely developed. There were 6 wild type, 11 heterozygous and 3 mutants.

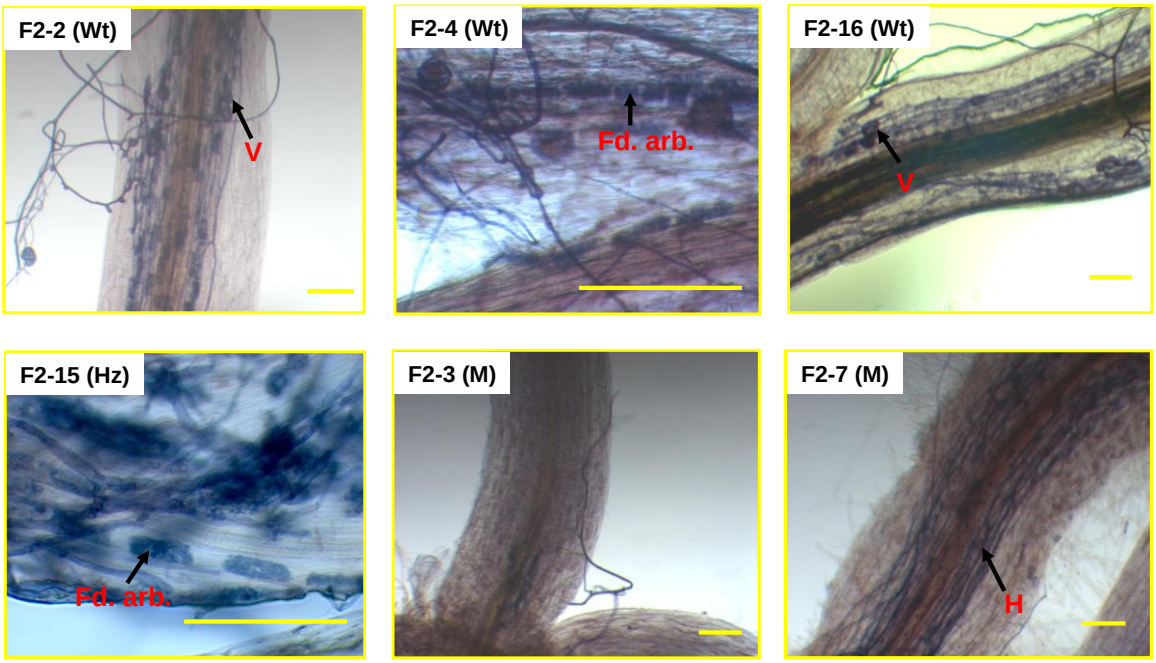


Fig. 4.17: Stained *M. truncatula* roots exhibiting fungal colonization in progeny of backcross in NF905. 20 root samples were analysed. Fd.arb: Fully developed arbuscules, H: Internal Hyphae. V: vesicle. Scale bar: 100 µm.

Chapter 5

Discussion

5.1 Transposon mutants exhibiting defects in the development of arbuscular mycorrhizal symbiosis

Mutants that exhibit defects in forming mycorrhizal association are a powerful tool to understand the mechanism of symbiosis. The identification of reduced fungal colonization lines of *Medicago truncatula* may be helpful to study the signalling events in mycorrhizal symbiosis. In the present research out of 28 mutant lines seven were identified as showing a reduced colonization phenotype (*ram1−2*, NF905, NF518, NF689, NF841, NF919 and NF940). It seems that two highly reduced colonization lines *ram1−2* and NF905 are impaired in arbuscule development function. This severe reduction of colonization was reminiscent of the phenotype in *ram1* in the preliminary experiment (Fig.3.1). Some discontinuous patches of vesicles and internal fungal hyphae were also observed in these lines. A similar phenotype was reported by Javot et al. (2007), where arbuscules were exhibiting premature degeneration in *MtPT4* mutants of *M. truncatula. MtPT4* is a phosphate transporter and short infection units were indicated in *MtPT4* mutants, hence fungal growth was aborted. A similar sort of aborted colonization was also observed in *ram1−2* and NF905. Previously David-Schwartz et al. (2001) reported a tomato mutant M161 showing very high resistance to fungal spores but not to hyphae emanating from colonized wild-type plant. In contrast to the results of David-Schwartz et al. (2001), the arbuscule development of *ram1−2* was not rescued by the use of colonized leek plants growing in the same compartment as the mutant. This gave an indication of the absence of some important step in the signalling pathway of arbuscular mycorrhizal association or a signal needed to form fully developed arbuscules may be abnormal in *ram1−2*. Unlike the phenotype of a maize mutant *nope1* where hyphal penetration was defective (Paszkowski et al., 2006), the initial stages of fungal entry were not found to be impaired in *ram1−2*. The fungi were able to penetrate the plant roots and showed few internal hyphae and vesicles, but further growth was not supported in mutant *ram1−2*. This aborted fungal growth resulted in severe reduction of total colonization. The reduced total colonization may be due to improper functioning of arbuscules because *ram1−2* is defective in arbuscule specific function. A similar sort of retarded fungal growth and defect in arbuscule formation was also reported by Murray et al. (2011) in a fast-

neutron mutant (FNB4) of *M. truncatula*. They also reported that mutant FNB4 and wild-type showed similar levels of colonization in a nurse pot experiment except for the presence of arbuscules. In the segregating population after back-crossing, the homozygous mutants of *ram1−2* were displaying more colonization compared to mutants tested in the screening experiment (Fig. 4.12 compare with 3.8). This may be the result of segregation of other mutations. However there was not even a single homozygous mutant plant with very low colonization therefore it was concluded that *ram1* gene is responsible for mycorrhizal mutant phenotype in mutant line *ram1−2*.

Total reduction of fungal colonization in mutant line NF905 also indicated defects in later stages of mycorrhizal association. A very reduced number of fungal hyphae, vesicles and arbuscules were observed in this line. Like *ram1−2*, mutant line NF905 also displayed incomplete arbuscule formation in a nurse pot experiment where colonized leek plants were used. Total colonization of NF905 was similar to wild-type in the nurse pot experiment but arbuscules were not fully developed. This indicates that *Tnt1* insertions had changed the ability of the mutant to fully develop arbuscules. A similar phenotype was reported in Petunia by Reddy et al. (2007) but they found the *Petunia* mutant to be fully devoid of arbuscules in nurse pot experiments.

The *Tnt1* insertion reduced the number of arbuscules, vesicles and fungal hyphae in other lines such as NF518, NF689, NF841, NF919 and NF940. Two mutant lines NF919 and NF940 are characterized by areas of seemingly wild-type colonization dispersed with areas of reduced colonization. The phenotype of these mutants seems weaker as compared to *ram1−2*. The overall growth, such as plant height and morphology were the same for all mutants studied except line NF447. Reduced fungal colonization in this line may be the result of poor growth, therefore NF447 was excluded from further analysis. Twenty-one plant lines were ruled out to have wild-type like colonization. Two highly reduced colonization lines *ram1−2* and NF905 were analysed further. Mutant lines NF518, NF689, NF841, NF919 and NF940 were not analysed further but they are still promising candidates for future analysis.

Host plants release a signal that is perceived by AM fungi. This signal stimulates hyphal branching prior to appressoria formation (Buee et al., 2000). It has been identified as strigolactone (5-deoxystrigol) by Akiyama et al. (2005). In addition to mycorrhizal association, strigolactones were reported to play a role in shoot branching. Application of strigolactones inhibited shoot branching in branching mutants of *Arabidopsis* (Gomez-Roldan et al., 2008; Rameau 2010; Umehara et al., 2008). It is possible that the levels of strigolactones increase as a result of deficiency of inorganic phosphorus. As a consequence of increase in level of strigolactones, the shoot branching is reduced and mycorrhizal association occurs. Stimulation of the production of strigolactones as a consequence of phosphorus deficiency was also reported by Yoneyama et al. (2007 a, b) in *T. pratense* and *S. bicolor.* The reduced strigolactone mutants may present a defect in the initial stages of symbiosis because branching of hyphae is reduced and there is reduced penetration of the plant root. The phenotype of mutant lines *ram1−2* and NF905 does not seem to be affected in the pre-symbiotic events between *Medicago truncatula* and AM fungi, however strigolactones have not been investigated in this project. Mutant lines identified in the present research project are impaired in interaction with mycorrhizal fungi but shoot branching was normal. This mutant phenotype does not appear similar to a strigolactone mutant of tomato where shoot branching of the mutant was increased compared to wild-type and the mutant exhibited greatly reduced fungal colonization (Koltai et al., 2010). Reduced fungal colonization can be due to defects in mycorrhizal signalling pathway or some important step needed for symbiosis may be absent.

5.2 Isolated flanking sequences from mutant line *ram1*-2

Applying a PCR based screening approach, flanking sequences were isolated from mutants in which colonization was strongly reduced. Two promising genes *ram1* and *Mtha1* were identified. *Tnt1* insertion on these genes was predicted to be responsible for the mycorrhizal phenotype. Transposon insertion on *ram1* gene in mutant line *ram1−2* resulted in severe reduction of fungal colonization. Identification of another allele (Oldroyd's lab) of the *ram1* gene in a deletion mutant *ram1* confirmed the present results. In this severely reduced colonization

mutant the whole *ram1* gene was deleted as a result of fast neutron mutagenesis. In *ram1−2* the mutation was located in the SAW motif whereby the last 25 amino acid residues of the protein are lost (Fig. 5.2). Previously Smit et al. (2005) reported a mutation in the SAW motif of a GRAS protein NSP1 which affected the nodulation process. Mutant showed highly reduced colonization of nodules suggesting that the SAW motif is required for the function of the transcription factor *NSP1*. Therefore, the *Tnt1* insertion in the SAW motif can be expected to abolish the function of *ram1* as well.

GRAS proteins have been named after the identification of the first three members of its family: Gibberellic acid insensitive (GAI), Repressor of GAI (RGA) and Scarecrow (SCR) (Pysh et al., 1999). These proteins have variable N terminus and a highly conserved C terminus that contains five identifiable motifs. These motifs are leucine heptad repeat I (LHR1), the VHIID motif, leucine heptad repeat II (LHR II), the PFYRE motif and the SAW motif (EMBL-EBI data bases) (Fig. 4.7 C) (Pysh et al., 1999). Portions of the PFYRE motif exhibit sequence similarity among all members of the family. The only common feature among the N terminus of Gras protein is the presence of homopolymeric stretches of some amino acids such as serine, threonine, proline, glutamine, glycine, glutamic acid and histidine. Many mutations of Gras transcription factors are caused as a result of substitution of valine, leucine and isoleucine at different positions in the VHIID motif. 33 Gras genes in Arabidopsis and 60 in rice have so far been identified (Itoh et al., 2005; Lee et al., 2008; Tian et al., 2004; Tong et al., 2009). Two other GRAS transcription factors *NSP1* and *NSP2* are also known in *M. truncatula* to play their role during symbiosis (Smit et al., 2005; Kalo et al., 2005). Previously Kalo et al. (2005) showed that *NSP2* from *M. truncatula* is essential for Nod-factor signalling. Mutation in the LHR1 domain of *NSP2* results in defects in nodule formation. *NSP2* may also be important in mycorrhizal symbiosis, as suggested by Maillet et al. (2011) who observed reduced mycorrhizal colonization in *NSP2* mutant as compared to wild-type. *NSP1* function in collaboration with *NSP2* to form nodules similarly it can be assumed that *ram1* may function with *NSP2* to develop AM association. The *ram1* mutants are defective in mycorrhizal development but not in nodulation,

hence the *ram1* gene is predicted to play its role specifically in mycorrhizal symbiosis.

Progenies of backcrosses of *ram1−2* were analysed for segregation of mutant phenotype. The overall higher fungal colonization in the homozygous mutants as compared to the mutant screening experiment might be the result of segregation of other mutations. This means that *Tnt1* insertions on two or more genes could possibly contribute to the observed phenotype. However if mutations of two genes were responsible for the phenotype, the plants which are homozygous for mutations in both genes among the offspring of backcross should show very reduced fungal colonization. We would expect one mutant out of sixteen or twenty plants in total. This did not seem to be the case because not even a single mutant plant was obtained with severely reduced fungal colonization in the progeny of backcrossed plants. However we cannot entirely exclude the possibility of contribution of other mutations because only twenty F_2 plants of the backcross were tested.

Fig.5.1 shows a possible model of signalling pathway for the mycorrhizal and nodulation pathway. The place of identified *ram1* gene has also been described in this model. During mycorrhizal association fungi produce signal molecules which are called Myc signals (Maillet et al., 2011). These Myc signals are diffusible sulphated and non-sulphated lipochitooligosaccharides that are structurally similar to Nod factors. The Nod and Myc factors are perceived by LysM receptor-kinases and symbiosis-receptor kinases (Myc receptor) and this perception occurs upstream of *DMI1* and *DMI2*. Depending on the specific structure of the LysM receptor-kinase it will perceive Nod factors, Myc factors, or both (Op den Camp et al., 2011). The genes involved in the early signalling pathway are shared between nodulation and mycorrhizal interaction. Protein phosphorylation may occur, due to which calcium is released into the cytoplasm from outside the cells and by intracellular calcium stores. This calcium may enter the nucleus and hence Nod factor induce calcium spiking (Shaw et al., 2003). *NFP, DM11* and *DMI2* are required for calcium spiking (Wais et al., 2000) and *DMI3* acts downstream of calcium spiking. Mycorrhizal and rhizobial-induced signalling diverge downstream of CCaMK. This divergence of symbiotic

pathway may possibly be the result of different nature of the induced calcium spiking (Kosuta et al., 2008; Oldroyd et al., 2009). However a recent report suggested that the nature of calcium spiking is infact the same in both nodulation and mycorrhizal development (Ratet personal communication) Calcium/Calmodulin dependent protein kinase (CCaMK) might be activated as a consequence of calcium spiking and this CCaMK then induce Gras transcription factor *NSP2* through phosphorylation, which could then induce the transcription of nodulin genes. The model also indicated that *NSP1* is involved in nodulation but not in mycorrhizal development. It was assumed that *ram1* gene may play a similar role in mycorrhizal development as *NSP1* does in nodulation. *NSP2* and *Ram1* may work is coordination with each to form mycorrhizal relationship is a similar way as was reported *NSP1* and *NSP2* do for nodulation.

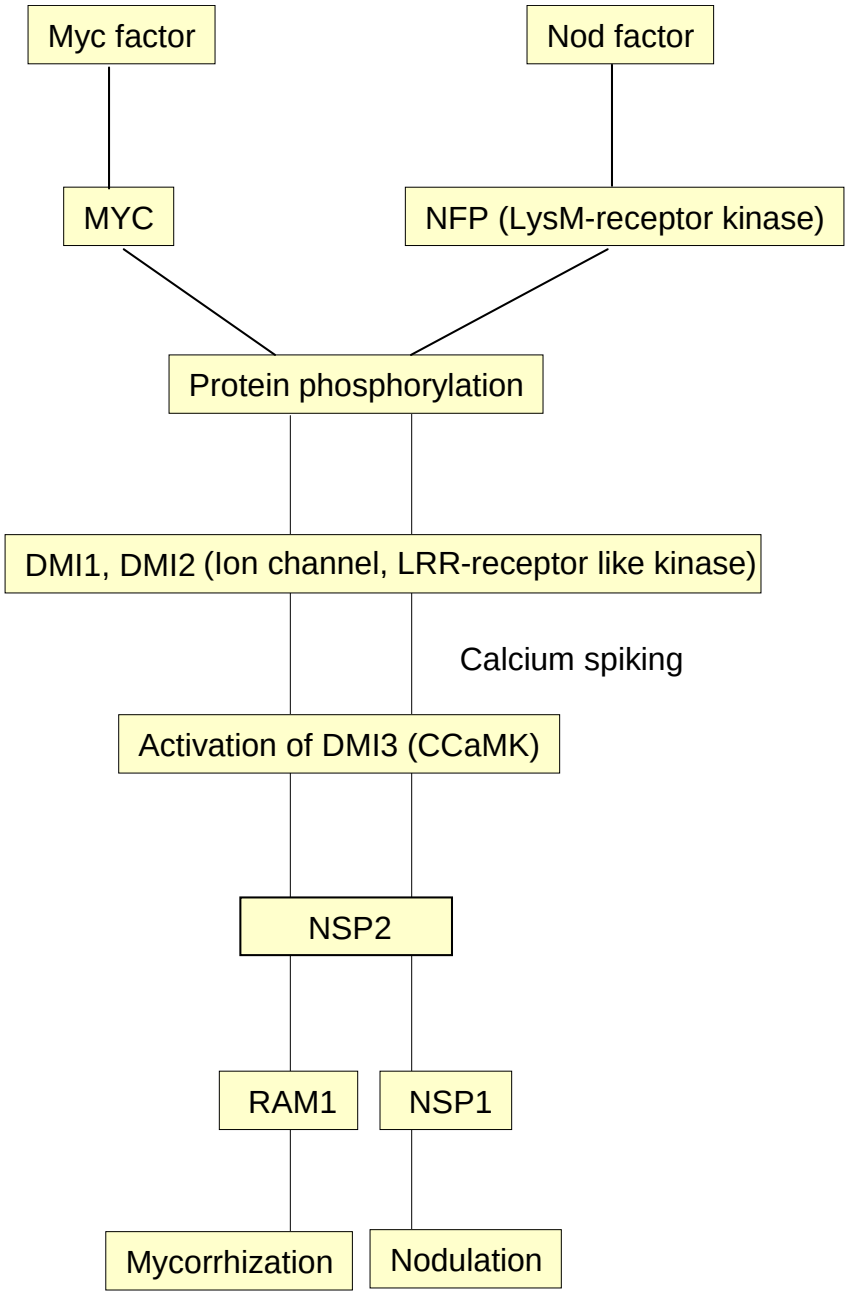


Fig. 5.1: Signaling pathway for mycorrhizal and nodule development. MYC: Mycorrhizal receptor.

Figure 5.2 shows the alignment of *ram1* protein with NSP2, NSP1, GRAS84, AI1 and Os11, indicating the conserved regions found in five motifs of these proteins. *Oryza sativa* (Os11), *Vitis vinifera* (unknown protein), *Selaginella moellendorffii* (unknown protein), *Populus trichocarpa* (GRAS 84), *M. truncatula* (NSP2 and NSP1) and *Ricinus communis* (GAI1) exhibited highest similarity with RAM1 protein. It was assumed that *ram1* gene might be playing a part to complete a step of symbiosis in these species. Phylogenetic analysis using proteins that showed highest similarity to RAM1 in a BLAST search places RAM1 in a group with related proteins from other plants, while *NSP1* and *NSP2* are more distantly related (Fig. 5.3). The RAM1 protein was highly similar to, *Vitis vinifera* (unnamed protein), *Populus trichocarpa* (GRAS 84), *Vitis vinifera* (unnamed protein), *Sorghum bicolor* (unnamed protein), *Ricinus communis* (GAI1), indicating that these sequences may share a common ancestor. This suggested that RAM1 gene may be playing a role for the establishment of symbiosis in these species.

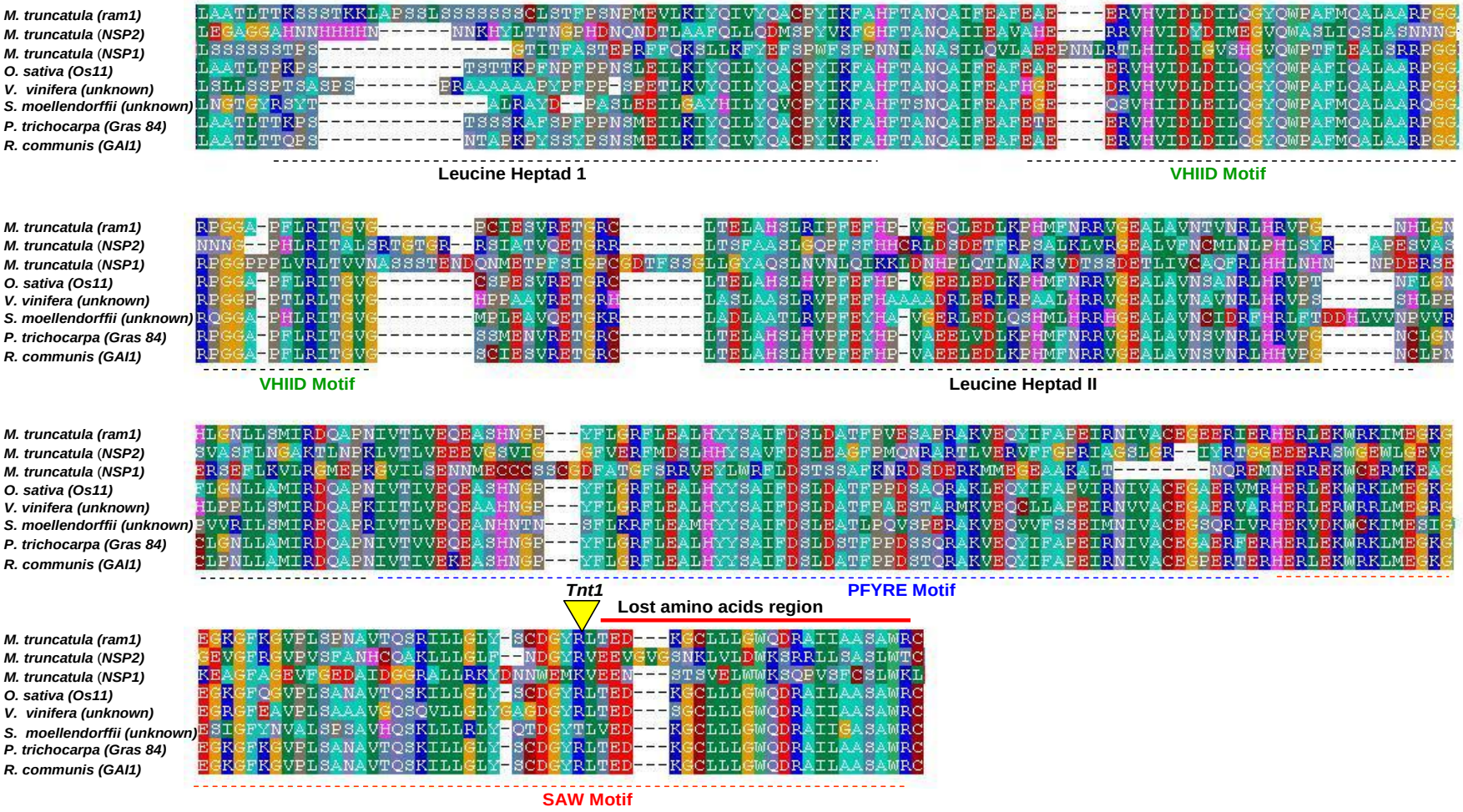


Fig. 5.2: Alignment of *M. truncatula* RAM1 protein with NSP2 and NSP1, *Oryza sativa (*Os11*), Vitis vinifera* (unknown protein), *Selaginella moellendorffii* (unknown protein), *Populus trichocarpa (*GRAS 84*), Ricinus communis (*GAI1*).* The sequences were aligned by using Bio-edit (Clustal W) programme. The triangle exhibits the position of *Tnt1* insertion, showing the deletion of region of last 25 amino acids.

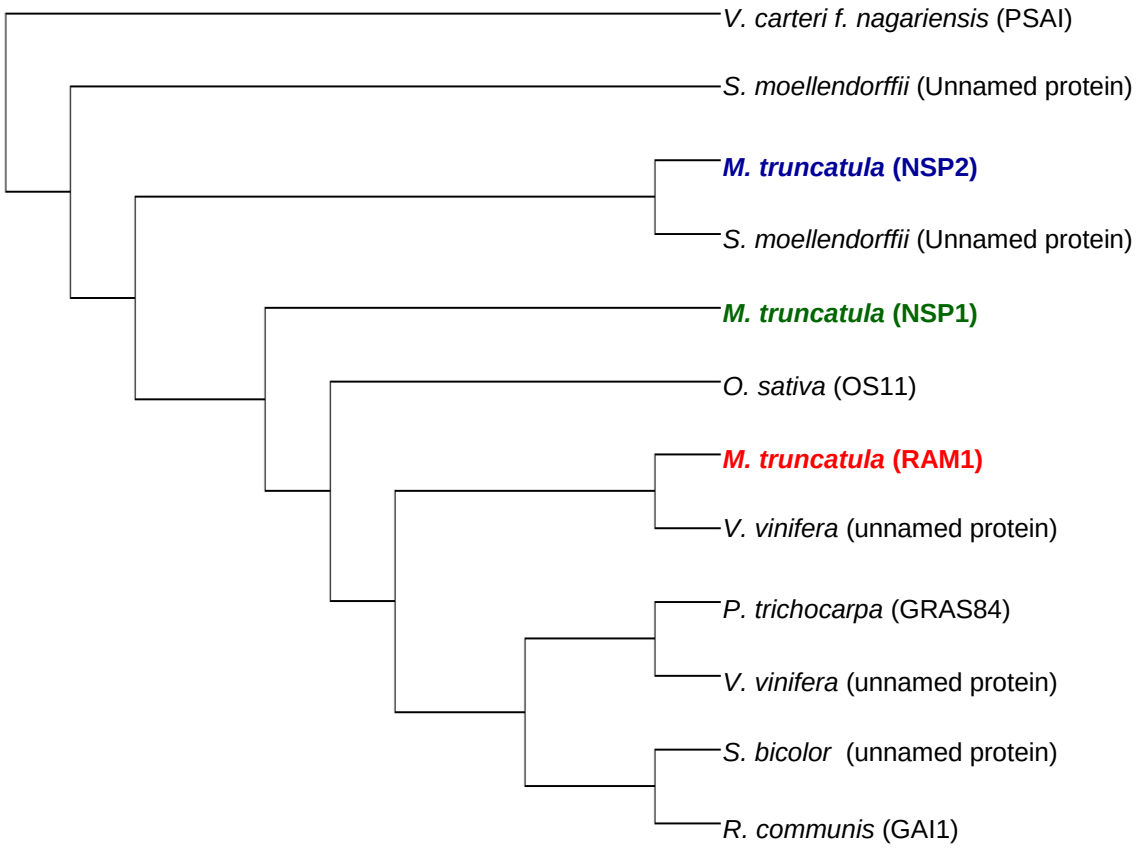


Fig. 5.3: Phylogenetic comparison of *M. truncatula* RAM1 protein with some others in database. *Medicago truncatula* (RAM1), *Vitis vinifera* (unnamed protein), *Populus trichocarpa* (GRAS 84), *Vitis vinifera* (unnamed protein), *Sorghum bicolor (*unnamed protein), *Ricinus communis* (GAI1) belong to three similar sub groups of a major group. *NSP2* (mycorrhiza and nodulation related) and *NSP1* (nodulating gene) form different groups. The outgroup used was psal from *Volvox carteri f. nagariensis*. ClustalX programme was used.

Figures 5.2 and 5.3 indicated that *ram1* was more closely related to *NSP1* than to *NSP2*. This is consistent with the idea that *ram1* performs a similar function in mycorrhizal development as *NSP1* does in nodulation.

5.3 Isolated flanking sequences from mutant line NF905

Recovery of flanking sequences in mutant line NF905 helped in the identification of some insertion sites. A flanking sequence was isolated with *Tnt1* insertion in the coding region of *Mtha1* gene. This gene was previously demonstrated to be expressed in arbuscule-containing cells of roots by Krajinski et al. (2002) and it encodes an H^+-ATPase. Present research demonstrated a mutation in this gene and the mutant phenotype of reduced mycorrhizal colonization. In NF905 a *Tnt1* insertion at the position of 602 amino acid in *Mtha1* gene resulted in loss of the last 364 amino acids. Hence intact protein is not produced and function of H^+-ATPase is affected in this mutant line.

Plants transport many molecules through membrane proteins and plasma membrane H^+-ATPases. These are widespread in plants for example some H^+-ATPases are present in guard cells and function in stomatal opening, in root hairs these function in transport of nutrients (Geisler and Venema 2010). They are highly concentrated in phloem and help in long distance transport. Their main function is energization of transport of nutrients (Geisler and Venema 2010). A plasma membrane H^+-ATPase contains ten transmembrane helices and a large cytoplasmic domain (Fig. 5.4). The cytoplasmic domain consists of four sub-domains: The nucleotide binding domain (N), the phosphorylation domain (P), the activator domain (A) and regulatory domain (R) (Geisker and Venema 2010). Mutation in the regulatory domain results in failure or alteration of activity of the H^+-ATPases.

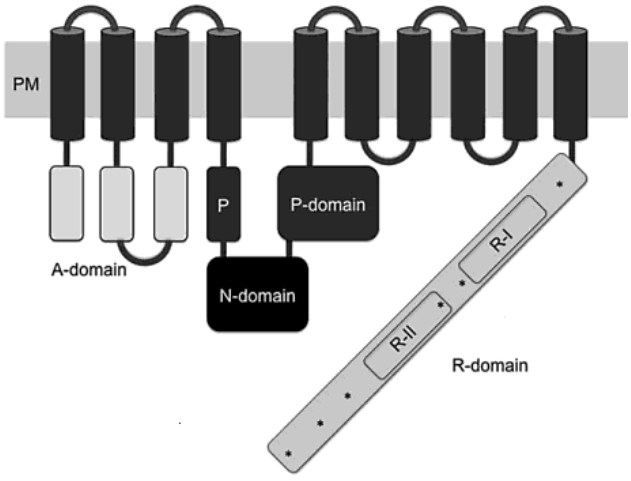


Figure. 5.4: Model of H^+-ATPase showing 10 helices and four domains (A, P, N and R). Picture from Briskin 1990.

H^+-ATPases produces an electrochemical gradient of $H^{+,}$ which drives a number of secondary transport systems and it results in the translocation of nutrients especially phosphorus in plants (Michelet and Boutry, 1995; Serrano, 1989; Sussman and Harper, 1989) (Fig. 5.5). Due to negative potential of plasma membrane towards inside, the uptake of cations and efflux of anions is favourable energetically, only the diffusion facilitator or channel protein is required. The plasma membrane H^+-ATPases facilitate the transport of nutrients, when the transport is not possible energetically, by providing the energy required for this transport. Fig. 5.5 shows the mechanism of the transport of phosphorus to the plant plasma membrane. Phosphorus is less in concentration outside the plasma membrane as compared to inside. The inside of membrane become negatively charged while outside is more positive as a result of H^+ produced by the activity of H^+-ATPase.

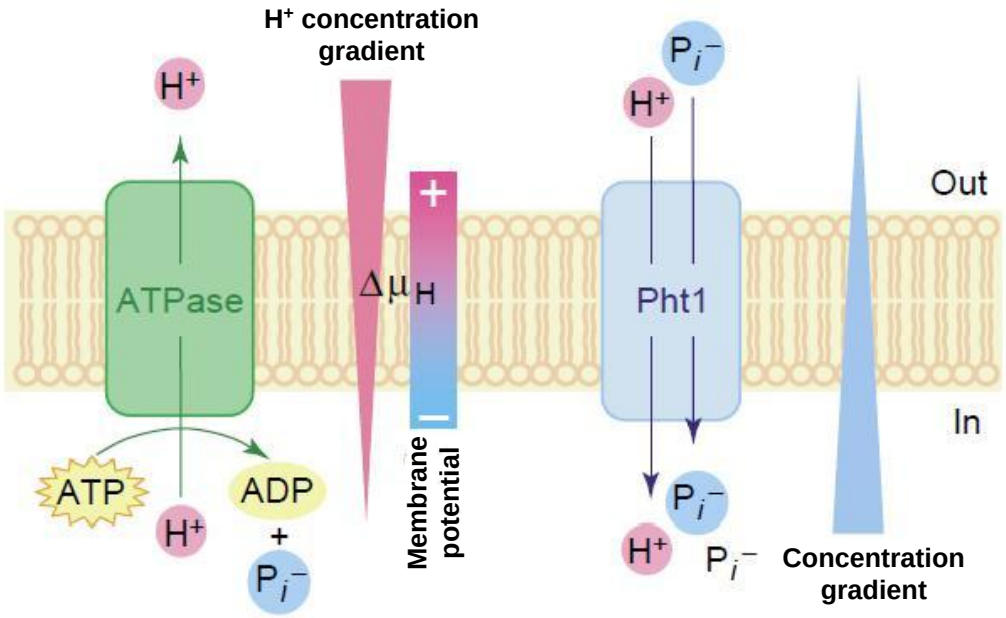


Fig. 5.5: Phosphate transport mechanism across the plant plasma membrane. H^+-ATPase extrudes proton on the expense of ATP. The electrochemical gradient (△μH) is generated across the membrane as a result of negative charge inside and positive outside of the plasma membrane. As a consequence of proton movement along concentration and electrochemical gradient, the transport of phosphate (Pi^-) occurs from out side of plasma membrane where the concentration of phosphorus is lower as compared to the inside. Picture from Karandashov et al. (2005).

H^+-ATPase activity was increased in the periarbusculer membrane surrounding arbuscules (Marx et al., 1982; Gianinazzi-Pearson et al., 1991). This high activity of H^+-ATPases suggests that H^+-ATPases may be associated with symbiotic nutrient transport activity in root cells containing arbuscules. The arbuscule specific expression of *Mtha1*, *NPha2* and *NPha4* results in the acidification of plant-fungus interface (Guttenburger, 2000) and an electrochemical gradient is generated. This gradient acts as a driving force for plant-fungal nutrient transport as suggested by Smith and Smith 1990. Phosphate transporters which are specifically expressed in arbuscule-containing cells would also need such as electrochemical gradient to facilitate phosphate transport. These transporters have been described in *M. truncatula* (Harrison et al., 2002), in rice (Paszkowski et al., 2002), in potato (Rausch et al., 2001). In addition of providing a driving force for nutrient uptake another role of H^+-ATPase be related to provide a suitable pH environment in the plasma membrane (Robsen et al., 1996) because acidification of matrix influences fungal growth.

The higher total fungal colonization in NF905 in the segregating population after backcrossing in homozygous mutants as compared to the screening experiment raised the question whether there was segregation of other mutations. However, similar to the segregation analysis upon backcrossing of *ram1* mutants, among the 20 plants analysed there was not one that showed a dramatically reduced colonization compared to the others. Hence, although it cannot be ruled out completely, it does not seem very likely that a single second mutation has been segregated out. Overall cosegregation analysis (average values of fungal colonizations) results suggested that the reduced colonization phenotype in mutant line NF905 segregates with insertion in the *Mtha1* gene.

Mtha1 expression pattern (Fig. 5.6) was obtained from *Medicago* gene expression atlas (Benedito et al., 2008; He et al., 2009). The presence of *Mtha1* expression in AM roots while absence in stems has already been reported (Krajinski et al., 2002). It was clear from Fig. 5.6 that *Mtha1* showed its expression in nodulating and mycorrhizal roots. In nodulating roots *Mtha1* was highly expressed under two days after nitrate treatment, indicating that this gene is expressed under certain conditions. It is possible that increased supply of

nitrate to plant may enhance nodulation but then nitrogen fixation is stopped in these nodules due to too much nitrate already supplied. *Mtha1* may perform a dual function in both nodulation as well mycorrhizal development. As NF905 plants did not show visible defects in nodule development it remains to be shown to what extent the function of nodules might be affected by the mutation in *Mtha1*.

Fig. 5.7 illustrates the alignment of *Mtha1* protein with H^+-ATPases induced in mycorrhizal roots in tomato and tobacco (Ferrol et al., 2002; Gianinazzi-Pearson et al., 2000). Owing to the induction of these genes in mycorrhizal roots, it can be concluded that all of them may be facilitating nutrient transport by producing increased proton pump activity. Previously it was indicated that the activity of H^+-ATPases was regulated by mycorrhizal colonization (Bago et al., 1997; Benabdellah et al., 1999). In colonized roots H^+-ATPases showed high activity as compared to uncolonized roots. Phosphate transport is clearly essential for AM symbiosis and without its transport by transporters (eg. *MtPT4*), arbuscules die prematurely and fungal growth is stopped (Javot et al., 2007 b). Phosphate transporters and H^+-ATPases work in coordination with each other and the transport of nutrients is facilitated as a result of the electrochemical gradient generated by H^+-ATPases. If the plant does not get enough phosphorus from arbuscules, then it does not benefit from the interaction with the fungus and by some unknown mechanism the fungal growth is not further stimulated by the plant. It was presumed that mutation of *Mtha1* gene resulted in disturbance in the process of generation of electrochemical gradient, hence phosphorus uptake is likely to be impaired. This resulted in reduced fungal colonization, probably because the plant does not support the growth of hyphae and arbuscules as much if there is little benefit from it.

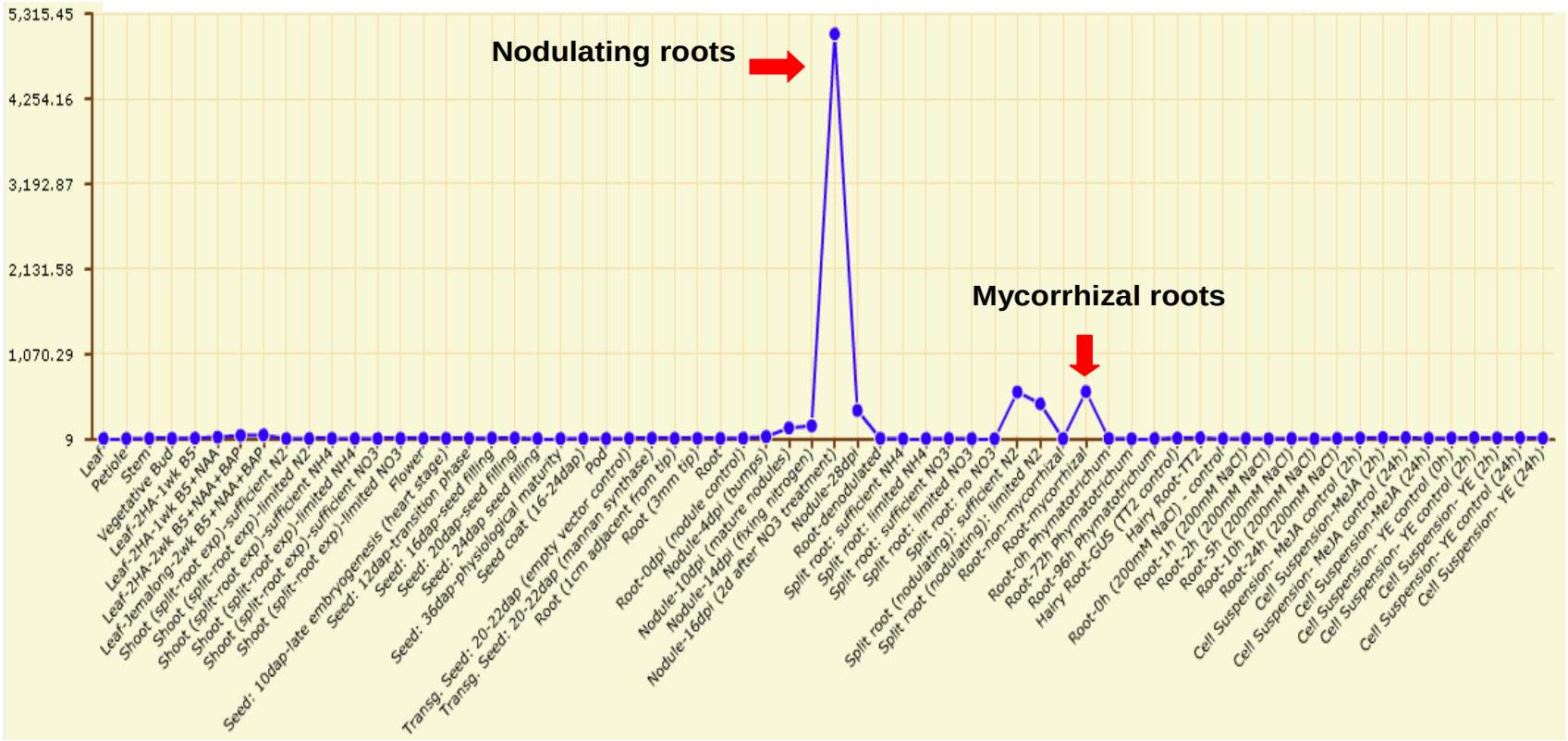


Fig. 5.6: Expression pattern of *Mtha1* (from gene expression atlas of *Medicago truncatula*). Arrow pointing to the expression of *Mtha1* in mycorrhizal root.

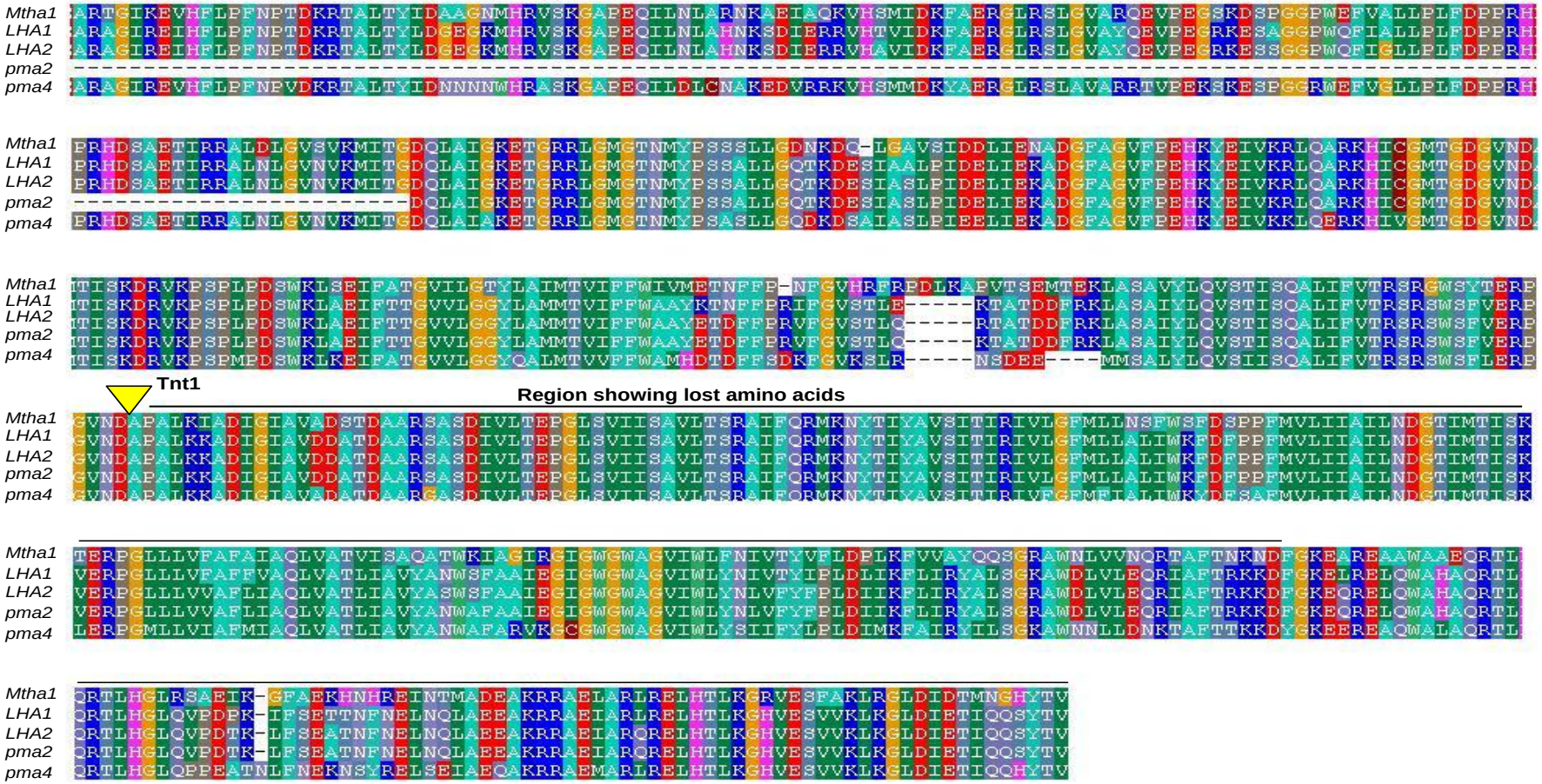


Fig. 5.7: Alignment of *Mtha1* protein with other H^+-ATPases as reported by expression studies in mycorrhizal roots. *Tnt1* insertion on *Mtha1* resulted in loss of 364 amino acids (loss of regulatory domain (see fig. 5.4) of 966 total.

Fig. 5.8 showed that *Mtha1* exhibits highest similarity to the ATPase Pma8 of *Nicotiana plumbaginifolia*, to the AHA7 of *Arabidopsis thaliana*, to the OsA1 of rice.

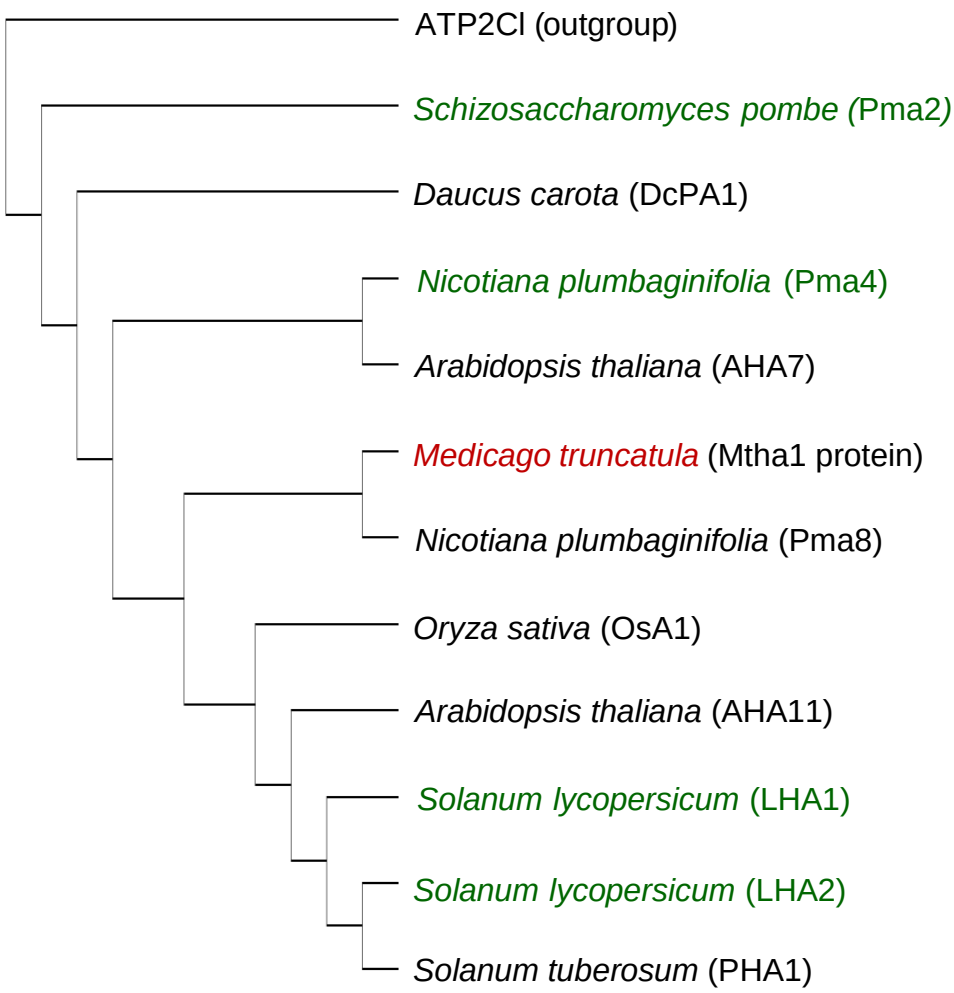


Fig. 5.8: Phylogenetic comparison of different plant H^+-ATPases. ATP2Cl from *Homo sapiens* was used as an outgroup. The H^+-ATPases represented by green colours are reported to be expressed in mycorrhizal roots. The programmes ClustalX2 and TreeView were used to construct the tree of comparison.

Interestingly the phylogenetic tree constructed with H^+-ATPases of different species indicated that *Mtha1* shows a different phylogenetic position from four other H^+-ATPases, which were previously known to be induced in arbuscule-containing cells (LHA1, LHA2, Pma2 and Pma4) (Ferrol et al., 2002; Gianinazzi-Pearson et al., 2000; Krajinski et al., 2002). A similar observation was made for phosphate

transporters (Krajinski et al., 2002). *MtPT4* in *M. truncatula* and *OsPT11* in rice show phylogenetic positions different from *StPT3* in potato, while all three genes are expressed in arbuscule-containing cells (Harrison et al., 2002; Paszkowski et al., 2002; Rausch et al., 2001). The different phylogeny of these genes suggests that corresponding genes and their regulation have evolved independently. It will be interesting to find other mycorrhizal associated H^+-ATPases to broaden the horizon of AM related genes.

In conclusion, the data provided in this work support the role of *Mtha1* in the function of mycorrhizal symbiosis suggested earlier by the expression in arbuscule-containing cells. To provide unequivocal evidence, further experiments are needed to demonstrate that the NF905 can be reverted to wild-type phenotype upon introduction of the wild-type copy of *Mtha1*. Also, it would be interesting to test the hypothesis that the mutation of *Mtha1* impairs phosphate uptake by directly measuring phosphate content in mutant compared to wild-type plants. If phosphate uptake is indeed diminished, then one would also expect that the growth of the plants is reduced provided the supply of available phosphorus in the soil is really limiting. The identification of two mycorrhiza related genes *ram1* and *Mtha1 in* this project has broaden the horizon of mycorrhizal research. The promoter Gus studies can be used for the better understanding of the role of these genes. Gene expression analysis and GFP fusion or in situ hybridization studies will further add our knowledge about the localization of these genes. Hence this work represents an interesting start for the molecular and physiological characterization of myc−nod+ mutants in *M. truncatula.*

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Appendix

Flanking sequences

NF788-1

5´

ATTAATAACTAGATTATTTCACACCATACCATATGAAAGGTGCAAAATGCTGATCGTGGTCTTCAACT
TTATGTGACAACAAACAAAACTTGCCTTTGTCTGTATACAGTTTATTACTTAGATCAACGATTGTTTA
ATTATTGTTTCTATATTCCCCTGCTACCACTACTATATAAATTAAAGCCCATTGTGAAGAAAAAAAGC
ATCCACCAAAGCCAAAGAAAAAGAAAATGGGATCCGAAGCAAACACTCATATACTTCTTATCTCTTAT
CCAGCACAAGGACACATAAACCCTCTTCTTAGACTAGCAAAGTGTCTTGCTGCAAAGGGTGCTTCTGT
CATCTTCATAACAACAGAGAAAGCTGGCAAAGACATAAGAACTGTTAACAACATCATTGAAAAATCAC
TCACTTCAATTGGTGATGGTTCTCTTACTTTTGAGTTCTTAGATGATGGTTTAGAAGATGATCATCCT
CTTCGAGGAAATtgatgat
488 bp

3´

cccaacaGAAATCTTTTAGGCTACATAGAACATCTCAAACTTGTTGGAAAACCCTTTGTTTCTCAAAT
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TCTTAGAGGGAATAAGTGAGAGAGGAAAAGTGGTGAAGTGGAGTCCACAAGAAGAAGTACTTGCTCAT
CCTTCAGTGGCATGCTTCATAACACACTGTGGTTGGAATTC
850 bp

NF788-2

cccaacaCAATTAAGAAAAAATATGTCAGTTTCTTTGATGGTAGTTTCACCACTGAGACTTGGCCTAG
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CAGATAAAATATGCAACTTTCAAACATTGTAGTTTTGTTTCATCATAAGATACCGGCTAAAAAATCAT
CTTATATCTGCAGACATCATGTGTTTCAAGAACAACCTCAACGATTTCATAACCTAAAATCACTTTCT
TCTAATTGCTAATCCAAAACAAATTAAT
293 bp

NF788-3

ATTAATGTTGGGGGGCAAGGTTGGTGAATGGTCGTTCACCTCTTTCTCTTTGGACACAACCATCtgat
gat
64 bp

NF788-4

cccaacaTCTTCATTTCTCTCATAACACCTTCACACTCAGCTCTCAAATGTGCTTCTCAAAAGCTTCC
ATCAAACCGTTCTTACACAAACTGCACAGATCCACAAACTGCGAAAATCCCTTTTTTTTCCTATCTCTA
TCCCgatc
137 bp

NF788-5

cccaacaCCATCTTCTCATAAGAAAAGAGAATCTTTGTAATTAACCAATTCAAAATATTAACTCATTT
GGATTGATCCCCGCTTTCGTGCTTTGGTGGGTCTTCGAGATGCTTTCGATGACCTATGTTGAAAAGAT
ATCTATCTATATgatc
145 bp

NF788-6 (from donor line)

ATTAATTGACCTCTCTCTAATGATATGATACACTTCACTCATTGTTGATTTAGAATAGAATTTTGCGA
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CTTCTCGTGGTTTTATGCAGTTGGAAATTTTGTATAGAATGAGGTTTTATTTTTTGATTAGTAAATAG
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gatgat
271 bp

NF788-7

cccaacaTAATGGAATTAGAGAGTACGTAGAAGAACAAAGTTACTTTCACTAATCACTATCATTGATT
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CATATTTAGGTAGCAATTATACAAGGCTGAATTC
163 bp

NF788-8 (from donor line)

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499 bp

NF788-9 (from donor line)

ATTAATTGAATTGTAAAACCCAACTATTTTTAACCTGCAGCGACTCTCTGTTGtgatgatg
53 bp

NF807-1

cccaacaACTTTGGTTGTGCAAAGATGATCGATGAAATTGTGCCGGCTGCCGGAACACCGATGTATAT
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GTTGCATATTCTGACGAAGTTCCTATGATTCCTTGTTTTTTATCTGAACAAGCAAAGGATTTCTTGGA
GAAGTGCTTAAGGAGGGATTCTAAGGAGAGGTGGAGTTGTAGTCAATTGTTGAAGCATCCATTTCTTG
ATTGTGGTGTTGAGAAAATTGAAGAATTTGATTCATGTTCACCAACTAGTATTCTTGAACAGGGCTTT
TGGAATTGTGATGAAGGGTCAGAAAGTTTCTTTTTTGATGATTTGGGTAAGATTAAT
458 bp

NF807-2

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TCTCTATCCTCTCTTCACCTTCACATGCCACGATGTTCCGAATCTCCGGTGCAAATATATACTGCTCC
ACCTTAGCCCTTGGTGCTGATTCCACAGGAAAAGTGGCGTCCAAGGAGTCGAAAATTGCGGAGTAGTA
ATGCAAAGCCTCGAGAAATCGGCCCAAAAAGTACGGTCCGTTATGGCTTGCTTCTTGCTCTACAAGTG
TTACTATGTTTGGTGCTTGGTCTCTTATCATGGACAACAAGTTCCCGAGATGATTCCCGGGAACACGG
TGGAGACGGTTAACGGTGTTAACCGCCAAAGCCTCGCCTACACGACGGTTAAACATGTGTGGTTTAAG
ATC
472 bp

NF807-3

cccaacaCGTTTTTCCTTCATATTCTTATGATACCATTTCCAAAGCTGCTTTTCCTATAGCAATACAT
TTAGCTCATGGAAATCAGATAGCTCTTAGACTTCAGAGAGGTTTCAAGCATTGACGCCACGCCCTTAT
GGAAAAGGGCATCATGGTGTACCAAAGTTGGCTAGATGGGGTGGAATAGAAATGATGAAAACTAAAGT
CTTGAAAAAGGTCATGGATTGTGCAGGATTTAATAATGAATTTCTGTGGAAGCCATACGAGAAATCAC
CATGCATTGAGGTTTATAATGAAAAGGATATGTGGAGATACTATGATAGTCCTTGTTTAGATGAAGAG
TTAGCGTCGTTTTCTTGATGTTTGAAAGTTTGTGAGTTGGTTGGTATGGAATGTAAAGAGAAGTACTT
CCCTCATCGTGTCGCAATGCAATTTGGTATGGATCAAGATATTCCTGCAGGTGAGGTTTTTCTTTGTA

AGAAGGATCCTTGGATGATTTATAACAAGCCGGTCGCAATACTAGATATAGATTTGCTCATTCAATTG
537 bp

NF905-1

cccaacaGCATCGTCCTTGGTTGATTAGAGTTACCCAAAACTCACATGGCAAAAAACTCTTCCACCTA
CTCTTCAAATCATTCTACTATTCCCATTGTTTTCCTCGTGTGCTTGACTTCAACAAACTCTCCGTCCT
CAATTTAGGAACAGATTTTATCATCGACCATGGAGATTTTAATCTTCGTAACTACTTCTTCTACAATA
ACAAGAACCGTGAAAAGTTCCTTGCTATCACATGCAATGGAGAGAAACCTATGGTTCTCGGCATTTCA
GAATATTTCCCCCATCCGATGCTGTTCTGTTACCTCAACAACTATTGGAAACCCATTTCTGACATGTC
AACTAAGCATGTAGATATCTGTGTGTTTAAAGGACAATTTTACTTGGTCGACAATACTGGTCGGACAG
TTGCAATTG
410 bp

NF905-2 5´

GATCTAATTATGTCTGCAAAACTTTCCATAGTTTCTGGTCTAAACTTGGAGGATATACCACTCTTAAA
ACAAGAAGAAAATAAAATTCAAGATTAAGTTTCATGTCATGATATAAAAGTATATAAAAGTAAGATTC
TAACTTCATGATATTAAGAGTAGGGGCCTGCATGGTAGAGCGACATATAGAATAGTTAAATTTAGAGA
GAAAGGAAATCTTCTCTATAAATTAAATGACTCGACGACCCCCAAGACCATACAACGGCCGTAAAAGA
TTCAAACCCGAGTGGATGGTATGACCCTTGGCATTCGGCTTTTAAATATGAAAGGAGCGCCTATAAAT
AGAAGTATGGCGAACATAAAAAGAATATTTATGGATATGAAACTAGACACCTTTTGTATGACCTGCTA
TGACATGATTTGCAATGTGTTATATGCTTCATCATTACCTTAACGGCCTTTTTGACTCAAAAACATTT
TGTATTGGTTAATATTTAATTCTAATGCAACACTTAGCCAAATTTATAAAAGCATAACGCAGATAGTT
AAAAATtgatgat
550 bp

3´

cccaacaAAAATGAGCATAAGGGATGCTAACCTGGTTTGATAACAATTCAAAAATCCCTCTAAGCTTG
TCAGAGAAAGAATAACCAAAGCTCAATCCACTGAGTGCTTCAATGAAGCTACCAAACTTAGTGATTCC
CACAGTTTCAATCACAGAAAGTTCTACTGGTAAAGGAGAAGGAACAACAAGAACATTTTTTGGACTAT
CTGCCATCCCTTGACCAACAAAAGGCTGATCCATAACCACCTGGATTCCATGTTCCTTAAGATGGTTA
GCAGGACCAATGCCACTCAGCATCAGTAACTGTGGACTTCCAATTG
311 bp

NF905-3

cccaacaCACACTCGTGGTGATGATGATGAAGAACAATTTGTGTAATGTAGTGCAAGGTTTGAAGCCA
ACGTTGTTAATGGTTATGGTGCAAATAGCATTTGCTGGTGTTAATGTTCTATACAAGTTAGCTGTAAA
CGACGGCATGAGTTTAAGGATTGTTGTTGCCTACCGTTTTATATTTGCAACTGCTTTCATAGCTCCAA
TTG
200 bp

NF905-4

cccaacaAAAGGGTTGAGAAAAAAGTGAAACCATAGTTAATGCAAGAGAAAGAAGGAAGTAAAGGTCG
GTGTTTTAACGTTTAACATAATAAGCAGAGAGTGAGCAGAAGAAAGATGTCATGTGTGTCAGTCATGC
AAGGCAATGCCAAGTACATTGTTGTCAATTTGAATTCGTCACCCGTCCCCTTCTTcCTTAACTGCCAG
AGTTACTGTACTACTATTGCTTTTAAAAATACTATTTAATATCCAGAAGTCCTGGCTCAACTGGCAAA
ATGCCGAAATTGTTAGGCCGGATGCCATGACCGGGGTTCGAACCCCGATACCGCCACTTGTGTGTGTG
AGTTTATAATGGCTTTGCCATTTCGTCTATCTAACAAAAAAAAAAAAAACTATTTAATGCCCTCATAAA
ACTTTCTGGTGTTCACATTGACATACCAGAATTAGTCACTTCAATTCCTCAATGAAAAAAGGAATAGT
CATTTAGTCTTTTTTTTTGGTGTAGACCGGGTATCCTCCGCGCGCAACTGCGGATACTAATCCCTCGA
GCATTGTGGGATCCAACTGGGCGGCAGGCTCTCCCTAAGAGTTTTAACACTGCTGCATGCCCAAGACT
GGATTCGAACCCAGGACCTTGGTTAAGCTAGAAGAGACCCGTTCCATCTCATCTAAGTGCTCTTGGGT
AATAGTCATTTAGTCTTTGAGTATAGAGAAATTGTAAAACAATTTTTGAAAATAGACTTAGTTGATCA
ATTTTAGGACTAAAGTGATTAAT
764 bp

NF905-5

cccaacaAGAAGAGTGATGTTTATAGTTTTGGGGTGGTCCTAGCAGAGCTACTGACAGGAAAGAAGGC
GCTATCTTTGGGCAGGCCAGAGGTTGATAGAAACCTTGCCGCATACTTGGTTTCTTCAATGAAAGAGG
GTCGGTTACTTCATATTTTGGACAAAAGTATAGATAATGCTGATATTGAGCAACTAAAGGAGGTTGCT
CTTATTGTAGAACGGTGTTTAAGGGTGAAGGGTGAGGACAGACCCACCATGAAAGAAGTGGCAATGGA

ATTAGAGGGAATATTAGTTAGTGAAGAGCGTCGTTGGGGAAGCGACAATTTATCTTCAGAAGAGACTG
AAAAGTTGCTTAAAACAGCACGGTCCATTAAAAACGTTGAAGATGTTGTTGGTGGAAGTGGCATTAAT
401 bp

NF905-6

cccaacaGGCCGTGGTTTCTATGAATATTCATTCTCCTCCGACAATGATGTGCGCACATCACTCGCAA
TGGGGACAATTAAT
75 bp

NF905-7

cccaacaTAAATAACATGTAGTTTTTTTATTAAT
27 bp

NF905-8

cccaacaGCACCTGCCTTAAAGATAGCTGACATAGGTATTGCAGTAGCAGATTCTACAGACGCTGCAA
GAAGCGCTTCTGACATAGTGCTGACAGAACCTGGTTTGAGTGTCATCATAAGTGCAGTTTTAACCAGT
CGAGCAATTTTCCAGAGGATGAAAAACTACACAGTAATCCCATGACTATAAATGCTGACATTTTTTAT
ATTTCATAGTGACAACAACTCATTATTTCTTTTCTCTTTTAATTTGCAGATATATGCTGTATCTATCA
CTATTCGTATTGTGGTAAGCTATTTGTGGAAATATATAGATTGTATTACAGGATCATCAACCATTTTC
TTATTTCCAGTTTGTTTATAATTTCAGTTGGGCTTCATGTTACTTAACAGCTTTTGGAGTTTTGACTT
TCCACCCTTTATGGTTCTTATCATTGCCATTCTCAATGATGGTAATGTCTGCTACTTTCTAGTACACT
AATCTCAAGTCATTATAATTTCATGGTCTATTTACTTTCTATTTTCTTTACGTGTTGGCTTTTTTAAT
TAAT
541 bp

NF905-9

cccaacaACACACGCAGCAGTCAATCTCAGCAATGATTTGAGATCGATCAACTTATATTACACCTTCA
CTAAATCATCAACAAATCATTTTCACCACTGGTTTCAGATCAGACCGCCAAAATCATTAGCCGCGTGA
CACAATTATCGTGTCTAGTCCAAATATGATTGATGGTTACTGGTTACCTCATGCCAGTATGTTTCATG
ATATTTGCATTTTTTTCTAGATAGTGAAATCCCACTTCATAATTTTCTGGGGTAAAGGAGGTGAAAAT
GATACTCTGCAGAACATTGTTATGGATTATACAGTAAAGGCCTCAGGAATCACTTATGTAAATATGAT
GTATTGATTAAT
345 bp

NF905-10

GATCAAAAGTCCCTCGTTTGAGATGGACTCCTGAACTTCATCGTTGCTTTGTTCATGCTATTCAAACC
CTTGGAGGTCATCATAGTAAGTTTCTTTCTTCACTCTCTCTATACATGTTTATTTGTTTCTTGTCCAA
GTTTTTCTTTCTATCAAGTTTTCTGATTTTTTTTTTACTTTGTATTTTTGGCAGAGGCTACTCCTAAA
CTTGTTCTTCAGTTGATGGATATCAAAGGACTCACTATTTCACATGTCAAGAGCCATCTTCAGGTTCT
TTTTTCATTTTTCTTTTCTTTTCTTTTGTACAAACACAAATAATTTTGTATCTTACTTGTTTTCCTTC
TTTATGATAACCAGATGTATAGAAGCATGAGAGGTGAATTAGGAAGACAAGGTACTAAACAAGTGTTT
TTTCTTTTCTTTTCTTTTCATGTCTTGTCATGAGTGAAAATTTTGCATGCTTTGTGCATTGAATGCAA
GGCATTTCTTCAGTCTATTATTTTTTCTTCTCTTGGTATTTATTTAAAGTTCCAAAATAATTCCCTTG
TGGATTTTCTTTATGTATAACAtgatgat
566 bp

NF905-11 (from donor line)

cccaacaGAGATGTACTTTTGGTCCTTGTACTTTATAAAAATAAGTTTTGTTTGGCCCTCTTCTTCAA
AGAAAATTGGTTTTTTGGGGTCATTATTTAACTTTTATCTGAATTTTGGTTTTGTCTCATTTTTGGAT
C
130 bp