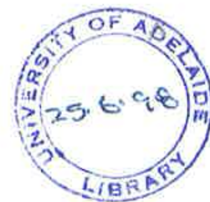

Molecular Characterisation of the Vesicular-arbuscular Mycorrhizal Symbiosis in *Lycopersicon esculentum* Mill.

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Abstract

Vesicular-arbuscular mycorrhiza (VAM) are symbiotic structures formed between plant roots and soil fungi. Both organisms benefit through bi-directional nutrient exchange; plants grown in poor soils gain better access to nutrients such as phosphate and zinc and the fungus obtains a carbon supply via the plants' photoassimilates. Physiological aspects of the mycorrhizal symbiosis in whole plants have been well documented, however, the inability to culture the fungi in the absence of plants has inhibited progress in many areas of mycorrhizal research. Advanced molecular biological techniques enable more detailed studies of the VAM symbiosis, with these techniques only beginning to be applied to this field.

The aim of this thesis was to clone and characterise plant genes that were differentially expressed in the root upon colonisation by a mycorrhizal fungus. To maximise differential expression of plant genes between mycorrhizal and non-mycorrhizal roots, an inoculation method was developed that produced a plant root system with abundant, rapid and near synchronous fungal colonisation units. Not only was this inoculation technique used to generate plant material for molecular experiments, but was also used to clarify the physiological question: Which stage of the colonisation process is inhibited by host plants with grown in high soil phosphate? The results indicated that there was a small decrease in the number of entry points formed on high soil phosphate treated plants, with the main inhibition in colonisation occurring during the intercellular hyphal growth stage.

The molecular studies utilised both targeted and non-targeted approaches to gene cloning. The targeted approach focused on the uptake of phosphate in the plant under mycorrhizal colonisation. An *Arabidopsis thaliana* expressed sequence tag (est) that showed high levels of sequence similarity to characterised transmembrane phosphate transporters was used to screen a phosphate stressed *Lycopersicon esculentum* cDNA library. A clone was obtained that also had high sequence similarity to phosphate transporters and was designated LePT1. Expression studies showed that LePT1 maintained a constant level of expression in mycorrhizal roots, while expression increased in non-mycorrhizal roots. Furthermore, *in situ* hybridisation studies revealed that LePT was expressed in arbuscule containing cortical cells, implicating LePT in uptake of phosphate from the plant.

The aim of the non-targeted approach was to isolate and characterise genes that were differentially expressed during the early stages of colonisation. To achieve this, cDNA libraries were screened with probes generated by labelling cDNA from pre-arbuscular mycorrhizal and non-mycorrhizal roots. Four different clones were isolated, and represented genes that had increased expression during the early stages of colonisation. The clones were designated *Lycopersicon esculentum* var Rio Grande interacting with the mycorrhizal fungus *Glomus intraradices* (LerGi) 1, 2, 4 and 5.

Sequence analysis showed that LerGi 1 contained the partial sequence of the S17 ribosomal protein. The LerGi 2 clone contained sequence that had previously been identified as being expressed in giant cells of the plant pathogenic interaction between *L. esculentum* and root-knot nematodes. LerGi 4 was identified as coding for the enzyme S-adenosyl-l-homocysteine hydrolase and LerGi 5 as a phospholipase D enzyme. DNA and RNA gel blot analysis was used to partially characterise each of the genes, and *in situ* hybridisation was completed on LerGi 2. The possible roles of each of these genes in the mycorrhizal symbiosis is discussed.

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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or any other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Garry Rosewarne　　　　10/6/98
Date

Abbreviations

Common abbreviations are listed in Current Protocols on CD-ROM (1993). Additional abbreviations are as follows:

Amino Acids

	A	Alanine
	D	Aspartic Acid
	E	Glutamic Acid
	G	Glycine
	K	Lysine
	L	Leucine
	P	Proline
	R	Arginine
	S	Serine
	T	Threonine
	X	Any Animo Acid

bp	base pair
BSA	bovine serum albumin
DEPC	di-ethyl-pyro-carbonate
DMSO	dimethly sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
g	gram
hr	Hour
IPTG	isopropyl ß-thiogalactopyranoside
kbp	kilo base pairs
M	molar
min	minute
ml	millilitre
mM	millimolar
NBT	4-nitroblue tetrazolium chloride
ng	nanogram
nm	nanometre
OD_{600}	optical density at 600 nm
PEG	polyethylene glycol
rpm	revolution per minute
s	Seconds
SDS	sodium dodecyl sulphate

μg	microgram
μl	microlitre
μM	micromolar
°C	degrees centigrade

Chapter 1

Literature Review and Research Proposal

1.1 Introduction

The overall aims of the research described in this thesis were to use molecular techniques to identify and characterise plant genes that are involved in the vesicular-arbuscular mycorrhizal (VAM) symbiosis. At the commencement of this research, there had been little published data relating to this field. Several relevant papers have since been published. However, this field of research is still in its infancy. The work described here adds a significant contribution to the understanding of the molecular mechanisms involved during the establishment and maintenance of the VAM symbiosis.

1.2 The Symbiosis - An Overview

VA mycorrhizas are a mutualistic symbiotic association between plant roots and soil fungi. Approximately 120 species of fungi within the order Glomales (Zygomycetes), and up to 80% of all plant species can form this association. Mycorrhizal associations are considered to have ancient origins, with fossil evidence supporting their existence in the earliest land plants, 400 million years ago (Kidston & Lang 1926; Nicolson 1975; Remy *et al.* 1994). This agrees with a molecular clock estimation by Simon *et al.* (1993) who used sequences obtained from nuclear genes encoding the ribosomal small subunit rRNA (SSU) to calculate the time of divergence between glomalean families and genera at 353-462 million years ago. In the symbiosis both organisms generally benefit by an increased supply of nutrients. The plant gains greater access to elements such as phosphate (P) and zinc (Zn), and the fungus receives carbon from plant photoassimilates. Plants show a characteristic growth response when colonised by VAM fungi, particularly with respect to an increase in shoot biomass. Colonisation by the fungus is limited to the root cortex, with the vascular cylinder and aerial parts of the plant remaining uncolonised.

The fungus can extract nutrients from the soil more efficiently than plants, primarily through more efficient soil exploration. As plants take nutrients from the soil, a zone of depletion of some of the more immobile nutrients (e.g. P and Zn) immediately around the root can form. The fungal hyphae extend beyond this

zone of depletion, taking up nutrients from previously unexplored soil. These are then translocated through the hyphae and transferred to the plant for subsequent use in growth.

The fungal symbiont requires a carbon source for its own growth. This is supplied in the form of photoassimilates produced in the plant shoot. The photoassimilates are translocated through the phloem and into the roots. The intimate contact between plant roots and hyphae enable the fungus to obtain a portion of this translocated carbon, although the mechanisms are not clear (see section 1.6).

1.3 Plant Control Over Fungal Growth

There are four lines of evidence that the plant has some form of genetic control over fungal growth. The first line of evidence is associated with the levels of colonisation within mycorrhizal roots when plants are grown under differing levels of nutrition. The observed growth response in mycorrhizal plants is greatest when the plants are grown under nutrient limiting conditions. Under nutrient sufficient conditions, the growth response is reduced, and a decrease in colonisation of the roots is also observed. This indicates that the plant nutritional status affects colonisation by the fungus. Recent evidence shows that plant phosphate transporter genes have lower levels of expression in the roots of both phosphate fertilised roots and roots colonised by VAM fungi (Liu *et al.* 1998). The lowered expression levels in mycorrhizal roots is presumably the result of improved P nutrition. Burleigh and Harrison (1997) investigated an unidentified gene, Mt4, that was induced by P starvation. This gene showed similar expression patterns to phosphate transporter genes in mycorrhizal roots, however, Mt4 had lowered expression in mutant plants that do not form arbuscules. Assuming that arbuscules are the site of phosphate transfer, this data indicates that a second signalling pathway that is mycorrhiza-specific may exist.

The second line of evidence for plant control comes from morphological differences in fungal development in different taxonomic groups of plants. The fungi have broad host ranges, but the structures formed within the root are host dependent. There are two broad morphological types of internal structures that are formed and these are termed 'Arum' and 'Paris' type mycorrhizas (Gallaud 1905). The 'Arum' types have extensive intercellular hypha from which lateral branches penetrate the cortical cells, forming arbuscules, whereas in 'Paris' type hosts grow the fungi predominantly intracellularly, forming coils within the cells. The coils

sometimes produce small arbuscules (Smith & Smith 1997). This indicates that the host influences the internal growth form of the fungus.

Thirdly, a number of legume nodulation mutants have been isolated that have a reduced capacity or complete inability to form the typical mycorrhizal symbiosis (Duc *et al.* 1989). Two phenotypes have so far been observed and are further discussed in section 1.5.2. The identification of these mutants indicates that plant genes are essential for the formation of the association.

A fourth line of evidence that plants have some form of molecular control over fungal colonisation is that the fungi are unculturable in the absence of plants. The plants must provide either essential nutrients or some form of trigger that enables the fungus to produce the large amounts of external hyphae and spores that occur in a functioning symbiosis and allows the fungus to complete its life cycle.

1.4 Colonisation

Extensive work has been carried out on the morphological changes observed throughout the colonisation process. This process can be divided into several developmental phases;

1) preinfection spore germination and growth,
2) appressorium formation and tissue penetration,
3) intercellular hyphal growth (Arum type),
4a) arbuscule formation and subsequent degradation (Arum type),
4b) cellular penetration and coil formation (Paris type)
5) vesicle formation (Glomineae only),
6) external hyphal growth and secondary colonisation.

Colonisation has been most extensively investigated in the Arum type interaction, although many of the steps can also be applied to Paris type interactions. Pre-infection stages of the fungal development have been investigated to determine how plants influence the early events associated with fungal growth. The fungus exists in the soil in the form of propagules consisting of spores or previously infected root pieces. Under appropriate conditions these germinate and hyphae grow through the soil. Both spore germination and hyphal growth are positively influenced by volatiles and root exudates from VAM host plants (Gianinazzi-Pearson *et al.* 1989). More specifically, CO_2 (Bécard & Piché 1989) and the flavonol, quercetin (Bécard *et al.* 1992) stimulated hyphal growth of germinated

spores. Giovannetti *et al.* (1993a) showed that exudates from plant roots can effect changes in hyphal morphology, inducing extensive branching and characteristic swellings. This occurred in very close proximity to the plant roots, suggesting that a concentration gradient of the signal molecules develops outward from the root surface, and that the fungus responds only at high concentrations of these signals. The observed morphological changes occur prior to appressorium formation, and are presumed to increase the likelihood of contact between fungal hyphae and the plant root.

Roots of different species vary in their effect on controlling precolonisation events. Several investigations have utilised plant species that do not form mycorrhiza to determine the effects of root exudates on pre-infective hyphal growth. Non-host plants of VAM fungi did not elicit the differential morphogenesis that was observed when the fungus was challenged with host root exudates (Giovannetti *et al.* 1994). As a consequence, no further infection structures were observed in the non-host plants. Root exudates from *Lupinus albus* L., a non-mycorrhizal plant, did not hinder mycelial growth but hyphal attachment and fungal recognition of roots was inhibited. However, excised *L. albus* roots were recognised by the mycorrhizal fungus and structures resembling appressoria were formed (Giovannetti *et al.* 1993b). This suggests that compounds are produced in the shoots of *L. albus* that inhibit appressoria formation. Additional evidence for this comes from grafting experiments where shoots from *L. albus* were grafted onto normally mycorrhizal pea roots. The roots of this intergeneric graft were unable to form normal VA mycorrhizas (Gianinazzi-Pearson & Gianinazzi 1992).

Appressoria form when fungal hyphae attach to the surface of the host plants and produce a swelling. From there, a penetration peg is formed, which forces its way through the epidermal cell layer and into the root cortex, probably through hydrostatic pressure (Garriock *et al.* 1989).

The timing of formation of different fungal structures has been best studied in the 'Arum' type plants, *Allium porrum* L. (Brundrett *et al.* 1985), *Triticum aestivum* L., *Avena sativum* L. (Alexander *et al.* 1988), *Allium cepa* L., *Phaseolus vulgaris* L. and *Lycopersicon esculentum* Mill. (Alexander *et al.* 1989). After initial contact, hyphae proliferate intercellularly throughout the root cortex. The fungus takes between two to four days after initial contact to form arbuscules where hyphae penetrate into cortical cells and undergo profuse dichotomous branching. During this process the fungal cell wall is modified and both plant and fungal cell

membranes stay intact, ensuring the cytoplasm of each organism remains separate (Bonfante & Perotto 1995). Due to the large surface area of contact between the plant and fungal membranes at the arbuscular interface, this is presumed to be the site of phosphate transport into the plant (Cox and Tinker 1976). The developmental cycle of individual arbuscules spans around 7-14 days (Alexander *et al.* 1988). However, new arbuscules are continuously forming as intercellular hyphae spread to uncolonised sections of the root.

Vesicles are formed in cortical cells by fungi in the Glomineae (but not by fungi in the Gigasporineae). They contain abundant lipid and numerous nuclei and are thought to be storage organs. Another characteristic mycorrhizal structure is the external mycelium, which grows out from the roots and ramifies through the soil. These hyphae serve several functions. They obtain nutrients from the soil, form infective propagules which colonise either neighbouring plants or other sections of the original host root system, produce spores and play a role in anchoring the plant.

The mycorrhizal symbiosis progresses through a series of developmental stages that result in the extensive colonisation of the plant root. However the various stages of development are not synchronously timed, so that any one root system is likely to contain all stages of development at any one time. This factor needs to be considered when undertaking any developmental molecular studies. As indicated by work with the pea mutants (1.3), molecular interactions occur at very early stages of colonisation. As there are generally few points of contact between plants and fungi at the primary colonisation site, it would be extremely difficult to identify genes that are differentially expressed by the plant in response to fungal contact. Furthermore, as the fungal biomass increases proportionately to root biomass during the later stages of colonisation, any changes in gene expression may be in response to better nutrition and increased plant growth. To investigate gene expression involved in either the early stages of colonisation or other developmental stages, the root material must have a high level of fungal material, whilst maintaining a degree of synchronicity in the stages of colonisation.

Although the physiology of the VAM symbiosis has been well studied, little work has been completed on the molecular mechanisms involved in either developmental or nutrient transport processes of this association. The application of molecular techniques will enable the extension of knowledge in many aspects of the symbiosis, giving insight into how the symbiosis is established and maintained, the mechanisms of fungal differentiation to form the various

mycorrhizal structures, and the means by which nutrients are transferred between symbionts. The application of molecular techniques will provide information on the fungus that has been previously unattainable due to the unculturability of the fungus. Before reviewing the rather limited literature on molecular aspects of the VAM symbiosis, it is useful to consider molecular work on related biological systems for which our understanding is more comprehensive, including ectomycorrhizas, nodule-forming bacterial associations and plant-pathogenic interactions.

1.5 Molecular Similarities Between the VAM Symbiosis and Other Plant/Microbe Interactions

1.5.1 Ectomycorrhiza

Ectomycorrhizas are symbiotic structures formed between a variety of soil fungi including members of the Basidiomycotina and Ascomycotina with the roots of many forest trees, including members of the Dipterocarpaceae, Fagaceae, Myrtaceae and Pinaceae (Smith & Read 1997). This symbiosis is similar to the VAM symbiosis in that both organisms benefit through improved nutrition, although the structures which facilitate nutrient exchange differ and the interaction is more specific. Ectomycorrhizal fungi do not penetrate through cortical cell walls as VAM fungi do during arbuscule development. Instead the hyphae aggregate around the host root to form a mantle. Hyphae then penetrate between the outer root cells to form the Hartig net (Smith & Read 1997).

A number of ectomycorrhizal fungi have been cultured in the absence of host plants. As a result, ectomycorrhizal fungi are better understood than VAM fungi. However, as the hosts of ectomycorrhizal fungi are tree species, they are generally not suited to molecular/genetic research, making a molecular understanding of the ectomycorrhizal symbiosis harder to achieve.

Earlier molecular work on ectomycorrhizas was directed at identifying symbiotically induced changes in both plant and fungal metabolism and involved the characterisation of both fungal and root proteins which were altered upon colonisation. Hilbert and Martin (1988) examined changes in protein composition as colonisation progressed in the *Eucalyptus globulus-Pisolithus tinctorius* interaction. Using two dimensional polyacrylamide gel electrophoresis (2D PAGE) they found three classes of proteins; those that accumulated upon colonisation, others that were less abundant in mycorrhizal tissues, and those that were mycorrhiza specific. The latter were termed ectomycorrhizins. Symbiosis

related (SR) proteins have also been shown to accumulate during early mycorrhizal development in the *Betula pendula-Paxillus involutus* association (Simoneau *et al.* 1993).

Some of the ectomycorrhizal fungi secrete specific glycoproteins which enable them to recognise and attach to the plant root surface. Lei *et al.* (1991) observed an extracellular layer of fibrillar polymers on the surface of *Laccaria bicolor*, and an increased secretion of these polymers at the site of contact between the plant and the fungus. Further work with 2D PAGE has identified a group of acidic polypeptides that accumulate in eucalypt ectomycorrhizas (Hilbert *et al.* 1991). Martin *et al.* (1995) indicate that these polypeptides are either located in the fungal cell wall or secreted extracellularly by the fungus. Although no role for these molecules has yet been proven, Martin *et al.* (1995) speculate that they may play a role in attachment of the fungus to the plant.

There have been a few successful attempts to clone symbiosis related (SR) genes. Tagu *et al.* (1993) differentially screened a *E. globulus-P. tinctorius* cDNA library using probes synthesised from polyA RNA isolated from colonised roots and from free living *P. tinctorius*. Approximately one third of the cDNAs screened represented genes that were differentially regulated, with a significant proportion of plant genes showing decreased expression. Characterisation of one of the fungal gene sequences revealed significant similarity to an inducible acid phosphatase. This particular clone represented a gene that was highly expressed in free living mycelia, but down regulated in symbiotic tissues. Continuing work on some of the other clones should soon give an indication of how the symbiosis is established and maintained.

Another molecular cloning approach by Tagu and Martin (1995) generated expressed sequence tags (ESTs) by randomly sequencing cDNA clones isolated from *E. globulus-P tinctorius* ectomycorrhizal tissues. Of approximately 100 clones, 42% showed significant sequence homology to previously sequenced genes. Nine of these sequences were originally identified from other fungal species and seven from plants. However, DNA gel blot experiments have not yet been carried out to confirm the origin of the clones in the *E. globulus-P. tinctorius* symbiosis.

Two of the ESTs cloned by Tagu and Martin (1995) showed high levels of homology to previously identified hydrophobin genes from *Schizophyllum commune* (Wessels *et al.* 1991). A hydrophobin from *S. commune,* Sc3p, is

excreted by the aerial hyphae and these molecules self-assemble at the cell wall to form a hydrophobic (air)/hydrophilic (cell wall) interface. The ectomycorrhizal hydrophobin genes *hydPt-1* and *hydPt-2* were expressed at low levels in free living mycelia. However, in roots that had been inoculated for three to seven days, expression levels of these fungal genes were very high (Tagu *et al.* 1996). This suggests that the hydrophobins play an important role in the early stages of differentiation and colonisation of roots.

Some of the findings from the work on ectomycorrhizas may also apply to VAM fungi. Sequences from both the hydrophobins and acid phosphatases could be used to isolate the corresponding VAM fungal genes. These genes could then be examined for their involvement in symbiotic development.

1.5.2 Symbiotic Nitrogen Fixing Bacteria

As is the case with some ectomycorrhizal symbioses, both partners of the *Rhizobium*-legume interaction are culturable, making these systems easier to study than the VAM symbiosis. The ability to culture *Rhizobium* increases the range of molecular techniques that can be applied and molecular research is much further advanced for this symbiosis than in either ectomycorrhizal or VAM symbioses. Although the *Rhizobium*-legume interaction is very different from that occurring in mycorrhizas, there are some common threads running between the different symbioses, as has been shown with the work on mutant legumes that cannot form nodules and are also non-mycorrhizal (1.3). A brief overview of the establishment of symbiotic nitrogen-fixing associations, particularly related to early interactions between plants and nodulating bacteria that may be similar to the VAM symbiosis follows.

Soil bacteria from the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (collectively termed rhizobia) colonise the roots of leguminous plants and form a specialised plant organ, the nodule. There are several specific steps in nodulation of legumes. The rhizobia are attracted to plant roots through the production of plant exudates. Upon contact with the root, the rhizobia attach to root hairs and induce curling of the hair. The root hair cell is penetrated by the bacterium and an infection thread forms and continues to grow towards the root cortex. In the root cortex, the bacteria are released into host cells which have been stimulated by the bacteria to form the nodule primordia. This process involves the surrounding of the bacteria with a peribacteroid membrane. As nodule formation continues, the

bacteria differentiate into bacteroids and proceed to fix nitrogen (Roth *et al.* 1988).

Nodule formation has many similarities to the VA mycorrhizal symbiosis. Table 1 summarises the common features identified so far, in the order they occur during colonisation. These similarities are expanded upon below.

Table 1. Similarities in the colonisation processes of the rhizobium/legume and VA mycorrhizal symbioses.

Process	**Rhizobium/Legume**	**VA mycorrhizal**
1) Diffusible Plant Signals	Flavonoids stimulate *nod* expression	Flavonoids stimulate fungal spore germination and hyphal growth
2) Attachment of micro-symbiont (mutant studies)	Nod$^-$ plants unable to form nodules	Some nod$^-$ plants are also myc^{-1} and prevent colonisation by fungus
3) Stimulation of plant gene expression	Plant ENOD gene expression regulated by nod factors	Plant ENOD genes expressed in response to VAM fungal colonisation
4) Arbuscule$^-$ (myc^{-2}) plant mutants	Some myc^{-2} mutants are also nod$^-$	myc^{-2} mutants cannot form arbuscules
5) Peribacteroid compartment glycoconjugates (MACs)	Bacterioids surrounded by plant membrane containing specific glycoconjugates	Arbuscules surrounded by plant membrane containing similar glycoconjugates

1) Diffusible plant signals

Plant flavonoids are involved in the earliest steps of the colonisation process. In the establishment of the rhizobial symbiosis, specific flavonoids induce gene transcription in free-living rhizobia. A series of bacterial genes involved in nodulation (*nod* genes) are transcribed, with the *nodD* gene being central to this process. This gene is constitutively expressed and its protein product binds to specific flavonoids, enabling the protein to act as a transcriptional activator of other *nod* genes (Goethals *et al.* 1992; Fisher & Long 1992). Mycorrhizal fungal spores also respond to specific flavonoids with increased levels of germination and hyphal growth (Gianinazzi-Pearson *et al.* 1989; Nair *et al.* 1991; Bécard *et al.* 1992), processes which indicate changes in fungal gene expression mediated by the flavonoids. It is possible that a receptor/transcriptional activator similar to

nodD may exist in VAM fungi. No fungal genes relating to this process have been cloned, and the unculturability of the fungus makes gene cloning difficult.

2) Attachment of micro-symbiont (mutant studies)

Mutant studies have revealed that similar processes are required for the successful colonisation of roots by both rhizobium and VAM fungi. A range of mutant legumes unable form a complete symbiosis with rhizobium (*nod*$^{-}$) have been screened for ability to be colonised by mycorrhizal fungi and a number of non-mycorrhizal (*myc*$^{-}$) genotypes identified. Such mutants have been described in a range of plant species including *Pisum sativum* L., *Vicia faba* L. (Duc *et al.* 1989), *Medicago truncatula* (Gaertn.) (Sagan *et al.* 1995) and *Phaseolus vulgaris* L. (Shirtliffe & Vessey 1996). The mycorrhizal mutants fall into two classes. The *myc*$^{-1}$ mutants prevent the penetration of hyphae through the epidermal cell layer, and in *myc*$^{-2}$ mutants, penetration and intercellular fungal growth occurs, but the fungi do not form arbuscules (Gianinazzi-Pearson *et al.* 1991a). One of the *P. sativum myc*$^{-1}$ mutants has been further investigated and it appears that a localised cell wall defence response at the site of the appressorium occurs, which is possibly responsible for the failure of colonisation (Gollotte *et al.* 1993).

3) Stimulation of plant gene expression

A further similarity between colonisation of roots by VAM fungi and rhizobium is the expression of a group of early nodulin (ENOD) genes in response to colonisation by the microsymbionts. The ENOD genes are expressed in legumes roots during the early stages of colonisation, in response to nod factors produced by rhizobium. Recent evidence provided by van Rhijn *et al.* (1997) indicates that a group of ENOD genes are expressed in VAM alfalfa roots. Furthermore, Frühling *et al.* (1997) showed that a *Vicia faba* leghemoglobin gene was induced in both mycorrhizal roots and root nodules.

4) Arbuscule-less (myc^{-2}*) plant mutants*

The *myc*$^{-2}$ mutants have so far only been isolated from *Pisum sativum* and although they can be colonised by mycorrhizal fungi, fully differentiated arbuscules and vesicles do not form (Gianinazzi-Pearson *et al.* 1991a). These mutants formed inefficient white nodules and were termed (*nod*$^{+}$, *fix*$^{-}$). Similar nodulation mutants have been found in other legumes (Duc *et al.* 1989; Sagan *et al.* 1995; Bradbury *et al.* 1991) but these are all *myc*$^{+}$. Although the picture is still

inconsistent, the late stage at which the mutation acts in both VAM and rhizobium interactions indicates genetic overlaps occur throughout the development of these symbioses.

5) Peribacteroid compartment glycoconjugates (MACs)

Both the arbuscules and peribacteroid units form specialised interfaces with the host plants. At these interfaces, similar plant glycoprotein epitopes have been detected using monoclonal antibodies specific for extracellular plant glycoproteins (Perotto *et al.* 1994).

It can be seen that both microsymbionts mentioned above utilise similar pathways to gain access to plant roots. Similarities are beginning to appear at all temporal stages of the symbiosis, from the first signalling molecules to the wall components that the plant produces in the symbiotic interfaces. There are clear differences, but through the analysis of the well researched rhizobium symbiosis, progress has and will continue to be made in understanding the VAM symbiosis.

1.5.3 Pathogenic Associations

Plants have evolved many defence reactions in order to prevent invasion by pathogenic fungi. However, VAM fungi avoid major plant defence responses and can colonise large portions of the root cortex. They appear to be similar to the other fungal biotrophs in that they are able to colonise roots over extended periods of time without the stimulation of significant defence responses. Before reviewing the specific and minor plant defence responses elicited by VAM fungi, it is useful to examine the main strategies adopted by plants towards invading fungi.

Biotrophic organisms have a slow and controlled release of cell wall degrading enzymes. They thus cause minimal damage and are able to feed off the living plant for extended periods of time in compatible interactions. In incompatible interactions specific plant defence responses such as cell wall thickening, phytoalexin production and a hypersensistive response (HR) can be elicited. The HR can be stimulated by elicitors originating from either the fungus or degraded plant material and stimulates a rapid localised cell death in the plant which inhibits further colonisation by the invading organism. Some biotrophic pathogens have developed races that no longer elicit an HR and can form a susceptible interaction with specific hosts.

There have been many studies of the defence responses associated with VAM fungal colonisation. Some have involved investigation of the expression of genes coding for enzymes likely to inhibit the fungi (e.g. endochitinases, β-1,3-endoglucanases). These studies gave somewhat conflicting results. Lambias and Mehdy (1993) found a slight suppression of a basic endochitinase polyA RNA and no difference in expression of an acidic extracellular chitinase mRNA in *Vicia faba* colonised with *Glomus intraradices* compared with non-mycorrhizal *V. faba*. Furthermore, enzyme assays of total chitinase activity showed that there was less activity in mycorrhizal roots. This contrasted with results of Dumas-Gaudot *et al.* (1992) who also used enzyme assays and showed that several chitinase isoforms were induced upon mycorrhizal colonisation in *Allium cepa*. Levels of β-1,3-endoglucanase enzyme have been shown to be both reduced (Lambias and Mehdy 1993) or unaltered (Dumas-Gaudot *et al.* 1992) in mycorrhizal roots. The differences between these findings could be attributed to differences between host species or to the lack of synchrony of colonisation in *A. cepa* (see below), so that mild defence responses to certain developmental stages were not detected because of overlaps with other stages of development. This highlights the need for model systems in which colonisation is synchronous and it is possible to obtain material enriched in particular developmental stages.

Phenylpropanoid metabolism involves a complex of biosynthetic pathways that produce compounds such as flavonoids and phytoalexins. These are involved in a wide range of plant processes, often being implicated with plant defence. Key enzymes in these pathways include phenylalanine ammonia-lyase (PAL), involved in the initial stages of the phenylpropanoid pathway, chalcone synthase (CHS) and chalcone isomerase (CHI), enzymes that occur subsequent to PAL in the flavonoid/isoflavonoid pathway, and isoflavone reductase (IFR) which is involved in the final stages in the production of the isoflavonoid, medicarpin (Harrison & Dixon 1994, and references therein).

A detailed study of expression of phenylpropanoid pathway genes in mycorrhizal roots of *Medicago sativa* was carried out by Volpin *et al.* (1994). An important feature of their work was the ability to produce roots that had high levels of near synchronous colonisation from which to isolate RNA for gel blot analysis. Whereas Dumas-Gaudot *et al.* (1992) and Franken and Gnadinger (1994) found no changes in expression of PAL genes and slight decreases in expression of chalcone isomerase (CHI) genes, Volpin *et al.* (1994) showed that at 14-18 days after colonisation, PAL transcripts were twice as abundant in mycorrhizal roots and those of CHI were six times as abundant compared with non-mycorrhizal

roots. Their conclusions were that defence related transcripts initially increased upon colonisation, but later dropped to levels below that found in control plants.

The Volpin study, using synchronously colonised roots, was able to identify large changes in transcription rates of certain genes. Further data have been obtained with use of *in situ* hybridisation and immunocytochemical techniques to identify specific plant cells that express genes and the localisation of the corresponding protein products. Although Blee and Anderson (1996) found no difference in gene expression of PAL and chitinase (CHT) through northern blot analysis, *in situ* hybridisation revealed that transcripts of both of these genes accumulated in root cells of *Phaseolus vulgaris* containing arbuscules. Those cells with young arbuscules contained greater levels of transcripts than cells containing older, degenerating arbuscules.

Using *Medicago truncatula*, Harrison & Dixon (1993) examined isoflavonoid accumulation, and RNA transcript accumulation of several genes involved in phenylpropanoid/flavonoid/isoflavonoid metabolism, namely PAL, chalcone synthase (CHS) and isoflavone reductase (IFR). PAL and CHS showed increased levels of transcription upon VAM colonisation, whilst IFR expression decreased. These changes in expression were accompanied by changes in flavonoid profiles within the root. The flavonoids formononetin malonyl glucoside (FGM) and medicarpin malonyl glucoside (MGM) accumulated in roots upon colonisation whereas the unconjugated isoflavone forms of FGM and MGM, formononetin and medicarpin, decreased at the later stages of colonisation. Further studies (Harrison & Dixon 1994) using *in situ* hybridisation revealed transcript accumulation of PAL and CHS in arbuscule-containing cortical cells, while IFR transcripts were more abundant throughout the root cortex of non-mycorrhizal roots than in mycorrhizal roots.

The function of the flavonoids is unclear, as different compounds can be associated with plant development, defence responses and regulation of gene expression in *Rhizobium* spp. Harrison & Dixon (1994) have speculated that flavonoids may play a role in carbon transport. As flavonoids can inhibit auxin transport (Yang *et al.* 1992) and auxin affects photosynthate transport in plants (Sturgis & Rubery 1982), the accumulation of PAL and CHS transcripts in colonised roots may be associated with facilitation of carbon transport to the fungus, rather than a defence response to fungal invasion. However, medicarpin is toxic to a number of plant pathogenic fungi (Blount *et al.* 1992) and its

suppression in mycorrhizal roots may be related to facilitation of mycorrhizal colonisation.

The available data show that plant defence genes are expressed in close association with specific developmental stages during mycorrhizal colonisation. It is therefore not surprising that confusing data was initially obtained from root samples that would have contained an array of infection units at differing stages of development and containing different fungal structures. More accurate timing of colonisation and localisation of both gene transcripts and translation products have clarified the interpretation of molecular studies on defence responses by mycorrhizal plants.

Much information has been obtained through approaches targeting defence related genes and proteins. We now know that these types of genes are expressed slightly and transiently upon colonisation, and that they are expressed in different cell types in the roots. Mycorrhizal researchers are now starting to apply targeted approaches to other aspects of the symbiosis such as nutrient exchange.

1.6 Nutrient Transport in Mycorrhizas

Mineral transport processes in plants are currently undergoing extensive investigation with the questions being asked relating to the types of transport proteins and solutes involved in nutrient exchange between mycorrhizal symbionts. The main solutes involved in VAM symbiosis are phosphate moving from the fungus to the plant, and an as yet unidentified carbon source being obtained by the fungus from the plant. The cloning of phosphate and hexose transporters is currently of much interest. As more of these genes become available, *in situ* hybridisation and immunolocalisation should enable the identification of proteins involved in nutrient transfer between the symbionts and the biochemical form in which nutrients are exchanged.

1.6.1 Carbon Transport

The photosynthetic plant tissues fix carbon to form sucrose. This is loaded into the phloem and transported to heterotrophic tissues such as the root. Carbon may be unloaded as either sucrose, or as its substituent sugar monomers, glucose and fructose (Sonnewald & Willmitzer 1992). It is unclear how the mycorrhizal fungi access organic carbon in the root cortex. Harrison (1996) cloned a hexose transporter gene from *Medicago truncatula* (Mtst1). When the gene was

expressed in *Saccharomyces cerevisiae,* an increase in glucose and fructose uptake over that of control *S. cerevisiae* was observed. This contrasted to sucrose uptake, which did not change in the transgenic yeast. The expression of this gene increased two to four fold in roots after colonisation by a mycorrhizal fungus. *In situ* hybridisation showed the transcript was expressed in cortical cells that were in close association with the fungus i.e. those cells containing arbuscules or cells adjacent to intercellular hyphae. As the Mtst1 protein was shown to be involved in uptake of hexoses, the protein is unlikely to be involved in transfer of carbon to the fungus (which would require a transporter involved in export) but could be controlling the level of hexoses in the apoplastic regions where the fungus in growing.

NMR data has shown that ^{13}C-glucose applied exogenously to mycorrhizal roots can be absorbed by the fungus (Shachar-Hill *et al.* 1995). Work by Saito (1997) also concluded that glucose was likely to be the form of carbon transferred to the fungus. The enzymes succinate dehydrogenase, trehalase, hexokinase and phosphofructokinase, all implicated with glucose metabolism, had greater activity in mycorrhizal roots, as well as activity in isolated intercellular fungal membranes. Glucose does appear to be the likely source of carbon transferred to the fungus, however the cloning of sugar exporter genes has not yet been accomplished in any species, so that probes are not available to facilitate the identification of proteins involved in carbon export from the plant to the interfacial apoplast, from which absorption by the fungus could occur.

1.6.2 Phosphate Nutrition

Improved phosphate nutrition is the predominant benefit to plants associated with mycorrhizal fungi (Smith and Smith 1996). Kinetic studies of phosphate uptake are useful in classifying uptake mechanisms and the cloning and subsequent molecular characterisation of phosphate uptake proteins have the ability to further confirm the kinetic studies.

Phosphate is an essential element for plant growth, being involved in a variety of essential metabolic processes (Marschner 1995). Therefore, its uptake and subsequent transport throughout the plant are undergoing much investigation. Phosphate is thought to be taken up by plants from the soil in the form of $H_2PO_4^-$ due to the good correlation between the effects of pH on both the rate of inorganic phosphate (Pi) uptake and the relative concentration of $H_2PO_4^-$ (Bieleski 1973). Ullrich-Eberius *et al.* (1984) observed a phosphate-induced membrane

depolarisation in *Lemna gibba*, consistent with energised transport across the membrane.

Transport is regulated at several different points in non-mycorrhizal plant roots. Initial uptake into the symplast is likely to occur at the epidermis or outer cortical cells. Poirier *et al.* (1991) characterised mutant *Arabidopsis thaliana* that showed an inability to transport phosphate to the shoot, and as root phosphate levels were unaltered, it was concluded that the mutant was deficient in activity of a protein required to load phosphate into the xylem. Compartmentalisation of Pi into the vacuole is an important mechanism for regulating cytosolic Pi concentrations, with the Pi stored in the vacuole, from which it is released under cytosolic Pi deficiency (Lee *et al.* 1990).

Plants have the ability to increase their capacity to absorb phosphorus in response to phosphate deficiency (Bowen 1970). Clarkson and Scattergood (1982), using tomato and barley showed that after addition of phosphate to phosphate stressed plants, uptake increased dramatically over the first two days, thereafter returning to control levels. Lee (1982) showed that the K_m for phosphate uptake in barley is little affected during phosphate stress, and increased uptake is probably the result of a larger V_{max}. As the phosphate transport systems become slowly repressed after phosphate resupply, it is expected that control may be exerted via turnover of carrier sites rather than a feedback or allosterically regulated mechanism.

A kinetic study of phosphorus uptake by vesicular-arbuscular mycorrhizal fungal germ tubes revealed the presence of two transport systems (Thomson *et al.* 1990). A high affinity system operated at a K_m 1.8-3.1 µM with a V_{max} of 3.1-3.6 nmol mg protein^{-1} h^{-1}. A low affinity system had a K_m of 10200-11300 µM and a V_{max} of 445-895 nmol mg protein^{-1} h^{-1}. This dual system for phosphate uptake has also been observed in plants (Clarkson 1984), yeast (Borst-Pauwels 1981) and other fungi (Burns and Beever 1977) with K_m values in the range of 1-10 µM and 100-1000 µM for the high and low affinity systems respectively.

The kinetic studies in plants and fungi have been further enhanced by molecular genetic work on the phosphate uptake (PHO) system in the yeast *Saccharomyces cerevisiae*. The *PHO* system involves a group of structural and regulatory genes, the products of which mediate phosphate uptake in relation to intracellular phosphate levels (Tamai *et al.* 1985). Integral plasma membrane proteins facilitate the uptake of phosphate in a biphasic manner. Bun-ya *et al.* (1991) showed that the product of the *PHO84* gene catalyses the high affinity transport of phosphate

in this system. Transport of a monovalent phosphate anion is coupled with the co-transport of 2-3 protons. Further studies have revealed that PHO84 activity is modulated by a protein encoded by the *GTR1* gene, although the mode of interaction is yet to be determined (Bun-ya *et al.* 1991).

Other high affinity phosphate transporters from a mycorrhizal fungus *Glomus versiforme* (Harrison & van Buuren 1995), *Arabidopsis thaliana* (Muchhal *et al.* 1996) and *Solanum tuberosum* (Leggewie *et al.* 1997) have been cloned and show sequence similarity to *PHO84*. Phosphate transporters from *Neurospora crassa* (Versaw 1995) have also been cloned through complementation of a yeast mutant deficient in high affinity phosphate permease activity. Through the molecular characterisation of these genes, an understanding of their structure and function is being developed. These factors are further discussed in Chapter 4.

The cloning of a *Glomus versiforme* phosphate transporter, GvPT, (Harrison & van Buuren 1995) is of particular interest in this study. Transmembrane phosphate transport by the protein was demonstrated by complementation of the yeast strain NS219, which carries the *pho84* mutation, severely impairing its ability to take up phosphate. RT-PCR revealed that GvPT was expressed in the external hyphae of the fungus, implicating it in uptake of phosphate from the soil. GvPT was not expressed in the plant root. In mycorrhizal systems net transfer of P from the fungus to the plant requires efflux from the fungus to the interfacial apoplast and subsequent (active) uptake by the plant cells. This would require activity of P transporters at least in the plant, and from nutritional studies we know that the rate of transfer is of the same order as the expected uptake step (Smith *et al.* 1994).

The work of Harrison and van Buuren (1995) was the first report of the cloning and characterisation of a gene involved in nutrient uptake by VAM fungi. The application of these procedures will lead to a greater understanding of nutrient acquisition by mycorrhizal plants. Questions of particular interest include: How is phosphate transferred from the fungus to the plant? What other nutrient uptake mechanisms are involved in the VAM system?

1.7 Non-targeted Molecular Approaches

Most research investigating gene expression in VAM fungi has used targeted approaches such as those already discussed. This work is starting to characterise mechanisms involved in defence responses and nutrient transport. However a non-

targeted approach is essential in identifying symbiosis specific genes or previously characterised genes that are not predictably involved in the symbiosis.

Methods used to isolate differentially expressed genes include 1) the screening of cDNA libraries, 2) a subtractive hybridisation approach which enriches for differentially accumulated products and 3) an approach which incorporates reverse transcription (RT) coupled with the polymerase chain reaction (PCR) to amplify sections of cDNA molecules for subsequent examination (Liang & Pardee 1992). This technique is commonly called differential display (DD).

Published work using non-targeted approaches in VAM research has focused on differential screening of libraries. The differential screening of a cDNA library is achieved by producing replica filters of the library and probing them with labelled, reverse transcribed RNA from each of the two tissue types. Clones representing genes that are differentially expressed will hybridise to one probe more than the other. This approach has been used by Murphy *et al.* (1997) to isolate four genes that had increased expression in *Hordeum vulgare* roots upon mycorrhizal colonisation. Sequence analysis revealed that one of these showed a high level of sequence similarity to proton-ATPases. This supported work of Gianinazzi-Pearson *et al.* (1991b) where cytochemical lead staining indicated there was membrane bound ATPase activity at the arbuscular interface, presumably supplying the proton motive force to enable proton coupled uptake of solutes including phosphate. Burleigh and Harrison (1997) also used a differential screening approach to isolate a gene induced by P starvation in the plant.

The differential screening and subtractive hybridisation approaches are most likely to identify plant genes that are differentially expressed because the relatively small fungal biomass within the roots would reduce the chance of detecting differentially expressed fungal genes. As DD incorporates a PCR step, the ability to isolate the scarce fungal transcripts is greatly enhanced. In a molecular study, the appropriate method should be chosen on the basis of whether plant or fungal genes are being investigated.

1.8 Conclusions, Outstanding Questions and Methodological Requirements

1) Targeted molecular approaches have so far revealed that defence responses to mycorrhizal colonisation are slight and transitory. The work highlighted problems relating to progressive colonisation of roots by mycorrhizal fungi and the need for a model system in which colonisation is almost synchronous and material enriched for particular developmental stages can be obtained. Brundrett *et al.* (1985) developed an approach to this problem and the requirement for this project was to refine this method and develop a reliable system for isolation of plant material suitable for molecular research.

2) Molecular approaches to understanding mycorrhizal transport were limited when this project started. The only gene cloned from mycorrhizal systems at that stage was a plant ATPase (Murphy pers. comm.) obtained using a non-targeted approach. Since then the plant hexose transporter (Harrison 1996) and fungal phosphate transporter (Harrison & van Buuren 1995) have been cloned and preliminary studies of expression made. The picture remains incomplete with regard to understanding the integration of plant and fungal processes. More is needed on both plant and fungal transporters and the way their expression is altered and co-ordinated at different stages of colonisation.

3) Non-targeted approaches to gene cloning can identify novel or unexpected genes that are differentially regulated upon colonisation. The use of mutant plants that form abnormal colonisation is a powerful technique at identifying genes that have important roles in the symbiosis, however the bioassay is expensive in both time and resources. Other non-targeted approaches use variations of the differential screening of cDNA clones and differential display. Theoretically, differential screening of cDNA libraries is likely to isolate clones of plant origin as large changes of moderately to highly expressed genes can be detected by this method. Differential display uses a PCR step and is more likely to identify genes that are present in one tissue and not in another, regardless of the initial template concentrations. This implies that fungal genes (not present in non-mycorrhizal tissue) are likely to be detected.

4) Several different host plants have been used in previous work, without sufficient attention necessarily being paid to characteristics which will facilitate molecular/genetic characterisation of the symbiosis.

The specific aims of this project were:

1) To choose an appropriate host plant.

2) To establish a reliable method of obtaining material with synchronous colonisation.

3) To use a targeted approach to identify clones representing genes with similarity to P transporters and study their expression.

4) To screen a cDNA library developed during an honours project, utilising a non-targeted approach for "unexpected" genes.

Chapter 2

Materials and Methods

2.1 Biological Methods

2.1.1 Growing Medium

All plants were grown in a sterile 1:9 mixture of Mallala soil and washed river sand. The Mallala soil was collected from Mallala, South Australia and contained 12 mg bicarbonate extractable phosphate (P) kg^{-1} soil (Colwell 1963). High P treatment of soil was achieved by adding sodium di-hydrogen orthophosphate to the growing medium, to give a final concentration of 0.8 mmol P kg^{-1}.

2.1.2 Plants

Leeks (*Allium porrum* L. cv Musselburgh) were used to establish mycorrhizal colonisation in "nurse" pots (Rosewarne *et al.* 1997; Brundrett *et al.* 1985). Tomato (*Lycopersicon esculentum* Mill. cv 76R Peto Seed Co., California) was chosen to monitor colonisation because of its high suitability for molecular studies, being diploid, self-fertilising and having a well-mapped genome. It has also been shown to responsive to vesicular-arbuscular colonisation (Kahliel & Elkhider 1987).

2.1.3 Fungus

Inoculum of dried "pot cultures" of *Glomus intraradices* Schenck and Smith grown on *Trifolium subterraneum* L. cv Mt. Barker (supplied by S.E. Smith) colonised to between 80% and 90% by the mycorrhizal fungus was incorporated into the growing medium at a 1:9 (w/w) ratio when required.

2.1.4 Surface Sterilisation of Seeds

Seeds of *L. esculentum* and *A. porrum* were surface sterilised by immersion in 0.05% Milton antibacterial solution (0.05% available chlorine) for two min; sterile reverse osmosis water for one min; 70% ethanol for 30 sec and sterile reverse osmosis water twice for one min. Seeds were germinated on water agar at 22°C for two to five days.

2.1.5 Plant Growth Conditions

2.1.5.1 Nurse pots

Nurse pots were used to establish soil conditions that facilitated rapid, synchronous colonisation in *L. esculentum* seedlings. The method of Brundrett *et al.* (1985) was modified to achieve the colonisation of roots suitable for molecular analysis. Germinated leeks were planted in 11 cm diameter non-draining white plastic pots (Polocup) filled with 1.4 kg of growing medium containing mycorrhizal inoculum. Twelve leek seedlings were transplanted to each pot; eight evenly around the circumference, and four in a central block. Control pots were also established in which leeks were planted into uninoculated growing medium. Plants were grown for three to six weeks and were watered twice weekly to 12% of soil dry weight.

2.5.1.2 Tomatoes

Non-mycorrhizal tomatoes were grown individually in black, compartmented seedling trays (Rite Grow Kwik Pots, Pyco Horticultural Products) with each compartment containing 50g growing medium (+ or - P) without inoculum and watered daily. In one experiment (see Chapter 3) growth was monitored up to 28 d.

2.1.6 Transplantation

After 14 days the tomato seedlings were transplanted into the nurse pots. The transplantation was accomplished by gently shaking excess soil from the tomato roots and placing them in a hole in the nurse pot. Soil from the hole was used to backfill around the root system. Four tomatoes from either the low soil P pretreatment (LSPP) or the high soil P pretreatment (HSPP) were transplanted into each pot.

A "traditional" inoculation method was also used where tomatoes were transplanted into the same inoculated growing medium used to produce nurse pots.

All plants were grown in a growth room with temperature controlled to a day maximum of 22°C and a night minimum of 17°C. Lighting was provided by 400 watt Lucalux lamps (high pressure sodium supplied by General Electric) for 14 h

day^{-1}. The average irradiance was 450 µmol m^{-2} s^{-1}. A nutrient solution minus P (Smith & Smith 1981) was applied at a rate of 7 ml kg dry soil^{-1} week^{-1}.

2.1.7 Harvesting of Plants

Plants were harvested by gently washing soil off the roots with running water, lightly blotting dry, separating roots from shoots and weighing them. Shoots and roots used for nucleic acid extraction were immediately frozen in liquid nitrogen and stored at -80°C. When required, shoots were dried and the P levels determined as by the method of Hanson (1950). Roots for microscopic evaluation of mycorrhizal colonisation were placed immediately in 10% KOH.

2.1.8 Microscopic examination

Root material was cleared by immersion in 10% KOH at 55°C overnight. After rinsing several times in water, roots were stained for 90 min at 90°C in a solution consisting of 0.05% trypan blue in lacto-glycerol (a modification of the method of Phillips & Hayman 1970). After washing several times in water, roots were stored in 50% glycerol containing approximately 0.05% Tween 20. Colonisation was determined by the line intersect method of McGonigle *et al.* (1990) under 400x magnification. External hyphae, internal hyphae, arbuscules and vesicles were scored, each as a percentage of total root intersects counted. The number of entry points per root system was estimated by careful microscopic examination of randomly selected 0.1 mm root sections. Over 5 mm of roots from each root system was investigated by this method.

2.2 Molecular Methods

2.2.1 Isolation of Nucleic Acids

2.2.1.1 Lambda DNA Preparation

A number of different procedures are available to isolate DNA from lambda cloning vectors. The following procedure gave good, consistent yields that were not dependant on the complete lysis of the bacterial starter culture.

A bacterial culture of *E. coli* C600*hfl* was grown in 5 ml LB (Appendix 1) supplemented with 10 mM $MgSO_4$, until the bacteria reached an OD_{600} of 0.4-0.6. A 100 µl aliquot of the starter culture was mixed with 50 µl of phage and inoculated into 5 ml LB containing 10mM $MgSO_4$ and grown overnight.

To this culture, 100 µl of chloroform was added and mixed. A 1 ml aliquot was taken and centrifuged at 4000 rpm for 10 min in an microcentrifuge. The supernatant (800 µl) was transferred to a fresh tube, 2 µl DNase (2 mg/ml) was added and the solution incubated at 37°C for 30 min. Diethylpyrocarbonate (DEPC) (2 µl) was added and mixed, followed by the addition of 200 µl of a solution containing 1M Tris; 1% SDS; 0.1M EDTA, pH 8.5. Solutions were mixed and incubated at 70°C for 5 min. 100 µl 5M potassium acetate was added, the solutions mixed again and incubated on ice for 30 min. Samples were centrifuged in a microcentrifuge for 15 min at 12000 rpm. The supernatant was collected and DNA precipitated with 500 µl isopropanol for 5 min at room temperature. The centrifugation was repeated, supernatant removed and the pellet resuspended in 100 µl TE buffer (Appendix 1) containing 0.2% DEPC. Solutions were incubated at room temperature for 5 min and heated to 65°C for 10 min. DNA was reprecipitated with the addition of 1/10 volume 3M sodium acetate, pH 5.2 and 2 volumes of ethanol. Samples were centrifuged for 15 min in a microcentrifuge, pellets washed briefly in 70% ethanol, dried and dissolved in 40 µl sterile water. Enough DNA was isolated for approximately 4 restriction enzyme digests (~1 µg).

2.2.1.2 Bacterial Plasmid Isolation

Standard alkaline lysis methods (Sambrook *et al.* 1989) were used for small and large scale bacterial plasmid DNA isolation.

2.2.1.3 Plant DNA Isolation

Total plant DNA was isolated from frozen leaf tissue using the minipreparation method outlined below.

Several leaves were frozen in liquid nitrogen, placed in a 1.5 ml microcentrifuge tube and crushed with a knitting needle to a fine powder. The crushed leaf was mixed with 600 µl DNA extraction buffer (Appendix 1) and vortexed vigorously. The DNA was extracted with phenol/chloroform, then chloroform and precipitated with isopropanol (Section 2.2.5). The samples were incubated for 1 min at room temperature and DNA pelleted by centrifuging for 5 min in a microcentrifuge. The supernatant was removed and the DNA pellet washed in 70% ethanol, dried then dissolved in 50 µl of TE. RNA was degraded by the addition of 4 µl RNase A (400 µg/ml) and incubated for 1 hr at 37°C. The DNA was stored at -20°C.

2.2.2 RNA Extraction

RNA was extracted from roots and leaves of both mycorrhizal and non-mycorrhizal plants using a method adapted from Logeman *et al.* (1987) by Delp *et al.* (1997). Briefly, the frozen root material was ground in liquid nitrogen and dissolve in a Guanidium-based extraction buffer followed by a phenol/chloroform extraction and an isopropanol precipitation. The RNA was dissolved in water and reprecipitated with LiCl and again dissolved in water. If the RNA was to be used for reverse transcription reactions, it was first treated with RNase free DNase 1 (Promega).

2.2.3 cDNA Library Screening

2.2.3.1 cDNA Library Production

Complementary DNA (cDNA) libraries were synthesised during an honours project carried out by this author (Rosewarne 1993). A Lambda gt10 vector (Pharmacia LKB Biotechnology) was used to produce the library. Briefly, total RNA was extracted from both uncolonised tomato roots and roots at a very early stage of VA mycorrhizal colonisation (28% total colonisation, 4% arbuscular colonisation), enabling the production of two cDNA libraries. The mRNA was extracted using the polyAtract mRNA isolation System (Promega, Maddison, WI, USA) according to the manufacturers' instructions. The cDNA synthesis and library production was completed using the cDNA Synthesis Kit from Pharmacia LKB Biotechnology, according to the manufacturers' instructions. Both libraries contained approximately 2×10^6 plaque forming units.

2.2.3.2 Plating of Phage

A single colony from a freshly streaked LB plate of *E. coli* C600*hfl* was used to inoculate 3 ml of LB medium supplemented with 0.2% maltose and 10mM $MgSO_4$. The culture was incubated with shaking at 37°C until the OD_{600} reached 0.6-0.8. An aliquot of the cDNA library was mixed with 100 µl of the bacterial culture and incubated at 37°C for 30 min. This was then mixed with either 3 or 8 ml of molten (45°C) TB top agarose (Appendix 1) and poured onto a 9 cm or 15 cm LB agar (Appendix 1) plate respectively. Once the TB top agarose had set, the plate was inverted and incubated at 37°C. Plaques were pinpoint in size after 5-6 hr incubation, and suitable for coring after an overnight incubation.

2.2.3.3 Preparation of Replica Membranes for Library Screening

Before plating the libraries for screening, the titre of the libraries was determined by using a 1:1000 dilution of the phage (in SM buffer, Appendix 1) and plating 100 µl as described above. An appropriate volume of the library was then plated out in the same way for the screening process. The clones were allowed to grow to pinpoint size before being transferred to replica filters.

Hybond N+ positively charged nylon membranes (Amersham Australia, Castle Hill, NSW) were used to produce the replica membranes using the protocol recommended in the suppliers' manual. Two replica membranes were produced from each plate, the first membrane was left in contact with the agar surface for 1 min, the second replica membrane left in contact with the agar surface for 2 min. The DNA was fixed to the membrane using the alkali fixation protocol recommended by Amersham, exposing the membrane to the alkaline conditions for 20 min.

2.2.3.4 Differential Screening of cDNA Libraries

The differential screening of the cDNA libraries involved the production of probes from mRNA isolated from mycorrhizal and non-mycorrhizal roots which were hybridised to the replica membranes. After hybridisation the membranes were stringently washed (2.2.7.8) and exposed to x-ray film. Clones that hybridised differentially to the probes were selected and rescreened with a second hybridisation. These processes have previously been described by Delp *et al.* (1997).

2.2.3.5 Purification of Clones

Plaques that contained DNA which differentially hybridised to the probes were cored from the plate, using a sterile pasteur pipette dipped into SM buffer. The agar plug was then expelled into 100 µl of SM buffer. This was left at room temperature for 30 min to allow phage particles to diffuse into the buffer. The eluted phage was used for replating, inoculation of a broth for DNA isolation or directly in a PCR reaction to amplify the insert.
After the second screening, the clones were further purified by replating the phage eluate and a number of isolated plaques were cored and used for PCR amplification.

2.2.3.6 PCR Amplification of Inserts

Inserts were amplified using primers (gt10f, gt10r; Appendix 2) designed to anneal to the vector either side of the *Eco*R1 restriction enzyme cloning site. After elution of phage particles, 5 µl of eluate (template) was boiled for 5 min and placed on ice for 5 min. The reaction was completed in a volume of 30 µl and comprised deoxynucleotide triphosphates (dATP, dGTP, dTTP, dCTP) at 167 µM each, forward and reverse primers at 3 ng/µl each, 1X PCR buffer IV and 0.5 units *Taq* polymerase (buffer IV and *Taq* polymerase from Advanced Biotechnologies, London, UK). The PCR was carried out with the following incubations in a GeneAmp PCR System 9600 thermocycler (Perkin Elmer, Norwalk, CT, USA): 95°C for 4 min; 35 cycles of 94°C for 1 min, 55°C for 2 min, 74°C for 2 min; finishing with 74°C for 5 min. A 5 µl aliquot was then analysed by agarose gel electrophoresis.

2.2.4 Sub-cloning

2.2.4.1 Vector Preparation

DNA fragments were subcloned into the vectors pGEM-3zf+ (Promega) or pZERO (Invitrogen, San Diego, CA, USA). The vector pGEM-3zf+ was used for both blunt ended and 5' overhang ligations, and pZERO used for blunt ended ligations only. Vectors were prepared by cutting 2 µg with the appropriate restriction enzyme in a 100 µl volume at 37°C for 2 hr. Where the restriction enzyme left a 5' overhang, the vectors were dephosphorylated with Calf Intestinal Phosphatase (CIP). This reaction was carried out in a volume of 112 µl using 10x CIP buffer and CIP enzyme supplied by Promega. The reaction was incubated at 37°C for 30 min. CIP stop buffer (Appendix 1) was added to 400 µl. The DNA was extracted with phenol/chloroform and precipitated with ethanol (Section 2.2.5).

2.2.4.2 Insert Preparation

DNA used for cloning was purified by gel electrophoresis (Section 2.2.6) and, if it was to be used in a blunt ended ligation, treated with DNA polymerase 1 large (Klenow) fragment (Promega) to end-fill the fragments. Klenow treatments were carried out in a 20 µl volume in Klenow buffer (Appendix 1) using 5 units of Klenow enzyme. The reaction was incubated for 10 min at room temperature and the enzyme inactivated by heating to 75°C for 10 min.

2.2.4.3 Ligation

The concentrations of the inserts were calculated by comparison with known amounts of marker DNA in ethidium bromide-stained agarose gels, following electrophoresis. The concentrations were converted to molarities and ligation reactions set up with vector and insert at a 2:1 molar ratio. The ligation was effected with T4 DNA ligase (Promega) in 1x buffer supplied by the manufacturer. The reaction mixture was incubated at 16°C for 4 hr.

2.2.4.4 Transformation

Ligated DNA was transformed into 50 µl DH5α or TOP10F' (Invitrogen) competent cells either prepared fresh or from a frozen stock (Section 2.2.4.5). All of the ligation mixture was added to the competent cells. Solutions were kept on ice for 10 min, then heat shocked for 50 sec at 42°C. The solutions were put back on ice for 2 min and 900 µl LB added. Samples were incubated at 37°C with shaking for 1 hr. Two LB agar plates containing the appropriate antibiotics were overlayed with either 100 µl or 860 µl of the transformation mix. Plasmid vector pGEM3zf+ enabled blue/white colour selection, and transformation mixes with this plamid were spread on the ampicillin plates (50 µg/ml) that had 40 µl 24 mg/ml IPTG and 50 µl 2% X-Gal previously overlayed. The pZERO vector was selected with 50 µg/ml Kanamycin and 1mM IPTG.

2.2.4.5 Competent Cells

Competent cells were prepared by inoculating 200 ml SOB (Appendix 1) with 2.5 ml of an overnight culture of DH5α or TOP10F' cells. Cultures were grown at 37°C with shaking for 3-4 hr until the OD_{600} reached 0.5. The broth was equally split between 8, 50 ml centrifuge tubes and incubated on ice for 1 hr. The tubes were centrifuged at 2,000 g for 15 min at 4°C. The supernatant was decanted and the pellets suspended in a total volume of 10 ml FSB (Appendix 1) and held on ice for 10 min. The centrifugation was repeated and the pellets drained and resuspended in 1.8 ml FSB. 65 µl DMSO was added dropwise to each tube and incubated on ice for 15 min. A further 65 µl DMSO was added followed by another 15 min incubation on ice. Cells were separated into 50 µl aliquots in cold microcentrifuge tubes before being frozen in liquid nitrogen and stored at -80°C.

2.2.5 Extraction and Ethanol Precipitation

To extract proteins from nucleic acid samples, an equal volume of phenol/chloroform/isoamylalcohol (IAA) (25:24:1) was added to the sample and mixed by inversion for 5 min. Samples were then centrifuged for 5 min in a microcentrifuge at 15000 rpm. The upper aqueous phase was collected to a fresh tube and an equal volume of chloroform/IAA (24:1) was added and mixed by inversion for 5 min. Samples were again centrifuged (as above) and upper aqueous phase transferred to a fresh microcentrifuge tube. DNA was then precipitated by adding 1/10 volume 3M sodium acetate pH 5.2 and either ethanol (2 volumes) or isopropanol (0.6 volume). The precipitate was incubated at -20°C before centrifuging in a refrigerated (4°C) centrifuge at 15000 rpm for 15 min. The supernatant was removed and the pellet washed with cold 70% ethanol, vacuum dried and dissolved in an appropriate volume of sterile distilled water.

2.2.6 DNA Extraction from Agarose Gels

DNA was separated by non-denaturing agarose gel electrophoresis (Section 2.2.7.1) and the band of interest cut from the gel with illumination by long wavelength UV light. Extraction of the DNA was completed with the "Bresaclean DNA Extraction Kit" according to the manufacturers instructions (Bresatec, Adelaide, SA, Aust.).

2.2.7 Gel Blot Hybridisation Studies

2.2.7.1 Non-denaturing Gel Electrophoresis

DNA was separated under non-denaturing conditions in horizontal submarine agarose gel units (Hoefer Scientific Instruments, San Fransisco, USA, Models HE99X and HE33). Gels were made from agarose (Promega LE, analytical grade) ranging in concentration from 0.7 to 1.5%, melted in 1x TAE buffer (Appendix 1), and run in 1x TAE buffer. DNA samples were mixed with 10x DNA loading buffer (Appendix 1) before loading into wells. After electrophoresis, gels were stained in ethidium bromide (1 μg/ml) for 30 min at room temperature and destained in water for 30 min. DNA was visualised with UV light and photographed using a Polaroid Land Camera.

2.2.7.2 Denaturing Gel Electrophoresis

RNA samples were electrophoresed in denaturing conditions using a submarine agarose gel unit (Hoefer Scientific Instruments). RNA (made up to 3.5 μl) was denatured by adding 2 μl 10x MOPS (Appendix 1), 10 μl deionised formamide, 3.5 μl 37% formaldehyde and heated to 65°C for 10 min. Samples were cooled on ice and 1 μl RNA loading buffer (Appendix 1) added. Samples were loaded into 1.2% agarose gels in 1x MOPS (from 10x stock) and 1.1% formaldehyde. The gel running buffer was 1x MOPS.

2.2.7.3 RNA and DNA Gel Blotting

Both RNA and DNA separated by gel electrophoresis were transferred to hybond N+ nylon membranes (Amersham) according to the manufacturers' instructions. Fixing of nucleic acid to membranes was accomplished by exposure to UV light for 5 min.

2.2.7.4 DNA Radio-labelling for Hybridisation Studies

Probes were prepared from DNA extracted from an agarose gel (Section 2.2.6). Approximately 100 ng of template DNA was mixed with 3 μl of 6mer random primer (Promega) and made up to 7.5 μl before heat denaturing by boiling for 5 min and cooling in an ice water bath for 5 min. To this, 12.5 μl of 2x reaction buffer was added and the sample briefly centrifuged. The radiolabelled nucleotide, α-^{32}P-dCTP, was added (3 μl) followed by the addition of 1 μl of Klenow enzyme (5U/μl) and the sample was incubated at 37°C for 1 hr.

2.2.7.5 Purification of Radio-labelled DNA

Synthesised probes were separated from unincorporated nucleotides by passing the probe through a column prepared as follows: A sterilised 6 inch pasteur pipette, with a small amount of glass wool inserted at the constriction, was filled with a slurry of sephadex G-100 in TE buffer and washed through with several volumes of TE. The probe was added to the top of the column and eluted with TE. Radioactivity was monitored in the eluate and the first of two peaks were collected. This first peak contained DNA with incorporated α-^{32}P-dCTP, whereas the second peak was the unincorporated α-^{32}P-dCTP.

2.2.7.6 DNA Gel Blot Hybridisation

Membranes were prehybridised in a 10 ml solution of DNA prehybridisation solution (Appendix 1) for at least 1 h. The labelled probe was heat denatured (boiled 5 min, ice water bath 5 min) and added directly to the prehybridised membranes. Hybridisation proceeded for 12-16 hr at 65°C. Both the prehybridisation and hybridisation took place in Ratek hybridisation bottles (Ratek Instruments, Boronia, Vic., Aust) in a Ratek hybridisation oven with constant rotation.

2.2.7.7 RNA Gel Blot Hybridisation

Membranes were prehybridised in a 10 ml solution of RNA prehybridisation solution (Appendix 1) for at least 4 hr. The labelled probe was heat denatured (boiled 5 min, ice water bath 5 min) and added directly to the prehybridised membranes. Hybridisation proceeded for 40-48 hr at 42°C. Both the prehybridisation and hybridisation were carried out in hybridisation bottles and a rotating hybridisation oven with constant rotation.

2.2.7.8 Membrane Washing

After hybridisation, the membrane was removed and washed twice with constant agitation in 2X SSC (Appendix 1); 0.1% SDS for 5 min at room temperature, twice again in 2X SSC; 0.1% SDS for 15 min at 65°C, twice in 1X SSC; 0.1% SDS for 15 min at 65°C, and twice in 0.1X SSC; 0.1% SDS for 15 min at 65°C.

2.2.7.9 Hybridised Signal Detection

The membranes were sealed between plastic sheets and exposed to x-ray film between intensifying screens at -80°C, with the film being developed in a Curix 60 (Agfa) autodeveloper. Alternatively, the membrane was exposed to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA, USA), with the signal detected on a STORM 860 scanner and visualised with Imagequant software (Molecular Dynamics).

2.2.7.10 Stripping of Membranes

Membranes were stripped by incubating in a freshly boiled solution of 0.1% SDS and 2mM EDTA, with shaking for 30 min. This was repeated and the membrane exposed to a phosphoimager screen to confirm complete stripping.

2.2.8 Sequence Analysis

DNA sequencing was performed using the dideoxy method (Sanger *et al.* 1977) in an Applied Biosystems Model 373A automatic sequencer at the Special Research Centre for Plant Biology, University of Adelaide. Double stranded plasmid DNA was purified as previously described (Section 2.2.1.2). The concentration was determined by UV absorption and 2.5 μg analysed per sequencing run. Electronic sequence data was analysed with Lasergene software (DNASTAR Inc., Maddison, WI, USA) on a Macintosh Power PC.

2.2.9 5' RACE (Rapid Amplification of cDNA Ends)

The 5' RACE procedure (Life Technologies, Gaithersberg, MD, USA) was utilised to obtain the full length mRNAs, following the manufacturers instructions. The gene specific primers GSP1 and GSP2 were designed from the original cloned sequence (Appendix 2). Primers were checked for dimer and loop formation with the Lasergene software.

The cDNA was synthesised using GSP1 as the primer. After cDNA synthesis, the sample was treated with RNase A and the cDNA was then purified with a Glassmax DNA Isolation Spin Cartridge. The purified cDNA was 3' tailed with poly-cytosine molecules and a product amplified with the primers GSP2 and Abridged Anchor Primer (AAP, Appendix 2) in a PCR reaction according to the 5' RACE Kit instructions.

A secondary PCR amplification using GSP2 and the Abridged Universal Amplification Primer (AUAP) was completed. The method used was different from that recommended by the supplier and is outlined below. The primary PCR product was diluted 1:100 with water and 5 μl was used as the template. The reaction contained 0.2 mM of each dNTP, 2 mM MgCl2, 0.5 mM of each primer and 0.5 μl *Taq* polymerase (0.5U) in 1x reaction buffer IV (Advanced Biotechnologies) in a final volume was 20 μl. The PCR was carried out with the following incubations in a Perkin Elmer thermocycler: 95°C for 4 min; 35 cycles

of 94°C for 1 min, 55°C for 2 min, 72°C for 2 min; finishing with 72°C for 5 min. The resulting product was separated by non-denaturing agarose gel electrophoresis. The gel was blotted and hybridised to the original clone to determine whether the PCR products had homology to the original sequence. To clone the PCR product, the DNA was gel purified (Section 2.2.6), and subcloned into pZERO (Section 2.2.4).
Plasmid DNA was prepared from the clones (Section 2.2.1.2), then the inserts were excised with the appropriate restriction enzyme and separated by agarose gel electrophoresis. The gel was blotted and hybridised to the original clone to confirm that fragments had significant sequence similarity to the 3' portion of the known sequence before any were selected for sequencing.

2.2.11 *In situ* Hybridisation

2.2.11.1 Probe Preparation

Clones were cut with restriction enzymes to enable the production of transcripts of both the sense and anti-sense strands from the SP6 and T7 transcription initiation sites in the vector. DNA was extracted and ethanol precipitated (2.2.5). RNA probes were synthesised with the DIG RNA Labelling Kit (Boehringer Mannheim Australia, Castle Hill, NSW, Aust.) according to the manufacturers instructions. The template was removed by the treatment with RNase-free DNase 1 and the probes were chemically hydrolysed. This hydrolysis was completed with freshly prepared hydrolysis buffer, mixed from stock solutions (Appendix 1) to give a final concentration of 60 mM Na_2CO_3 and 40 mM $NaHCO_3$ and incubated at 60°C for the appropriate time. The time of incubation was determined according to the following formula:

$t = (Lo-Lf) / (k).(Lo).(Lf)$

Lo = Starting Length (kb)
Lf = Final Length (kb)
k = 0.11

The Lf required was between 0.075 and 0.1 kb.

The reaction was stopped by transferring the tubes to ice and adding 3 μl 3M sodium acetate and 5 μl 10% glacial acetic acid. 1 μl of each reaction was examined for degradation by agarose gel electrophoresis. The RNA was precipitated with 1 μl 10mg/ml tRNA, 8 μl sodium acetate and 250 μl ethanol at -80°C overnight. After centrifugation (Microcentrifuge, 15 000 rpm, 15 min,

4°C), the pellet was washed in 70% ethanol and dissolved in 25 μl TE. The RNA was quantified by spotting a dilution series of the probe onto nylon membrane, UV-fixed for 5 min and detected using the DIG Nucleic Acid Detection Kit according to the manufacturers instructions (Boehringer Mannheim).

2.2.11.2 Fixation of Plant Material

Plant roots were washed so as to remove any soil and cut into 1 cm lengths before being placed into scintillation vials and immersed in 4 ml of fixative solution (Appendix 1). Roots were vacuum infiltrated with fixative for 30 min, the vacuum released and reapplied for a further 10 min. Samples were dehydrated under vacuum with the following washes: 2 x 30 min washes in 0.05M Na cacodylate; 1 x 30 min wash in each of the following ethanol solutions; 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, and 70%.

Dehydrated roots were wrapped in a kimwipe tissue and inserted into plastic embedding containers and infiltrated overnight in a Shandon Citadel Tissue Processor (Shandon Southern Products Ltd., Cheshire England) wax embedding machine with the following incubations:

70% Ethanol	1h
80% Ethanol	1h
95% Ethanol	30 min
95% Ethanol	1h
Abs. Ethanol	2h
Abs Ethanol	2h
Histoclear/Ethanol 50%/50%	1h
Histoclear	2h
Histoclear	2h
Wax (Histoplast)	2h
Wax (Histoplast)	2h

The processed samples were mounted in wax blocks using a Tissue-Tek Tissue Embedding Centre (Ames Miles, Indiana USA). After mounting, 8 μm thick sections were cut to form a ribbon with a Leica 2055 Autocut Microtome (Jung, Heigleberg, Germany). The wax ribbon was layered onto a 45°C waterbath, floated onto silicone coated slides (Sigma) and incubated at 45°C overnight.

A staining dish was filled with xylene and the slides immersed in the xylene with constant agitation for 10 min. This was repeated once with fresh xylene. Four

staining dishes were set up with the following ethanol solutions; 100%, 100%, 70%, 50%. The slides were removed from the xylene and placed in the first 100% ethanol solution, dipping up and down approximately 15 times. This was repeated for each of the ethanol solutions. Finally the slides were dried at room temperature for 20 min.

2.2.11.3 Proteinase K Treatment

A solution of 100mM Tris (pH 7.5) and 50mM EDTA was prewarmed in a staining tray to 37°C. Proteinase K was added to a final concentration of 1 µg/ml. Slides were placed in the solution and incubated at 37°C for 15 min. The slides were removed and washed twice in 2X SSC and once in water before being dried on a 65°C hotplate.

2.2.11.4 Acetylation Reaction

Slides were equilibrated in 100mM triethanolamine pH 8.0 in a staining dish. A second staining dish was set up containing 500 ml of 100mM triethanolamine with a stir bar at the bottom. The solution was stirred quickly and 1.25 ml acetic anhydride was added and mixed for 5 sec. The slides were added and incubated at room temperature for 5 min. This acetylation reaction was repeated once.

2.2.11.5 Dehydration

Slides were washed twice (5 min each wash) in 2x SSC before being dehydrated through a series of ethanol washes as follows; 30%, 50%, 70% and 100%. The slides were air-dried for 30 min.

2.2.11.6 Hybridisation

Enough *in situ* hybridisation solution (Appendix 1) was made to add 150 µl to each slide. The probe was added to the hybridisation solution to give a final concentration of 0.25 ng/µl, heated to 70°C and quickly chilled on ice. The hybridisation solution was warmed to 42°C, 140 µl added to each slide and gently spread over the surface of the slide with the side of a Gilson yellow pipette tip. Slides were gently covered with coverslips and incubated at 45°C overnight in a sealed box containing filter paper soaked in 50% Formamide and 300 mM NaCl.

Coverslips were removed by holding slides lengthwise in RNase buffer (no RNase A) until the coverslips sliped off. The slides were briefly rinsed in RNase buffer

and incubated at 37°C for 30 min in fresh buffer containing 25 μg/ml RNase A. Washes to remove unbound probe were as follows: 2X SSC at 42°C for 1 hr with gentle shaking, 1X SSC at 42°C for 1 hr with shaking, 0.5X SSC at room temperature (RT) for 30 min without shaking, 0.2X SSC at RT for 30 min without shaking. The slides were rinsed in PBS (Appendix 1) and stored overnight at 4°C.

2.2.11.7 Colour Detection

Colour detection was carried out using a modification of the DIG Nucleic Acid Detection Kit (Boehringer Mannheim). Slides were incubated for 5 min in DIG buffer 1 (Appendix 1) followed by a 1 hr incubation in DIG buffer 2 (Appendix 1). The antibody conjugate was mixed in buffer 2 (1:2500) and 300 μl was dropped onto each slide and incubated with gentle shaking for 30 min. Two 15 min washes with buffer 1 were then carried out on the slides followed by a brief wash in DIG buffer 3 (Appendix 1). A solution of 0.34mg/ml nitroblue tetrazolium and 0.175mg/ml 5-bromo-4-chloro-3-indoyl phosphate toludinium salt in DIG buffer 3 was added to each slide (150 μl), covered with a cover slip and allowed to incubate in a humid chamber in the dark.

When the signal had developed to an appropriate strength, the coverslips were removed by soaking in TE for 5 min and the sections dehydrated by passing through an ethanol series with a 30 sec incubation in each of the following solutions: 30%, 50%, 70%, 90%, and 100% ethanol. The slides were dried under vacuum and mounted with 5 drops of Biomount Mounting Medium (Goldmark Biologicals). Coverslips were gently laid on top and slides allowed to sit flat for 48 hr.

Slides were examined and photographed under either bright field or differential interference with a Zeiss Axioplan Photomicroscope (Zeiss, Germany) using Kodak 160T film.

Chapter 3

Synchronous Colonisation of *Lycopersicon esculentum* by the Vesicular Arbuscular Mycorrhizal Fungus *Glomus intraradices*

3.1 Introduction

Developmental studies of the vesicular-arbuscular mycorrhizal (VAM) symbiosis are complicated by the nature of the colonisation process. A typical mycorrhizal root system contains infection units at a variety of different stages of development (Smith & Read 1997), making molecular studies of different colonisation stages difficult. Many molecular techniques also require highly colonised roots to discriminate changes in gene expression. To achieve this level of colonisation in a natural environment, the roots must be grown with inoculum over a period of weeks, precluding any synchrony in mycorrhizal development. Previous attempts to overcome the problem include using very large amounts of dried, pot culture inoculum (Murphy *et al.* 1997) and an axenic system for cotton (Ridgeway *et al.* 1994). Neither method fully overcame the problem. As discussed in the Introduction, a model colonisation system that ensured rapid, synchronous colonisation is a pre-requisite to effective molecular studies of colonisation.

Brundrett *et al.* (1985), in a microscopic developmental study of the time course of colonisation, developed near synchronous colonisation by transplanting 5-7 day old leek seedlings into established pot cultures consisting of one-year-old mycorrhizal 'nurse' leek plants. Fungal hyphae growing from the mature leek roots rapidly colonised the leek seedlings at numerous sites simultaneously. External hyphae contacted the roots within one day after transplantation, with penetration of hyphae into the roots occurring by the following day. Arbuscules formed within 3-4 days and vesicles by 4-5 days. This appears to be an extremely efficient inoculation procedure. However there are some drawbacks in utilising it for the generation of plant material for molecular investigations. The age and repeated use of the same nurse pots can lead to problems with pathogens and the nurse plants become root bound, making harvesting of roots of test plants difficult. Studies of gene expression require the exclusion of pathogens and a harvesting process that is rapid and gentle is essential because disease and wounding are known to induce expression of defence genes, which might obscure expression of important genes involved in the symbiosis. In addition, the amount of root material obtained from very young seedlings, while suitable for microscopic studies, may be insufficient for molecular studies. The primary aim

of the experiments described in this chapter was to develop a method of inoculation which can produce abundant, synchronously colonised root material to be used in molecular research. The work followed up a preliminary study of the use of nurse pots carried out during an Honours project (Rosewarne 1993).

The rapid, synchronous colonisation of roots is a useful tool in the study of many aspects of mycorrhizal colonisation. One aspect that has generated much debate in the past concerns the low colonisation observed in plants grown under phosphate (P) sufficient conditions. Synchronous colonisation methods would allow determination of stages at which mycorrhizal colonisation is inhibited in P sufficient plants. This was a secondary aim of the experiment.

The stages of colonisation that may be affected when plants grow in high P soil are:

1) hyphal growth through the soil,
2) formation of entry points,
3) intercellular hyphal growth and
4) the length of the lag phase between primary entry point formation and secondary entry point formation.

Amijee *et al.* (1993) investigated primary colonisation in *Allium porrum* L. and *Trifolium repens* L. grown in high and low P soil. Plants grown in high P soil took longer to form primary colonisation units, and this was attributed to a delay in the root encountering fungal hyphae and the subsequent fungal penetration into the root cortex. Jasper *et al.* (1979) noted that decreased VAM colonisation after P addition was correlated to the P status of the plant and not to direct effects of soil P on VAM fungal growth. Schwab *et al.* (1983) found no changes in the rate of intercellular hyphal growth between high and low P treated sudan-grass, with lower colonisation of high P plants being the result of fewer secondary colonisation units. This contrasts with the results of Amijee *et al.* (1989) and Braunberger *et al.* (1991) who concluded that colonisation was reduced in high P plants primarily due to a reduction in intercellular hyphal growth. Bruce *et al.* (1994) also found a reduction in intercellular hyphal growth, no change in the number of primary entry points, but (in agreement with Schwab *et al.* 1993) a large reduction in the number of secondary entry points in P sufficient plants.

The complex nature of the colonisation process makes analysis of different stages difficult and may have contributed to the apparently conflicting observations. Carefully timed harvests are required to eliminate variations in measurements due

to different processes occurring as colonisation progresses. Two main problems confuse the issue of the stage at which VAM colonisation is limited in P sufficient plants. Firstly, high soil P could have an effect on fungal growth in the soil. This effect could be eliminated if plants grown in high P soil had the soil removed prior to transplantation into inoculum. This would enable the determination of the direct effect high tissue phosphate has on colonisation. Secondly, the timing of secondary entry point formation may be altered by P nutrition. Transplanting a root system into actively growing inoculum would allow the exclusive examination of multiple primary infection units. Furthermore, the synchronous nature of colonisation events would allow other developmental processes, e.g. arbuscule formation, to be targeted.

This chapter describes the adaptation of Brundretts' method of synchronous colonisation to develop root material suitable for molecular analysis, and also addresses the question of the stage at which the colonisation processes are inhibited in P sufficient plants.

3.2 Tomato Growth Prior to Inoculation

The growth of tomato seedlings in seedling trays (2.1.5) was monitored over a period of 25 days. Two P levels (termed LSPP, low soil P pretreatment and HSPP, high soil P pretreatment) were used (2.1.1) and four plants from each treatment were harvested at 5 day intervals after germination. The purpose of this experiment was to determine an appropriate time for transplanting tomatoes into nurse pots for colonisation, as well as to investigate any growth response due to P fertilisation.

No significant difference in root fresh weight between the two P treatments was observed up to day 25 (Fig. 3.1). Bruce *et al.* (1994), using similar P levels with *Cucumis sativus* L. found fresh weights between treatments began to vary 15 days after germination. This contrasts with the more slow growing *L. esculentum*, where different soil P had no effect on root fresh weight over the 25 day period.

Although there was no fresh weight difference between the two P treatments, the HSPP tomatoes appeared healthier, with brighter green leaves. The LSPP tomatoes were turning purple, showing symptoms of P deficiency by 25d. It was decided that the optimal time for transplantation was after 14 days in the seedling trays, when the average root fresh weight was approximately 0.1 g. At the later

harvests, the root systems were larger but were also more easily damaged, with much of the root growing through a filter at the base of the seedling tray.

3.3 Transplantation and Harvesting

LSPP and HSPP tomatoes were transplanted into nurse pots and harvested at two day intervals over 28 days (2.1.6). Four tomatoes (one nurse pot) were harvested at each time point for both the LSPP and HSPP tomatoes (2.1.7). Fresh weights were recorded for both shoot and root samples. The shoots were dried and analysed for P content and the roots cleared and stained for microscopic examination (2.1.8). The inoculation procedure was compared to the "traditional" inoculation method, where tomatoes were transplanted into soil containing dried inoculum. "Traditional" inoculation pots were harvested at days 12 and 24 after transplantation.

3.4 Root Fresh Weight

Root fresh weight initially increased rapidly after transplantation into nurse pots (Fig. 3.1). This growth spurt was most noticeable with the LSPP tomatoes, with the growth of HSPP tomatoes lagging by approximately 2 days. The larger soil volume available to the plant after transplantation may have stimulated root production. That the growth of HSPP tomatoes lagged by two days could be an indication that the mineral deficiency was not as acute, with resources not being partitioned into the roots until some time later.

3.5 Shoot Phosphate

Shoot P was measured by the method of Hanson (1950). At the transplantation date, HSPP tomatoes had a relatively high P concentration that decreased over the course of the experiment (Fig. 3.2a). As total shoot P did not change over the same time period (Fig. 3.2b), the P initially present in the seed must have been utilised throughout the growing period. The LSPP tomatoes had a much lower P concentration at transplantation, which did not change over the period of the experiment (Fig. 3.2a). The total shoot P content also did not change in these plants (Fig. 3.2b), indicating that the plants absorbed little or no P from the low P medium. The HSPP tomatoes had bright green leaves, whereas the LSPP tomatoes had darker green leaves with purple veins underneath. This is symptomatic of P deficiency in tomatoes. (Jones 1991).

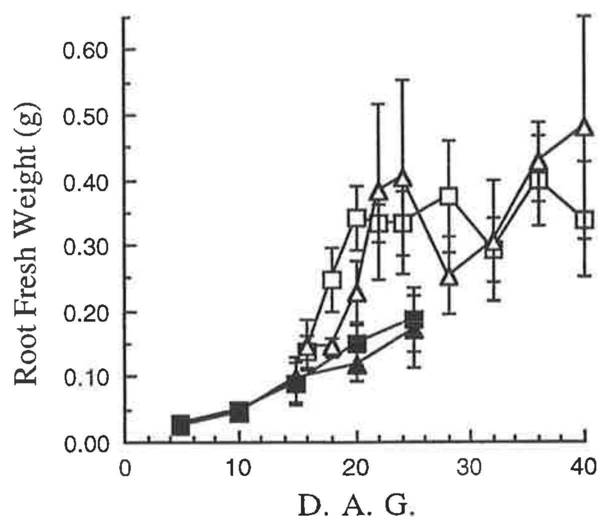

Figure 3.1 Root fresh weight per plant in LSPP (■) or HSPP (▲) *L. esculentum* Mill. grown in seedling trays. At day 14, LSPP (□) or HSPP (Δ) plants were transplanted into nurse pots. Means and standard deviations (n=4). D. A. G., days after germination.

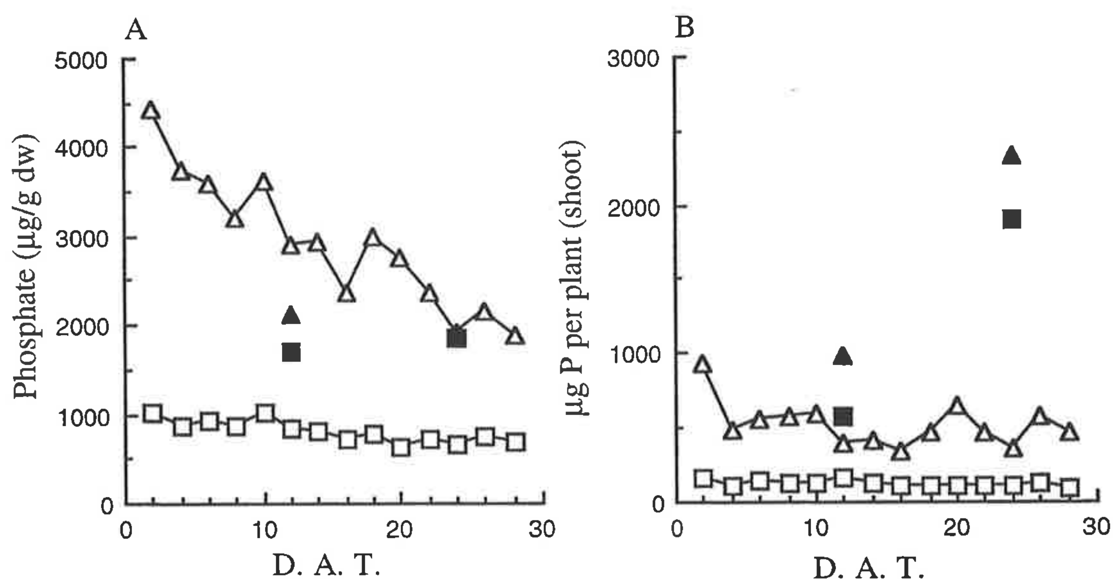

Figure 3.2 Changes in concentration of P (3.2A) and total P content (3.2B) in shoots of *L. esculentum* Mill. grown at LSPP (□) and HSPP (Δ) after transplantation into mycorrhizal nurse pots. Shoot P concentration is also shown for LSPP (■) and HSPP (▲) tomatoes transplanted into the traditional inoculum mix and harvested at 12 and 24 days after transplantation. P determinations were made on pooled plants from each pot (2.1.7). D. A. T., days after transplantation.

Tomatoes transplanted into the traditional inoculum mix grew much more rapidly than those transplanted into nurse pots, although the concentration of P in the shoots did not exceed that of the HSPP tomatoes. This indicates a sufficient level of P contained in the shoot to allow growth. Total P content increased, indicating absorption from the medium. Better growth of tomatoes transplanted to traditional inoculum mix compared with plants grown in nurse pots could be due to several different factors. These could include nutrient deficiency in the nurse pots, as the leeks had been growing in them for 6 weeks prior to transplantation, with consequent depletion of nutrients. The leeks had well developed root systems, which could have physically inhibited the growth of the tomato roots. Furthermore, the leeks may have exuded compounds that directly inhibited tomato growth. No evidence to distinguish between these hypotheses was obtained during the investigation.

3.6 Root Colonisation

The number of entry points formed by *Glomus intraradices* per unit length of root was determined over the first five harvests up to 10d (see Fig. 3.3). This corresponded to the period of enhanced growth of the root systems. There was a trend for more entry points to be formed on the roots of LSPP tomatoes during the early stages of colonisation, compared with HSPP tomatoes. At day six the number of entry points was significantly different between the two P treatments. However no differences were apparent at days eight and ten. This shows that entry point formation is inhibited in plants growing under P sufficient conditions. Similar results have been observed in onion by K. Tawaraya (pers. comm.). This contrasts with the results of Bruce *et al.* (1994) who found similar densities of entry points in cucumber grown in soils with different P levels. Braunberger *et al.*, (1991) compared entry point formation in *Zea mays* L. grown at three different soil P concentrations. In their study, the lowering of soil P lead to slightly more entry points being formed, however differences were not significant. The initial harvests of Bruce *et al.* (1994) and Braunberger *et al.* (1991) were four and eight days later (respectively) than the day six harvests in this study. Also, they used traditional inoculation methods which do not result in the rapidity of colonisation which can be achieved with nurse pots. This may explain the conflicting observations. In all investigation, differences in entry point formation between different soil P treatments were small. These differences probably have relatively minor importance in the overall inhibition of colonisation in P sufficient plants.

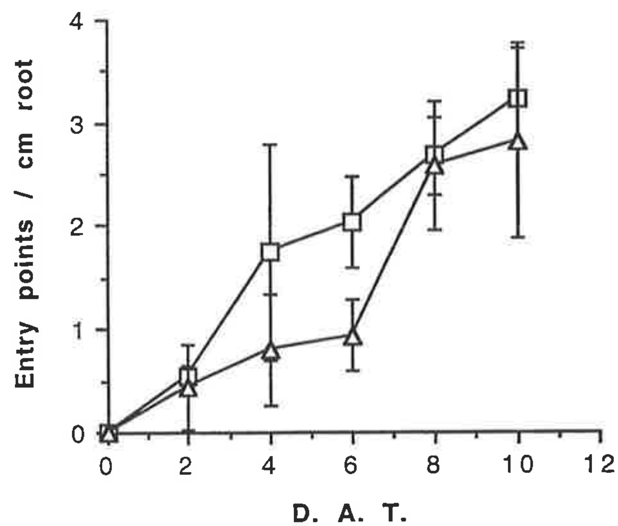

Figure 3.3 Development of entry points on roots of *L. esculentum* Mill. grown at LSPP (□) or HSPP (Δ) *L. esculentum* Mill. transplanted into nurse pots. Means and standard deviations (n=4). D. A. T., days after transplantation.

The rapidity with which interactions occur between the symbionts in the nurse pot system is emphasised by the data for fractional colonisation (Table 3.1) and for the presence of external hyphae on the surface of the root (Fig. 3.4a). After only four days the fraction of the root associated with hyphae was 0.6.

Table 3.1. Comparison of fractional mycorrhizal colonisation of plants of *Lycopersicon esculentum* Mill. grown at LSPP and transplanted either into nurse pots or inoculated by a traditional method, using dried pot culture inoculum.

Fractional	Day 12 Harvest		Day 24 Harvest	
Colonisation	Nurse	Traditional	Nurse	Traditional
Total	0.87	0.006	0.93	0.19
Arbuscular	0.44	0	0.26	0.01
Vesicular	0.23	0	0.38	0

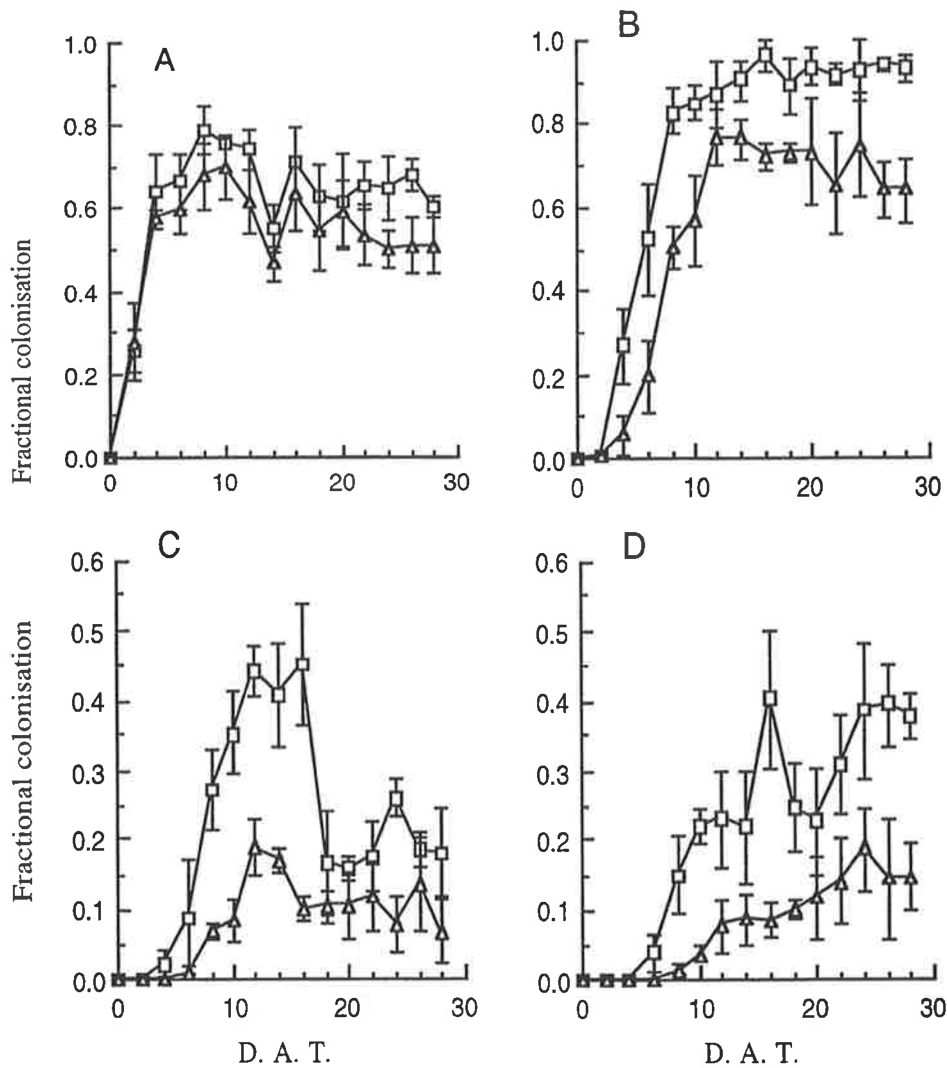

Figure 3.4 Colonisation roots of *L. esculentum* Mill. by *Glomus intraradices*. LSPP (□) or HSPP (Δ) plants were transplanted into nurse pots. Fraction of total root length associated with external hyphae (A), intercellular hyphae (B), arbuscules (C) and vesicles (D). Means and standard deviations (n=4). D. A. T., days after transplantation.

Intercellular hyphae, an indication of total colonisation of the roots, developed to different extents in the two P treatments (Fig. 3.4b). The initial lag in intercellular hyphal growth in the HSPP tomatoes correlates with the lower number of entry points observed at the earlier harvests (Fig. 3.1). The difference in entry point formation was only transient, and as total colonisation remained consistently lower in the HSPP tomatoes, higher P status must also have limited the growth of intercellular hyphae. This agrees with results of Bruce *et al.* (1994), Braunberger *et al.* (1991) and Amijee *et al.* (1989), which indicated that the primary factor

limiting mycorrhizal colonisation in P sufficient plants acts during intercellular hyphal growth, with any inhibition of entry point formation playing a secondary role.

The synchronous nature of the colonisation is consistent with the patterns of development of internal hyphae and arbuscules. The fraction of root length colonised by hyphae increased rapidly up to eight or ten days (depending on the treatment) after which plateau levels of 0.7 to 0.85 were maintained for the remainder of the experiment. In both treatments colonisation by hyphae was followed by a sharp peak in development of arbuscules (Fig. 3.4c). The first appearance of arbuscules occurred six days after transplanting (two days after hyphal colonisation), followed by a peak at 12 days and rapid decline at 16-18 days. The timing of arbuscule degeneration in this study is slightly delayed when compared to the work of Cox & Tinker (1976) and Alexander *et al.* (1989). Their studies indicated that arbuscules begin to form after two days and continue to develop for a further four to 4.7 days. The use of high powered microscopes (X750-X1000) enabled Alexander *et al.* (1989) to determine the exact time in which arbuscules begin to degrade and form clumps. In the study described here, a X400 magnification was used, making the early detection of degenerating arbuscules difficult,. This may have lead to inaccuracies in recording arbuscule collapse and possible broadening of the peak of maximum arbuscule colonisation. However, the existence of a clear peak in arbuscular development indicates that colonisation was synchronous during the first arbuscular cycle.

A second peak in arbuscule formation was observed in LSPP tomatoes, with a peak 24 days after transplantation. This was much lower and of shorter duration than the first peak, but is of interest given the near synchronous colonisation of roots, high levels of internal hyphae and the lack of growth of new roots at the later harvests. Unfortunately, it was not possible to determine whether, in this investigation, the same cells formed arbuscules twice, or if the new arbuscules are only formed in previously uncolonised cells.

The arbuscular cycle is poorly understood, particularly with respect to reasons for arbuscule degeneration. Some workers consider degeneration is due to "digestion" of the arbuscules related to transfer of P to the plant (see Cox & Tinker 1976). More recently it has been proposed that a mild defence response results in arbuscule degeneration (Gianinazzi 1991; Harrison & Dixon 1994). This could be further verified by examining expression of defence related genes during this

decline and the synchronous colonisation method is ideal for production of material to test this hypothesis.

The numbers of vesicles increased throughout most of the experiment, although the variable values from the last few harvests indicate a plateau may have been reached in both treatments. Further harvests would be required to confirm this. The LSPP tomatoes contained higher numbers of vesicles (Fig. 3.4d). This was consistent with the differences in all aspects of colonisation observed between the two treatments throughout the experiment.

Table 3.1 compares colonisation at 12 and 24 days after transplantation into either nurse pots or soil containing dried inoculum. Establishment of colonisation using the nurse pot method is much more rapid than the traditional inoculation procedure. After 12 days, there was little evidence of colonisation in the traditional mix, whereas the colonisation in nurse pots had reached a peak with respect to levels of both total and arbuscular colonisation. Colonisation in the traditional mix increased between the 12 and 24d harvests, but the increase was much less than that observed in the nurse pots over the same time period.

3.7 Generation of Root Material for Molecular Analysis

Later chapters discuss the identification of genes that are differentially regulated upon colonisation. To facilitate these studies, the nurse pot technique was employed to generate this root material in bulk. Mycorrhizal *L. esculentum* roots were developed in the same way as described above for the LSPP tomatoes. Uncolonised roots were required to make molecular comparisons between mycorrhizal and non-mycorrhizal roots. Non-mycorrhizal nurse pots were established (2.1.5) in the same soil as mycorrhizal nurse pots, but with the omission of inoculum. It was important that the mycorrhizal and control tomatoes were transplanted, grown and harvested in the same way so that changes in gene expression caused by the transplanting and harvesting procedure were the same in both types of root material.

LSPP tomatoes were transplanted into the inoculated and uninoculated nurse pots and maintained as described above. Ten pots of each treatment were harvested at days four, eight and twelve after transplantation (2.1.7). The harvests were timed to obtain material that was enriched for specific developmental stages in the colonisation process. At day four there was a significant level of intercellular hyphae, with very few arbuscules. By day eight, the level of intercellular

colonisation had reached a peak, and there were large numbers of developing arbuscules. Finally, by day twelve, arbuscular colonisation had reached a maximum.

The fresh weight of both mycorrhizal and non-mycorrhizal roots was recorded at the three harvests (Fig. 3.5). By day twelve, the roots of mycorrhizal plants had stopped growing, whereas the those of non-mycorrhizal plants continued to grow. A decrease in the root:shoot ratio is commonly observed in mycorrhizal roots (Smith & Read 1997), and the results presented here may indicate a similar response. These results need to be kept in mind in the analysis of data on gene expression in the following chapters as it is unknown how a reduction in root growth would affect gene expression.

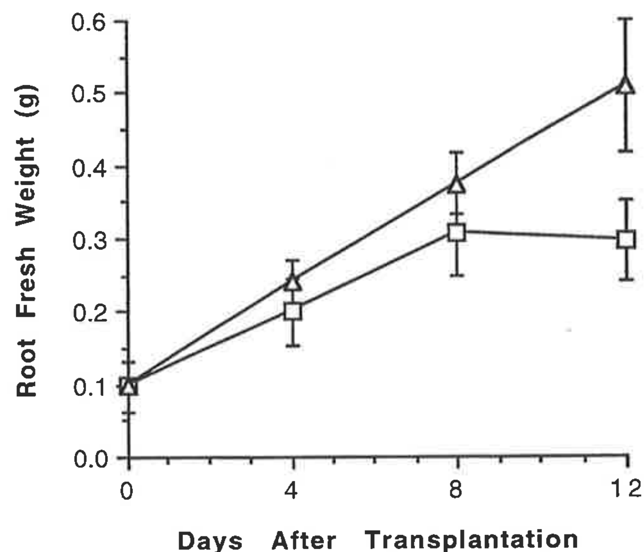

Figure 3.5 Fresh weight per plant of mycorrhizal (□) and non-mycorrhizal (Δ) roots of *L. esculentum* Mill. grown in nurse pots for use in molecular experiments. Means and standard deviations of nine replicate pots containing four plants each.

3.8 Conclusion

Young nurse plants (6 weeks old) are as effective as older ones (one year old in Brundrett *et al.,* 1985) in initiating near synchronous colonisation in seedlings transplanted to them. The nurse pots can be considered to be "disposable", allowing for rapid but gentle harvesting of roots, so that damage is kept to a minimum. The root fresh weight of tomatoes at various stages after colonisation ranged from between 0.15 to 0.4 g per root system, providing ample material for both developmental and molecular studies. The advantages of this colonisation

method over others include the production of a relatively large amount of root material with uniform colonisation. Furthermore, it is possible to obtain material selectively enriched in different structures. Harvesting plants at four days provided material with appressoria and intercellular hyphae, but few arbuscules, whereas harvesting between days eight and twelve maximised the proportion of roots containing arbuscules. Recently this method has been successfully used in our laboratory for molecular studies of the VAM symbiosis in barley, with similar timing of development of structures (G. Delp pers. comm.). It has also been used to study stages of colonisation in a mutant *L. esculentum* in which the fungus is restricted to development on the root surface, with very slight root penetration (S. Barker pers comm.) Our observations agree with those of Brundrett *et al.* (1985) and Giovannetti *et al.* (1994), which indicate that the mycorrhizal symbiosis is a developmental process with uniform, reproducible staging. The ability to apply molecular techniques to study material at specific stages of the mycorrhizal symbiosis will lead to an increased understanding of mechanisms involved in the establishment of the symbiosis and how this varies from parasitic fungal infection of roots.

Chapter 4

A Targeted Approach to Mycorrhizal Function: Cloning and Characterisation of a *Lycopersicon esculentum* Phosphate Transporter

4.1 Introduction

Phosphate (P) is required in relatively large amounts for plant growth, being taken up by the root from the surrounding soil. The concentration of P varies considerably in soils, ranging from 1 μM to 10 μM (Marschner 1995). Furthermore, as P is relatively immobile in the soil, depletion zones form around the roots of growing plants. The symbiotic association formed between plants and VAM fungi alleviates this problem as the fungal hyphae extend beyond the zone of depletion and allow a greater soil volume to be explored for P uptake.

Physiological studies show that an observed growth response in VAM colonised plants is correlated to increased plant P content. However little is known of the molecular mechanisms involved in P uptake from the soil by either the plant or the fungus, nor of the mechanisms of P transfer from the fungus to the plant (see Chapter 1).

Molecular mechanisms of P uptake have been best characterised in yeast, and appear to function as a multi-component system, with a number of proteins interacting with the PHO84 high-affinity phosphate transporter (Bun-ya *et al.* 1991, Bun-ya *et al.* 1996). A P transporter from *Glomus versiforme* (GvPT) shows significant sequence similarity to *PHO84* and the expression of GvPT in the external hyphae of the fungus implicate it with uptake of P from soil (Harrison and van Buuren 1995). When this research was initiated, no other plant P transporters had been isolated. Since then, a number of plant P transporters have been cloned and appear to form a family of genes that are also closely related by sequence similarity to *PHO84*. All probably function as proton-coupled high affinity phosphate transporters within the plant (Muchhal *et al.* 1996, Leggewie *et al.* 1997). However the possible roles played by these genes when the plant forms a mycorrhizal symbiosis have not been investigated.

This chapter describes the cloning and expression analysis of a phosphate transporter gene from *Lycopersicon esculentum* and investigations of the expression of this gene in the plant during mycorrhizal colonisation.

4.2 Screening of cDNA Library

A cDNA library constructed from polyA RNA from uncolonised *L. esculentum* roots (2.2.3) was screened with an expressed sequence tag (EST) (GenBank accession no. H36767) from *Arabidopsis thaliana* showing significant sequence similarity to high affinity phosphate transporters characterised in *Glomus versiforme* (Harrison & van Buuren 1995), *Saccharomyces cereviciae* (Bun-ya *et al.* 1991) and *Neurospora crassa* (Versaw 1995). Recently, several plant phosphate transporter genes have been characterised (Muchhal *et al.* 1996; Leggewie *et al.* 1997), and the EST used in this investigation also has high levels of sequence identity to these, confirming that the EST is likely to code for a phosphate transport protein. Expression of high affinity phosphate transporters has been shown to increase in response to phosphate starvation (Versaw 1995; Muchhal *et al.* 1996). Consequently, a cDNA library produced from roots of P-limited non-mycorrhizal *L. esculentum* was screened with the expectation that clones representing phosphate transporters would be better represented than in a library constructed from phosphate-sufficient roots.

A primary cDNA library was plated out on twelve, 11cm plates to give approximately 20,000 pfu per plate. Duplicate membranes were prepared from each plate and hybridised to the probe (2.2.3). After high stringency washes (2.2.7.8), the membranes were exposed to x-ray film at -80°C for 2 weeks. A number of plaques gave signals of varying intensity, and the twelve most intense were cored from the master plates with the large end of a sterile pasteur pipette and eluted in 1ml SM buffer. These were designated P1-P12. The numbers of pfu per ml from the eluate ranged from 4.7×10^6 to 3.0×10^7. Each original phage extract was replated to give 400 pfu per plate. Duplicate plaque lifts were made from each plate, and rescreened with the clone from *A. thaliana* .

Eleven of the twelve primary eluates contained clones that hybridised to the probe with the secondary screen. Two plaques that hybridised to the probe were cored from each plate with the narrow end of a pasteur pipette and eluted in 100 µl SM buffer. Phage DNA preparations were then completed on each of the 22 eluates (2.2.1.1). The DNA was digested with *Eco*R1 and the insert separated from the vector by agarose gel electrophoresis. The insert was purified from the gel (2.2.6) prior to cloning into the *Eco*R1 site of the vector pGEM3zf+ (Promega) (2.2.4). The *E. coli* strain DH5α was transformed with the recombinant DNA. Four of the 11 phage were subcloned and used for further analysis.

4.3 Sequencing of Clones

The *Eco*R1 restriction enzyme site is located in the multiple cloning region of the vector pGEM3zf+ (Promega). The vector is designed to allow sequencing of inserts in both directions with the use of M13 forward and reverse primers. Sequencing using the M13 primers (2.2.8) gave a primary sequence of between 200-400 bases from each end of the clones (Table 4.1). The sequence obtained from each clone enabled a prediction of open reading frames and subsequent searches of the Genbank database using the Blast algorithm.

The sequences of P7 and P11 overlapped and were identical in the overlapping region, indicating they were probably partial length cDNAs from the same gene. These sequences showed strong similarity to previously characterised phosphate transporters as well as to the *A. thaliana* EST used to screen the library. P4 and P8 (Table 4.1) did not show significant sequence matches to any sequences in the database and were not characterised further. As P7 was internal to P11, it was decided to pursue the analysis of the P11 clone. Internal primers (Appendix 2) were designed from the P11 sequence to facilitate the complete double stranded sequencing of this clone (Fig. 4.1).

Table 4.1. Primary forward (F) and reverse (R) sequencing lengths and results from database search of predicted amino acid sequences from the putative phosphate clones.

Clone	Insert Size	Initial Sequence Length		Homology
		F	R	
P4	2300 bp	454	346	None
P7	900 bp	401	279	Phos. Trans.
P8	800 bp	478	356	None
P11	1152 bp	329	340	Phos. Trans.

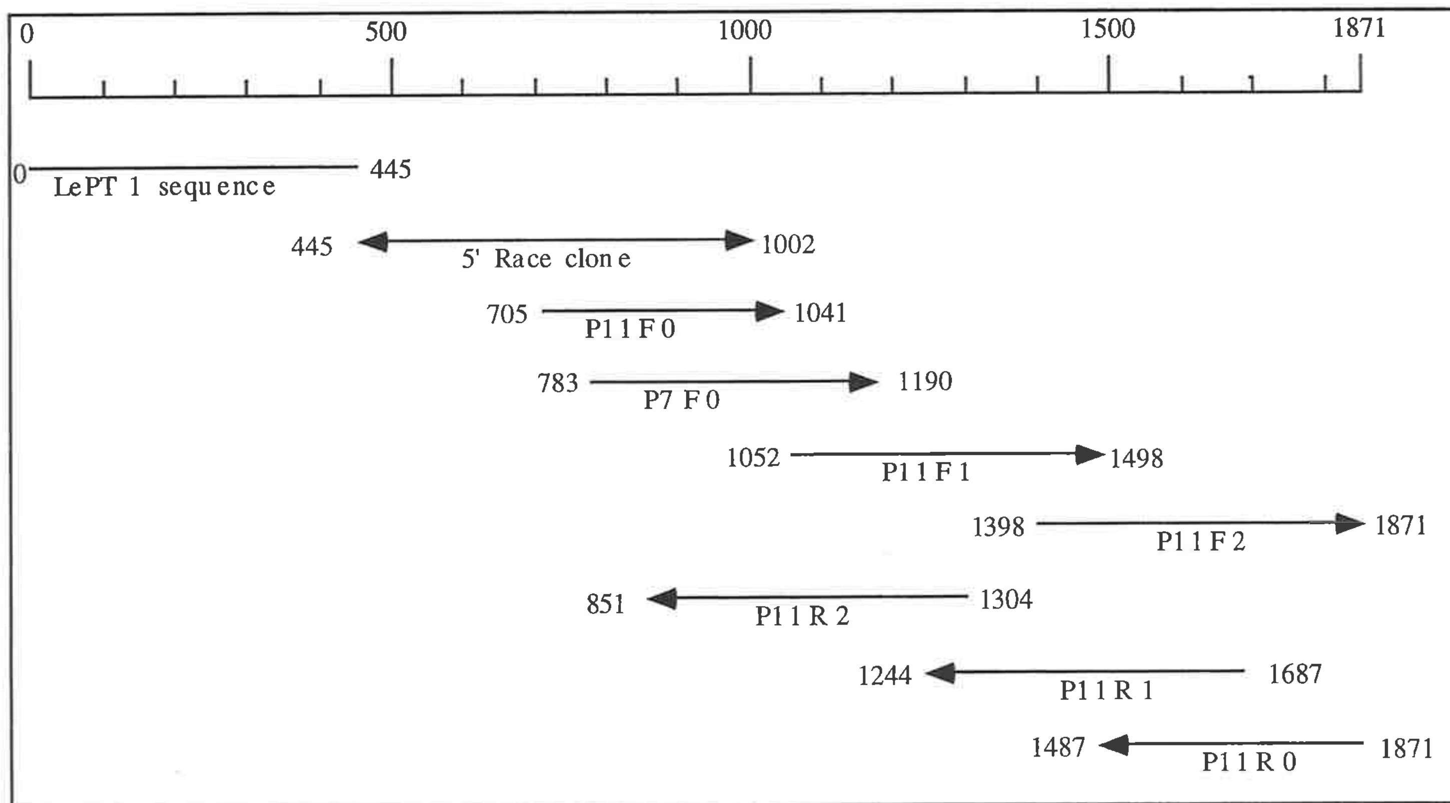

Figure 4.1 Sequencing strategy for LePT 1. The majority of the LePT 1 sequence was contained in the P11 clone and a 5' RACE clone. Both P11 and the 5' RACE clone were sequenced fully in both directions. Initial sequences were generated from the M13 forward and reverse primers (sequences designated P11F0 and P11R0 respectively in the P11 clone). Internal primers were designed to extend sequencing products further into the P11 clone. The remaining sequence of LePT 1 was obtained from a recent entry in the Genbank database (accession no. Y14214).

4.4 Complete Sequence Determination of the *Lycopersicon esculentum* Phosphate Transporter (LePT) cDNA

The clone P11 was 1152 bp in length. Sequence comparison to plant phosphate transporter genes indicated that P11 was not a full length clone. The sequence aligned to the 3' end of other known phosphate transporters. The full length cDNAs coding for phosphate transporters in *Solanum tuberosum* and *A. thaliana* are between 1754 to 1856 bp and therefore P11 is probably between 600 and 700 bp short of the full length sequence. To obtain the remaining sequence, the procedure of 5' RACE (Rapid Amplification of cDNA Ends) was adopted (2.2.9).

4.5 Amplification of the 5' Region of a Putative Phosphate Transporter

The 5' RACE technique is outlined in Fig. 4.2. The partial cDNA (Fig. 4.2 A) was sequenced and two Gene Specific Primers (GSP1 and GSP2) (B) were designed to anneal to the mRNA at a position close to the 5' end of the P11 sequence. (C) First strand cDNA synthesis was completed on DNase treated total RNA, priming the polymerase with GSP1. (D) The 3' end of the single stranded cDNA was tailed (TdT tailing) with a series of cytosine nucleotides. (E) A primary PCR using the abridged anchor primer (AAP) and GSP2 was completed to amplify the cDNA. The sequence design of the AAP facilitates both the binding of the primer to one end of the cDNA and abridging a new primer site to the 5' end of the DNA strand being synthesised. (F) A secondary PCR was completed with GSP2 and the abridged universal amplification primer (AUAP), the later primer binding to the primer binding site created by the AAP. The subsequent amplification products were cloned and analysed.

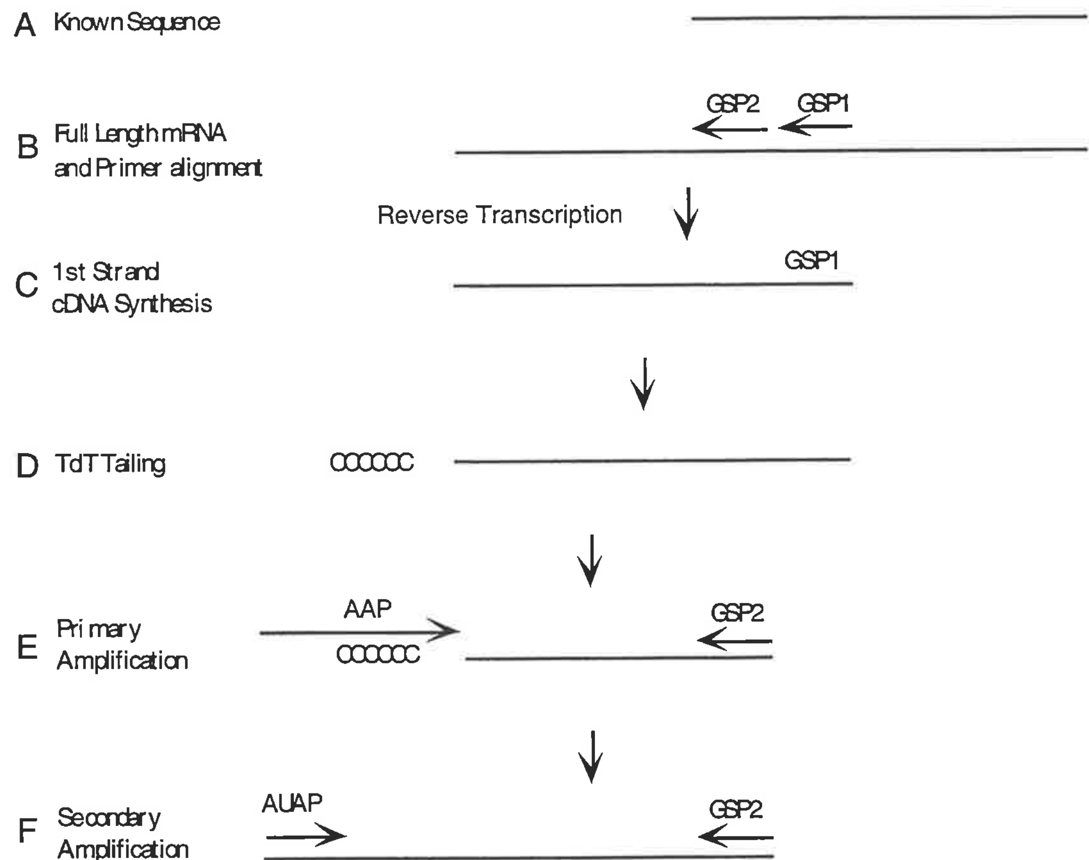

Figure 4.2 The 5' RACE procedure (see text).

Reverse transcription was carried out (2.2.9) on 1.3 µg total RNA using the primer GSP1 (Fig. 4.2C). The cDNA was purified and TdT tailed (Fig. 4.2D). Primary PCR (Fig. 4.2E) was completed with the GSP2 and the abridged anchor primer (AAP). A secondary PCR again utilised the GSP2 primer, this time in combination with the abridged universal anchor primer (AUAP) primer. Several controls were also completed to check critical steps in the 5' RACE procedure as follows:

1) To ensure cDNA synthesis was effective on the RNA sample, an additional reverse transcription (RT) reaction using the P11R1 primer was completed. This allowed the PCR amplification of cDNA using the primers P11R2 and P11F1 (for primer location, see sequencing details Fig. 4.1). This PCR did not rely on TdT tailing amplify a product.

2) According to the manufacturers instructions of the 5' RACE kit (Life Technologies), PCR artefacts caused by non-specific priming of the AAP is a common problem in this procedure. Therefore, a primary PCR reaction using a

template consisting of cDNA that had not been TdT tailed was completed to indicate amplification of non-specific DNA.

3) A negative PCR control where template DNA was omitted ensured there were no amplifiable contaminants in the PCR mix.

4) As a positive PCR control, a reaction containing cloned DNA of P11 as template and primers internal to the P11 sequence (P11F1 and P11R2) was included to ensure the PCR amplification steps worked.

The primer combinations used for these PCRs are outlined in Table 4.2.

Table 4.2 Primers used in the primary PCR reaction for amplification of 5' Race products. * indicates templates that were produced by reverse transcribing cDNA from polyA RNA annealed to the specified primer

Reaction Number	Template	Primers (Primary PCR)
1	GSP1*	GSP2; AAP
2	GSP1 (-TdT)*	GSP2; AAP
3	P11R1*	P11R2; AAP
4	P11R1 (-TdT)*	P11R2; AAP
5	P11 (cloned DNA)	P11R2; P11F1
6	Control (no template)	P11R1; AAP

The amplified products were too dilute to be visualised after agarose gel electrophoresis and ethidium bromide staining, and were reamplified with a secondary PCR. The secondary PCR used the same primer combination as the primary PCR (Table 4.2), with the exception that AUAP replaced AAP. AUAP is a truncated version of AAP (Appendix 2).

Amplified products from the secondary PCR were separated by agarose gel electrophoresis. The only evidence of amplification that could be observed with ethidium bromide staining was a smear produced in the GSP1 and GSP1 (-TdT) reactions. The smears ranged in size from 0.4-0.8 and 0.4-0.6 kbp for these primer combinations respectively. The PCR products were transferred to a nylon membrane (2.2.6) that was subsequently hybridised to a DIG labelled P11 probe. Only the smear from the GSP1 reaction hybridised to the probe, indicating that this product contained sequences homologous to some of the coding sequence of

P11 (data not shown). The smear produced in the -TdT reaction was a PCR artefact and did not hybridise to the P11 clone. Although not sufficiently abundant to be detected by ethidium bromide staining, hybridisation bands were visible in reactions 3 and 5, in which the templates consisted of cDNA synthesised with the primer P11R1 (reaction 3) and DNA from the P11 clone (reaction 7). The PCR primers in both template reactions were P11F1 and P11R2, and were expected to amplify DNA homologous to the P11 probe.

DNA amplified from the secondary PCR was again separated by agarose gel electrophoresis and the region covering 550-800 bp was excised, purified with geneclean and subcloned (2.2.4) into the pZERO vector. This was accomplished by endfilling the insert DNA with the Klenow fragment and ligating into pZERO cut with *Eco*RV. A number of clones grew and 20 were selected for plasmid DNA preparations (2.2.1.2). The insert was excised with *Hind*III and *Xba*1, and separated by agarose gel electrophoresis. Sixteen of the twenty clones had inserts that ranged in size from approximately 300 to 600 bp. The DNA was transferred to a nylon membrane and probed with the DIG labelled P11. All inserts hybridised to the probe.

Three of the largest clones (approximately 600 bp) and a fourth (approximately 300 bp) were sequenced. The sequence of all four inserts commenced with the GSP1 sequence and was immediately followed by the remaining known sequence of P11 (297 bases). A further 290 bases of sequence 5' of P11 was determined (Fig. 4.1) and showed significant sequence similarity to other characterised phosphate transporters (Fig. 4.3).

While this research was in progress, a sequence almost identical to P11 and its 5' extension was isolated from an *L. esculentum* root hair cDNA library and was designated LePT 1. (GenBank Accession no. Y14214). The nucleic acid sequences of P11 (including the 5' extension) and LePT 1 had 99.8% sequence identity and as they were considered to be the same gene, the further sequencing of the phosphate transporter was not pursued.

4.6 Sequence Analysis of an *L. esculentum* phosphate transporter (LePT 1).

The complete sequence of the LePT 1 (from GenBank) was highly conserved at the predicted amino acid sequence level with previously characterised phosphate transporters in *Sacchromyces cereviciae*, *Neurospora crassa, Glomus versiforme, Arabidopsis thaliana* and *Solanum tuberosum* (Table 4.3).

Table 4.3. Sequence identity of *L. esculentum* phosphate transporter with characterised phosphate transporters in other systems.

Organism	Accession Number	Amino Acid Similarity (%)
A. thaliana	U26330	75.6
A. thaliana	U26331	77.1
S. tuberosum	X98890	90.6
S. tuberosum	X98891	74.9
G. versiforme	U38650	33.4

There are several characteristics common to this group of presumed transmembrane phosphate transporters. These proteins are thought to be located in the membrane and to contain 12 membrane spanning helices, predicted using hydrophobicity plots. Consensus sites for N-linked glycosylation, phosphorylation by casein kinase II and by protein kinase C have also been identified (Muchhal *et al.* 1996; Harrison & van Buuren 1995). Fig. 4.3 shows details of these regions as well as sequence alignments to the five phosphate transporters outlined in Table 4.3

Sequence similarity indicates that it is highly likely that LePT 1 codes for a transmembrane high-affinity phosphate transporter. LePT 1 has over 90% amino acid sequence identity to 90% to a phosphate transporter from *S. tuberosum*. The phosphate transporters fall into a large family of membrane spanning transport proteins, being characterised by twelve membrane spanning helices, with a large hydrophilic region dividing the membrane spanning regions into two groups of six. A Major Facilitator Superfamily (MFS) was defined by Marger and Saier (1993) and included over 50 transporters that contained this motif. They identified

five clusters in the MFS facilitated transmembrane transport of a large range of substrates. The statistical sequence analysis indicated that these five groupings were homologous, that is the transporters were derived from a common evolutionary ancestor. Griffith (1992) identified four clusters of transporters in the MFS and Versaw (1995) subsequently located the *Neurospora crassa* Pho5 gene (a high affinity phosphate transporter) as a member of cluster one. This cluster contains transporters whose substrates are predominately sugars, including 5 and 6 carbon monosaccharides and the disaccharides maltose and lactose. It is predicted that the LePT 1 gene will also fall into that grouping.

Highly conserved amino acid sequences are common to all members of the MSF. Griffith *et al.* (1992) identified a characteristic motif between the second and third transmembrane helices whose secondary structure is predicted to form a β-turn. The motif contains one of the following amino acid sequences; GXXXDR, KXGRR or KXGRK (see abbreviations for full amino acid names). Transmembrane helix 4 contains a sequence RXXXGXXXG. LePT 1 is predicted to have forms of both of these motifs. Furthermore, basic residues (either arginine or lysine) are conserved at the beginning of the 6th transmembrane helix in all proton-coupled symporters, and LePT 1 also has an arginine in this location. It is assumed that these amino acids are associated with the specificity of proton binding. Finally, substrate export proteins show a motif GXXXGPXXG in the 5th transmembrane helix. As expected this is absent from LePT 1, which is likely to be involved in substrate uptake, rather than export.

Group 1 MFS of Griffith (1993) contains characteristic motifs of PESPRXL and PETKGXXXE at the ends of the 6th and 12th transmembrane helices respectively. The phosphate transporters have similar motifs, namely PETARXT and PESKGXXXE. Changes observed in these motifs at position three (S to T and T to S) are exchanges of aliphatic amino acids, and are not expected to result in significant alterations of protein structure or function. However, position four of the 6th transmembrane conserved region shows a substitution of a proline with an analine. This substitution could have a distinct effect on the secondary structure of the protein. Another substitution of leucine to threonine at position seven of this domain results in an aliphatic amino acid replacing a hydrophobic amino acid. These changes are likely to alter the structure of the proteins, which may relate to substrate specificity of the phosphate transporters.

It is interesting to note that the C-terminal half of members of sub-families of the MFS are generally more conserved than the amino-terminal half. Homologous

sugar transporters have conserved C-terminal regions, possibly indicating a region of the protein involved in substrate binding. This hypothesis is supported by inhibitor and photoaffinity labelling studies that locate sugar binding to the C-terminal domains of sugar transporters (Carruthers 1990). Phosphate transporters have a highly conserved 38 amino-acid sequence in the C-terminal domain. More specifically this conserved region spans parts of the predicted 10th and 11th transmembrane helices and an intervening hydrophilic loop. This conserved region may be involved in determining substrate specificity. As it is predicted to be located on the cytoplasmic side of the membrane, intracellular phosphate may interact either directly or indirectly with this region to limit phosphate passage through the transporter.

The presence of consensus sites for phosphorylation by casein kinase II and protein kinase C, as well as an N-linked glycosylation site, suggests that post-translational modifications may occur. Phosphorylation of glucose transporters has some regulatory effect (Lawrence *et al.* 1990), although this has not yet been shown for phosphate transporters.

Given that P11 is clearly a partial sequence of LePT1, it was used as a probe for expression studies

4.7 DNA gel blot analysis of LePT

Samples of total DNA (5µg) isolated from *L. esculentum* leaves were separately digested with the restriction endonucleases *BamH1*, *Dra1*, *EcoR1* and *HindIII*, and the products separated by agarose gel electrophoresis. The DNA was transferred to a nylon membrane and probed with P11 (2.2.6). The probe hybridised to a single band (Fig. 4.4) in each of the four digests, indicating the gene, LePT 1, is unique in the genome and is not part of a multi-gene family. This finding is somewhat surprising as Muchhal *et al.* (1996) and Leggewie *et al.* (1997) have cloned two high affinity phosphate transporters from *A. thaliana* and *S. tuberosum* respectively. Two more phosphate transporters have recently been cloned from *A. thaliana*, indicating that even in this species, with a very small genome, several different transporters are involved in phosphate transport. As the genome of *L. esculentum* is genetically more complicated than that of *A. thaliana* and as there is a high degree of genetic similarity between the closely related *S. tuberosum* and *L. esculentum*, it seems likely that more isoforms of the phosphate transporter will be found in *L. esculentum*. The hybridisation was carried out at a

very high stringency in this study and should be repeated under less stringent conditions to determine the size of the gene family.

4.8 RNA gel blot analysis of LePT 1

Total RNA was extracted from low P mycorrhizal and non-mycorrhizal *L. esculentum* roots harvested at four, eight and 12 days after the plants were transplanted into nurse pots and from leaf material taken from non-mycorrhizal plants. Approximately 10 µg of RNA from each sample (4 µg leaf RNA) was loaded onto a 1.2% denaturing agarose gel and separated by electrophoresis. The RNA was transferred from the gel to a nylon membrane and probed with P11 DNA (2.2.7). After high stringency washing (2.2.7.8), the hybridisation signal was detected with a phosphoimager screen (2.2.7.9). The RNA gel blot was repeated with RNA prepared from different plants to confirm the results.

Fig. 4.5a shows that the P11 probe hybridised to a band of approximately 2200 nucleotides in length. The sequence length of LePT 1 extracted from the data base was 1924 bp, indicating that it is not the full length of the gene transcript. The intensities of the bands from different samples were quantified, and data normalised according to the signal generated when the same membranes were hybridised to a ribosomal probe. To accomplished this, the membranes were stripped (2.2.7.10) and reprobed with an 18S rRNA probe derived from the BamHI-EcoRI fragment of the plasmid PHA1 (Gerlach & Bedbrook 1979). The normalised data of the P11 hybridisations were averaged and represented graphically in Fig. 4.5b. Hybridisation levels of the phosphate transporter gene in mycorrhizal roots remained fairly constant, whereas hybridisation increased two-fold by day 12 in non-mycorrhizal roots. A hybridisation signal was also observed in leaf RNA of non-mycorrhizal plants.

The difference in expression patterns between mycorrhizal and non-mycorrhizal roots is likely to be due to differences in P nutrition. Clarkson and Scattergood (1982) observed an increase in the Vmax of P uptake in tomato plants under P stress, which was attributed to either a protein modification or to an increased number of transporters in the plant root. The latter could be achieved by an increase in gene expression. Muchhal *et al.* (1996) also noted increased expression of phosphate transporter genes in *A. thaliana* grown under phosphate stress, and it appears that expression of these genes may be regulated in response to phosphate availability. Non-mycorrhizal plants grown in low P soil of nurse pots are likely to become progressively more P stressed as P depletion zones

develop around their roots (Smith and Read 1997). In contrast, mycorrhizal plants have a relatively constant supply of P due to uptake by the fungal hyphae from soil and the constant level of expression of LePT 1 is consistent with this.

To investigate the effect of P nutrition on LePT 1 expression further, RNA was isolated from both the roots and leaves of non-mycorrhizal tomatoes grown under phosphate deficient and sufficient conditions (2.1.1). RNA gel blot analysis showed expression was higher in both tissue types when the plants were grown under P limiting conditions (Fig. 4.6). Induction of P transporters under P stress has previously been observed in roots (Muchhal *et al.* 1996; Leggewie *et al.* 1997; Liu *et al.* 1998), however this is the first report of differential expression of P transporters in leaf material of plants grown under different P conditions.

```
LePT   MA--N-------D-LQVLNALDVAKTQLYHFTAIVIAGMGFFTDAYDLFCISMVTKLLGRLYYHHDGALKPGSLPPNVSAAVNGVAFCGTLAGQLFFG--WLGDKMGRKKVYGMTLMIMV 108
StPT1  ..--.-------.-.............................................I....N.................................--.................. 108
StPT2  ..VED-------NN.......L.........I..............S..L.........TKPDL...T..ARVGLRH.C.LV.........CAR.A-............VL.. 112
AtPT1  ..--E-------QQ.G..K............................V.L......I..FNPESA......H.A......L...S......--....L.......L..VM.I 109
AtPT2  ..--R-------EQ.............W.....I...............L......I...VE..Q...T....A.......................--....L...........V.. 109
GvPT   .STSDRVTIDVDKRRAA.KEI.D..FGWQ.IR.CLV..T...M.....AVNFASTMI.YV..-------G.KT.A.IDLGLKVIGSI...L......--Y.A.RL...RM..VE...II 111

LePT   ICSIASGLSFGHTPKGVMTTLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGMVAIIVSAAFKGAFPAPAYEVDAIGSTVPQADFVWRIILMFGAIPAGLT 228
StPT1  ..............S...............................................................S..............LA...S.................. 228
StPT2  V..V.....L.N...V..........................................................FS.I..L.TA.G.DH.YK..TFAEN.AV.......YI........SL..A.. 232
AtPT1  L..V.......EA...............................................V......F..LA..SI.DKK....T.A.NRAL..P..V.YI....V....L..A.. 229
AtPT2  L...........E..A..A.......................................VS..........M...IF...I.S..EAK..S...AD..L...I....L.......A.....AM. 229
GvPT   VATV..A..GESRAVT.VG.IM.W.VIM.V.........I.T..F.T..R...MM.S.........GSAI..LA.L.G.R----------NE.IKD.SAV.YC...V.GC..V.GLAA 221

LePT   YYWRMKMPETARYTALVAKNLKQAANDMSKVLQVEIEAEPEKVTAISEAKGANDFGLFTKEFLRR-HGLHLLGTASTWFLLDIAFYSQNLFQKDIFSAIGWIPPAQT---MNALEEVYKI 344
StPT1  .....................................A...V.N...E....S.....-.............................................---.......... 344
StPT2  ................DA.R..Q..GR.......S.EA.IEQ..RDET-.Q....SW..V..-.....F..C......................V...V....K.P.---...VQ.L... 347
AtPT1  ..................I...TA.......TD..L.ERVEDDVKDP.--QNY...S......-........T............................K.A.---...TH..FR. 343
AtPT2  ..S.S.............DA....S...........P.QQ.LEE..KE.S-KA....S...MS.-........T.............................S---...IQ..F.. 344
GvPT   L.F.LTI..P..MD.EHDVNK.TS.ITSY..KNDVN.DDTN.GNHVGVPKASWSD.VSY.GKWSN.KV....SMS..A......GIG.NNGI.L....YSETHEADLNLR.YNSLKNM 341

LePT   ARAQTLIALCSTVPGYWFTVAFIDKIGRFAIQLMGFFFMTVFMFALAIPYHHWTLKDHRI-GFVVMYSFTFFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAMVGAFGFLYA 463
StPT1  ............................R.....................L.........N..-.......L..........................................  463
StPT2  ............................I.....................I.........EAN..-..I....L...............................I...Y.....  466
AtPT1  ............................T....K...N...M.......I.F..N..IKPEN..-.......L........I..........................I..........  462
AtPT2  ............................V.......M..................N...H.EN..-...I...L.................F..........S..L...........L 463
GvPT   .VGNII.TIMG.A....V.A.LV.SW..KP.....GVL.ALFIVMGAAFNP--..E.S.PA.IILFTLLQ..Q.....T...I..G.V..T.Y...G......S..L..I.AQV..--- 456

LePT   AQPTDPTKTDAGYPPGHWCEELVDRPWL----C-NFLGMLFTFLVPESNGKSLEDLSRENEGEEEIVAEIRATSGRTV--PV 538
StPT1  ......K.......A.-----IGV.NS.IVLG.V..............K....EM.............M........LFKF 540
StPT2  ..SK..M.......A.-----IGIKNS.IVLGFI.A...VC..C....K....EA.Q.TISTG.A 527
AtPT1  ..SQ.KA.V.......-----IGVKNS.IMLGVL..I..........PK....E..G.A.VSHDEK 524
AtPT2  ..NP.KD.........-----IGV.NS.IVLGVV....I.........K....EM.G...DN.NSNNDS.TVP------I. 534
GvPT   ----SKL.DIG.-------SNAFVG.L.LIFSAWM.I.G..SI.I..TK.L...E.AN.EHTYDVEERKE.IKAD------A 521
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Figure 4.3 Deduced amino acid sequence alignment of *L. esculentum* phosphate transporter (LePT1)with amino acid sequences from *S. tuberosum* (Leggewie *et al.* 1997), *A. thaliana* (Muchhal *et al.* 1996) and *G. versiforme* (Harrison and van Buuren 1995). (.) represents amino acids conserved with the LePT sequence and (-) inserted spaces to allow for correct alignment. Boxed sequences indicate putative phosphorylation sites and the shaded boxed sequence is a consensus site for N linked-glycosylation.

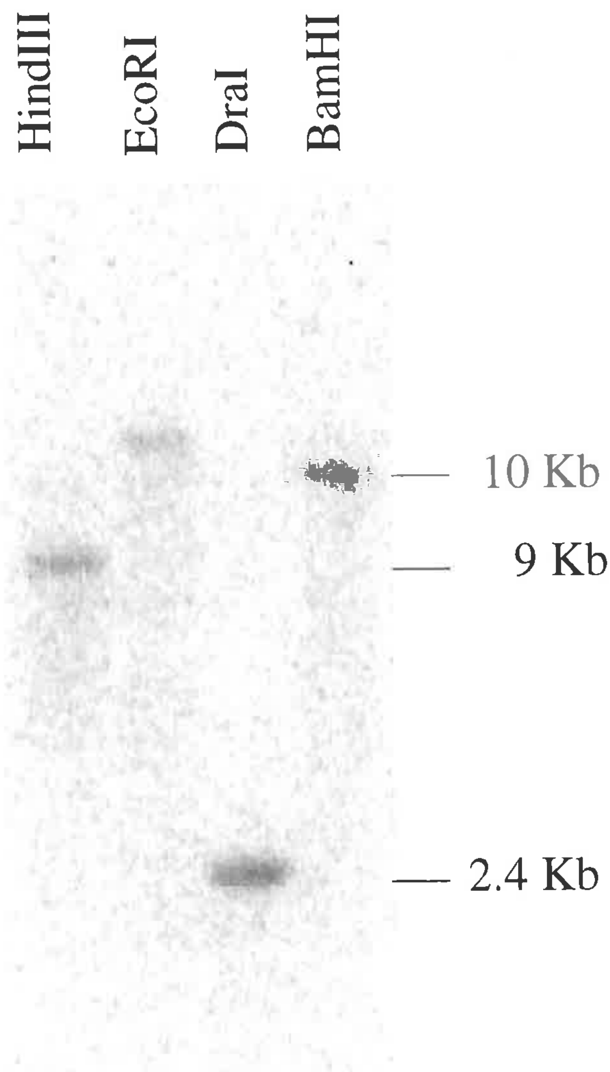
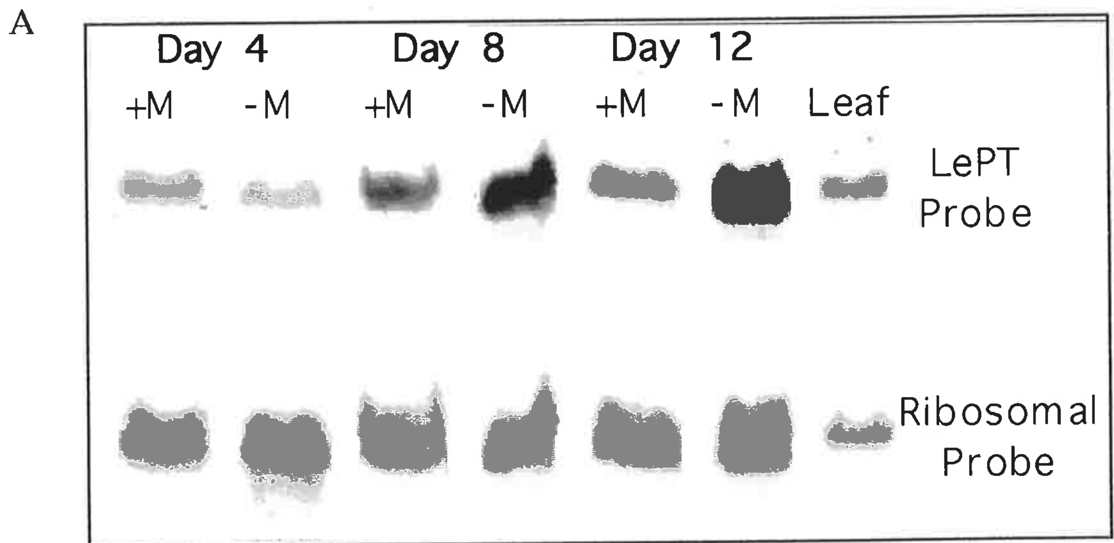


Figure 4.4 DNA gel blot hybridisation of total genomic *L. esculentum* DNA and the putative phosphate transporter clone, LePT 1. High molecular weight DNA (5μg) was extracted from leaf material from *L. esculentum* and digested with the restriction endonucleases *Bam*HI, *Dra*I, *Eco*RI and *Hind*III. The fragments were fractionated by size using agarose gel electrophoresis and the DNA transferred to hybond N+ membranes. The probe was prepared by incorporating ^{32}P-dCTP into DNA synthesised from the P11 insert in a randomly primed, DNA polymerase I (Klenow) reaction. After hybridisation the membrane was stringently washed (0.2% SSC, 0.1% SDS, 65°C 2x15') and exposed to a phosphorimager screen for 5 days. DNA size markers are shown on the right hand side.

A



B

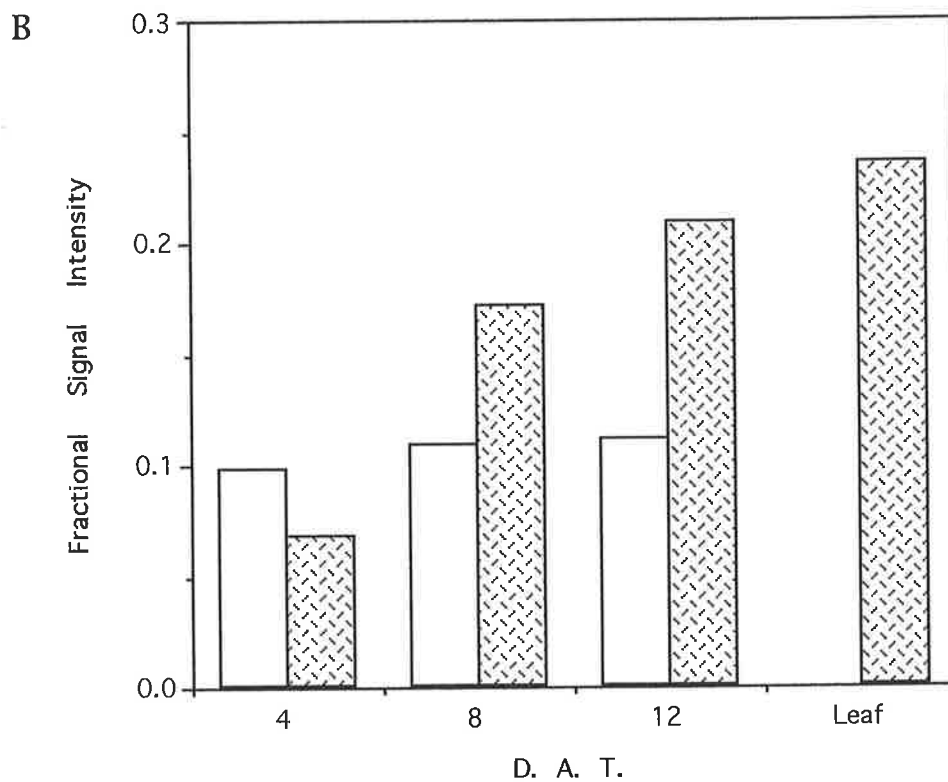


Figure 4.5 RNA gel blot analysis of the expression of the LePT 1 gene. A) Total RNA blots from mycorrhizal roots, control roots and control leaf were hybridised in duplicate (only one shown in A) to the P11 clone. The membranes were stripped and reprobed with a ribosomal probe to quantify the loading of RNA between samples. B) The signals generated by the P11 probe were quantified and normalised with respect to RNA loading (as determined by the ribosomal probe). Normalised signals were converted to fractions of the total signal generated in each blot. Data from two blots were averaged and plotted. Data from; ☐ mycorrhizal RNA samples, ⛆ non-mycorrhizal RNA samples.

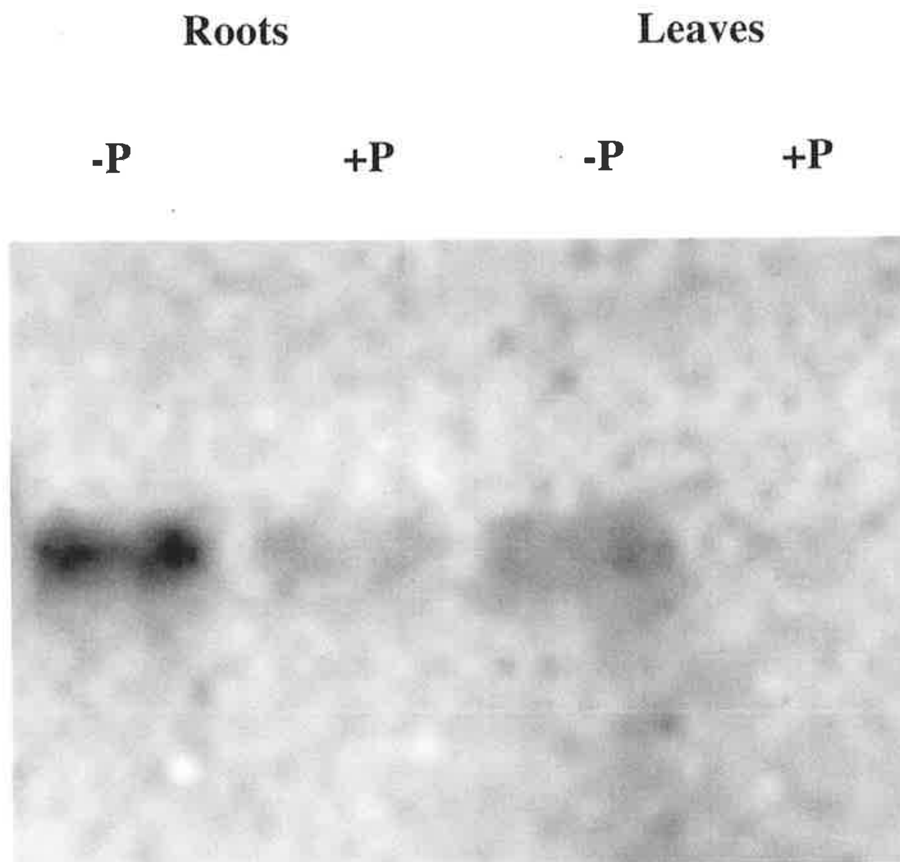


Figure 4.6 RNA gel blot analysis of RNA extracted from roots and leaves of non-mycorrhizal *L. esculentum* grown with high and low P and probed with the P11 clone.

4.9 *In situ* hybridisation of LePT 1.

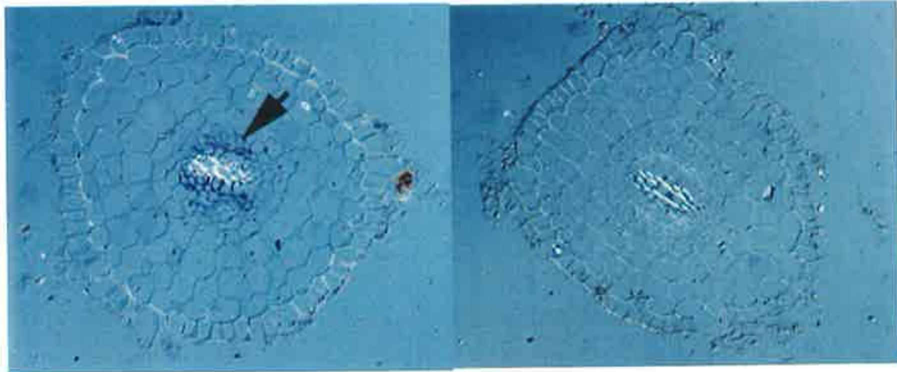
To determine which cells express LePT 1, the technique of *in situ* hybridisation was used. Roots of both mycorrhizal and control plants at four and 12 days after transplantation were sectioned and probed with sense and anti-sense RNA probes synthesised from the P11 clone (2.2.11). Fig. 4.7 shows the hybridisation patterns observed in the root sections. In uncolonised roots, there was slight hybridisation of the anti-sense signal throughout the root cortex, with little hybridisation in the epidermis. Phloem tissue within the vascular cylinder hybridised strongly to the anti-sense probe. A similar pattern was observed in the mycorrhizal roots, with strong staining also occurring in cortical cells containing arbuscules. Roots probed with the sense probe stained weakly indicating that no hybridisation occurred in either mycorrhizal or non-mycorrhizal roots. As detection of a hybridising signal was visualised through the enzymic reaction of an alkaline phosphatase, the lack of reaction with sense probe shows that any alkaline phosphatases present in the fungal material had been inactivated and therefore could not account for the signal observed with the anti-sense probe. This is important because mycorrhizal fungi show high activity of alkaline phosphatase in hyphae and arbuscules within the root (Smith & Read 1997).

The intense signal observed in the phloem tissue may be the result of high activity and relatively large cytoplasmic volume of these cells. The observations are similar to those made in *Medicago truncatula* roots probed with a hexose transporter (Harrison 1996) and *Phaseolus vulgaris* roots probed with phenylalanine ammonia-lyase (PAL) and chitinase (CHT) (Blee and Anderson 1996).

A faint signal was observed in all cells of the cortical parenchyma of both mycorrhizal and non-mycorrhizal roots. These cells contain large vacuoles with relatively little cytoplasm which would reduce the apparent strength of the signal. The high level of expression observed in arbuscule-containing cortical cells is of particular interest as these cells are thought to be the site of phosphate uptake by the plant from the fungus. The expression of a phosphate transporter in the plant membrane would be essential for P uptake by the plant from the interfacial apoplast.

Figure 4.7 Root sections of *L. esculentum* hybridised with antisense and sense DIG labelled RNA generated from the P11 clone (2.2.11). Non-mycorrhizal roots (A and B) and mycorrhizal roots (C and D) were hybridised with antisense (A and C) and sense (B and D) probes. Single arrow indicates that hybridisation of the probe occurred within the vascular cylinder. Double arrows show hybridisation of the antisense probe but not the sense probe, to arbuscule containing cortical cells.

A) B)



C) D)

4.10 Conclusions and Future Work

A putative phosphate transporter from *L. esculentum* has been cloned and sequenced. The high degree of sequence similarity to other characterised plant phosphate transporters means that the assumption the LePT 1 gene product does transport P is highly likely to be correct, although a complete characterisation is needed to confirm this.

The transporters listed in Table 4.3 have been characterised by their ability to complement a *pho84* yeast mutant that lacks a high-affinity phosphate transporter. One such mutant yeast strain is NS219 and when it is complemented with a functioning P transporter, the yeast strain shows increased growth on low P media when compared to the parent NS219. Furthermore, NS219 constitutively produces an acid phosphatase. This enzyme is repressed in both phosphate transporter-complemented and wild-type yeast strains grown on phosphate sufficient media. Production of this enzyme is determined by a simple staining technique that turns NS219 red, while leaving wild-type strains pale (Toh-E & Oshima 1974). Confirmation that LePT1 1 is a phosphate transporter would require studies of its role in the *pho84* yeast mutant.

Phosphate uptake kinetics have been examined in NS219 complemented with the phosphate transporter gene cloned from the mycorrhizal fungus *Glomus versiforme* (GvPT) (Harrison and Van Buuren 1995) and plant phosphate transporters from *S. tuberosum* (StPT) (Leggewie *et al.* 1997) and *Medicago truncatula* (MtPT1) (Liu *et al.* 1998). When complemented with the fungal phosphate transporter, an apparent *K*m of 18μM for P uptake was estimated. This value was similar to the *K*m of 8.2μM estimated from wild type yeast cells (Horikawa *et al.* 1993). A direct comparison of the rate of P uptake between the *G. versifome* and *M. truncatula* phosphate transporters used to complement NS219 was made. NS219 transformed with GvPT absorbed P at a rate approximately 30% higher than NS219 transformed with MtPT1 (Liu *et al.* 1998). Similarly, Leggewie *et al.* (1997) noted that although NS219 yeast was complemented when transformed with StPT, the rate of phosphate uptake was lower than that of NS219 transformed with the *PHO84* gene from yeast. The conclusion could be drawn that the plant phosphate transporters are less efficient than the phosphate transporter from the mycorrhizal fungus or from yeast. However, P uptake in yeast may require several proteins that interact together to form the functional uptake molecule. As the G. *versiforme* P transporter has a

greater amino acid sequence similarity to PHO84, than does the *M. truncatula* P transporter (Liu *et al* 1998), the former could interact in a more efficient way with other components of the yeast uptake mechanism and lead to higher uptake rates. This does not necessarily mean that there would be differences in efficiency naturally occurring in intact plants and intact fungal systems.

The LePT 1 gene is expressed in arbuscule-containing cortical cells, indicating that it may play a role in uptake of P from the apoplast surrounding the fungus. RNA gel blot analysis showed that expression remained constant in mycorrhizal roots, but increased in control roots. This finding needs careful consideration. Uptake of P via the fungus ultimately results in delivery to specific cortical cells containing arbuscules and hence to the apoplastic region between fungus and plant. Specific increases in expression of a P transporter in these cells, as shown by *in situ* hybridisation, would not necessarily result in increased expression at the level of the whole tissue, especially if the cortical cells were not P stressed. A further complication is the lack of detail of epidermal cells and root hairs in sections cut from wax mounted tissue. As previously mentioned, during the course of this study, LePT 1 was also cloned from a root hair cDNA library (GenBank Acc. No. Y 14214). This indicates that the gene is expressed in root hairs and expression in this tissue may be reduced as whole root P increases. The higher concentration of P at the symbiotic interface would allow P transporters to function more efficiently, therefore reducing the total number of transporters, and hence transporter gene transcripts, required in mycorrhizal roots.

In situ hybridisation indicates the cells in the root where this gene is expressed. The next step in characterisation would be to complete immunolocalisation using antibodies to the protein product of the gene to determine where the protein is placed within the cell. Further support for the involvement of LePT 1 in phosphate uptake by the plant would be obtained if the protein was similarly localised to the plant membrane in arbuscule containing cortical cells.

Chapter 5

A Non-targeted Approach Mycorrhizal Function

5.1 Introduction:

Unicellular and complex organisms respond to the wide array of changing conditions in the external environment through changes in gene expression and consequent alterations in biochemical and physiological processes. In an effort to understand the molecular-genetic responses to external stimuli, differentially expressed genes are cloned and characterised. As little is known of the molecular interactions occurring in the partners in the VAM symbiosis, the identification of genes that are differentially expressed in both the plant and the fungus as colonisation progresses must be a useful strategy in developing a greater understanding of this system. Of particular relevance to understanding how the symbiosis is established are those genes that are differentially expressed in the early stages of colonisation, as they are likely to be involved in developmental processes of mycorrhiza formation and could be involved recognition of the fungus by the plant, signal transduction or inhibition of defence related responses (Chapter 1). Fully developed mycorrhizal roots will not only exhibit changes in gene expression relating to the symbiosis but also have the complication of improved nutrition when compared to non-mycorrhizal plant roots. Genes identified from fully developed mycorrhizal plants may show patterns of differential expression due not only to interactions with the fungus, but also to the changed nutrient status, the later only indirectly related to VAM fungal colonisation.

There are several different approaches to cloning differentially expressed genes. The first approach adopts a "brute force" strategy, in which as many cDNAs as possible are cloned from the different tissue types. These are sequenced and the number of occurrences of particular sequences are tallied. A differentially expressed gene will show a significantly greater frequency in the cDNA of one tissue type than the other. Due to the large number of sequences used, this technique has been termed electronic subtraction (ES). In practice, between 1000 and 3000 cDNAs need to be sequenced from each cDNA library to identify differentially expressed transcripts (Adams *et al.* 1992; Okubo *et al.* 1992). This approach, although very labour intensive, has the advantage of providing a huge number of expressed sequence tags (ESTs) to the scientific community.

A second, less labour intensive and more direct approach to gene cloning involves the differential screening of cDNA libraries. Probes are made from labelled single stranded cDNA from the different tissue types and hybridised to replica membrane blots of the cDNA libraries. Clones are selected that hybridise differentially to the two probes. An advantage of this method over ES is that the many clones that are not differentially expressed are discarded early in the procedure. Advances in this method involve the treatment of cDNA prior to cloning by subtracting sequences common to the two RNA pools. There are numerous methods available to generate a subtracted library. The general principal incorporates a solution hybridisation step in which the target cDNA is mixed with at least a 20 fold molar excess of control tissue polyA RNA. The molecules that form duplexes (representing RNAs in common to both samples) are removed, and the remaining molecules are used either in probe production or to synthesise a subtracted library. With this approach, a large number of clones in the subtracted library represent genes that have increased expression in target tissue. However, due to the excess of control material used for subtraction, genes whose expression is increased in the target sample can still be subtracted from the library and therefore not identified. Also, genes that have lower levels of expression in the target sample will be lost.

A third approach is that of reverse transcription PCR (RTPCR), also known as differential display (DD) (Liang & Pardee 1992). RNAs from two tissue types is reverse transcribed and the resulting cDNA is used as a template in a PCR. The products are separated by gel electrophoresis, and novel bands derived from the different tissue types are likely to represent sections of genes that are differentially expressed. This approach has the advantage of being able to identify low abundance genes. However there is a high rate of false positives and abundant polyA RNAs may be missed as amplification is primer sequence dependent.

In this study, clones were identified by differential hybridisation of a cDNA library. This approach was successfully used by Murphy *et al.* (1997) to identify four genes of plant origin that were differentially expressed in roots upon mycorrhizal colonisation. This approach is likely to identify genes of plant origin that are moderately to abundantly expressed in root tissue, and have significant changes in expression upon mycorrhizal colonisation. This method was chosen for an initial study of the mycorrhizal symbiosis as relatively little is known about the likely differences in transcript levels between tissue types, and the risk that genes might be lost in a subtractive approach was considered significant. Furthermore, as fungal structures contribute little to the total root biomass, particularly as the

library was synthesised at an early stage of mycorrhizal colonisation, most cDNAs are likely to represent polyA RNAs that originate from the plant, with only a few derived from the fungus.

5.2 Purification of Clones

The five clones that were examined in this study were chosen on the basis that they represented genes with increased expression in mycorrhizal roots from a previous differential screening of a cDNA library as outlined in section 2.2.3 (Rosewarne 1993). Phage DNA was purified (2.2.1.1) and the inserts subcloned into the *Eco*RI site of the vector pGEM3zf+ (2.2.4) for further analysis. The clones were given the names LerGi 1, 2, 4, 5 and 6; LerGi being an acronym for the interaction between roots of *L. esculentum* cv Rio Grande, and the mycorrhizal fungus, *Glomus intraradices*.

Plasmid DNA was prepared from isolated colonies of each of the LerGi clones (2.2.1.2) and digested with *Eco*RI for size fractionation by agarose gel electrophoresis to confirm that the insert size in the plasmid vector corresponded to that in the phage vector from which it was cloned.

5.3 Primary Sequencing of Clones

The primary sequencing of clones was the same as outlined for the P11 clone in the previous chapter (4.3). The DNA sequence and any predicted open reading frames were submitted to Blast search on the GenBank and EMBL databases. The results of sequence searches are summarised in Table 5.1.

Table 5.1 Blast search results summarising the main database sequence similarities to each of the five clones.

Clone	Similarity
LerGi 1	Ribosomal protein S17
LerGi 2	Root knot nematode induced plant gene
LerGi 4	S-adenosyl-L-homocysteine hydrolase
LerGi 5	Phospholipase D
LerGi 6	Ribosomal protein S17

The sequence data showed that LerGi 1 and LerGi 6 were identical clones. LerGi 6 was therefore not analysed any further. The remaining four clones were studied through the techniques of DNA gel blot hybridisation and RNA gel blot hybridisation. Part of the LerGi 2 sequence had been previously identified as belonging to a gene that is expressed in giant cells that are formed in *L. esculentum* upon parasitism by the root knot nematode, *Meoidogyne incognita* (Bird and Wilson 1994). The finding that this gene appeared to show increased expression in both a symbiotic and parasitic interaction was intriguing, and the expression of LerGi 2 was further analysed by *in situ* hybridisation. Experiments pertaining to the characterisation of each of the LerGi clones are outlined below.

5.4 LerGi 1

5.4.1 Sequence Analysis of LerGi 1

The LerGi 1 sequence was 645 nucleotides in length and primary sequencing reactions in both directions gave an overlap of 118 nucleotides (Fig. 5.1). The nucleic acid sequence is shown in Appendix 3. The nucleotide sequence had extremely high levels of sequence similarity to the human S17 ribosomal protein. Between 60-80 ribosomal proteins associate with 3-4 rRNA molecules to make the ribosomes. The ribosomal proteins make up to 15% of the total protein content of the cell. The cloning of the S17 ribosomal gene in this study probably reflects the high level of transcript abundance expected for this type of gene.

5.4.2 RNA Gel Blot Analysis of LerGi 1

Confirmation that the cloned sequences represented genes that were differentially expressed in mycorrhizal vs. non-mycorrhizal roots was required. RNA gel blot hybridisations were completed to investigate the steady state levels of LerGi 1 RNA in various tissue types and at various stages of mycorrhiza development. The RNA gel blot analysis of LerGi 1 was the same as outlined in Section 4.8 for the P11 clone with the exception that only one gel blot was hybridised to the probe.

At the earliest time point (day four), transcript levels were much higher in the mycorrhizal roots than in the non-mycorrhizal roots. As time progressed, expression decreased in mycorrhizal roots (Fig. 5.2a and 5.3a). There could be two reasons for this decrease in expression over time. Firstly, the expression levels could be related to altered growth rates, as it was shown that mycorrhizal root growth slowed by the day 12 harvest (Fig. 3.5). This reduction in growth is

likely to be associated with a reduction in gene expression, and it has been shown that altered expression of ribosomal protein genes occurs under changes in cell growth (Nomura *et al.* 1984). Secondly, as colonisation increased, there would be an increasing contribution of fungal RNA to the total RNA pool. The rRNA gene used to quantify RNA loading would hybridise equally to plant and fungal transcripts, resulting in LerGi 1 transcripts appearing to be less abundant as time progressed.

Starkey and Levy (1995) identified an number of ribosomal proteins including a S17 homologue from a differential screen between healthy cat thymic tissue and cat thymic tumor tissue. A number of other ribosomal genes were co-ordinately upregulated in the tumor tissue and were proposed as regulatory mediators of growth, proliferation and neoplastic change. The mechanisms of regulation of the ribosomal protein genes have not clearly been defined, however as a number of these genes are co-ordinately regulated under changing cellular conditions (Shi & Tyler 1991, Bird D. McK. pers. comm.) additional members of the translational apparatus in *L. esculentum* may also be expected to have increased expression levels in roots upon mycorrhizal colonisation.

If other ribosomal genes are found to have expression co-ordinately increased during the early stages of mycorrhizal colonisation, future work could focus on the promoter elements involved in the transcriptional regulation of these genes.

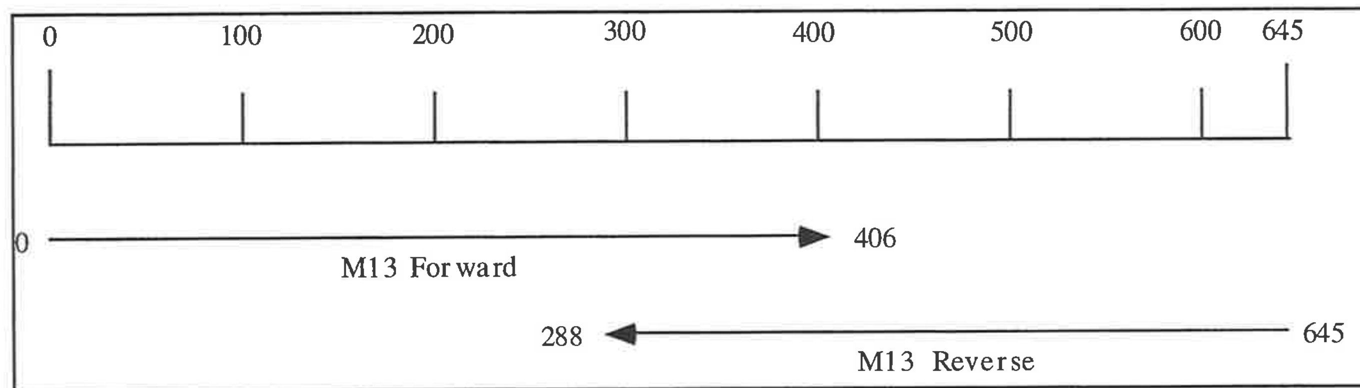
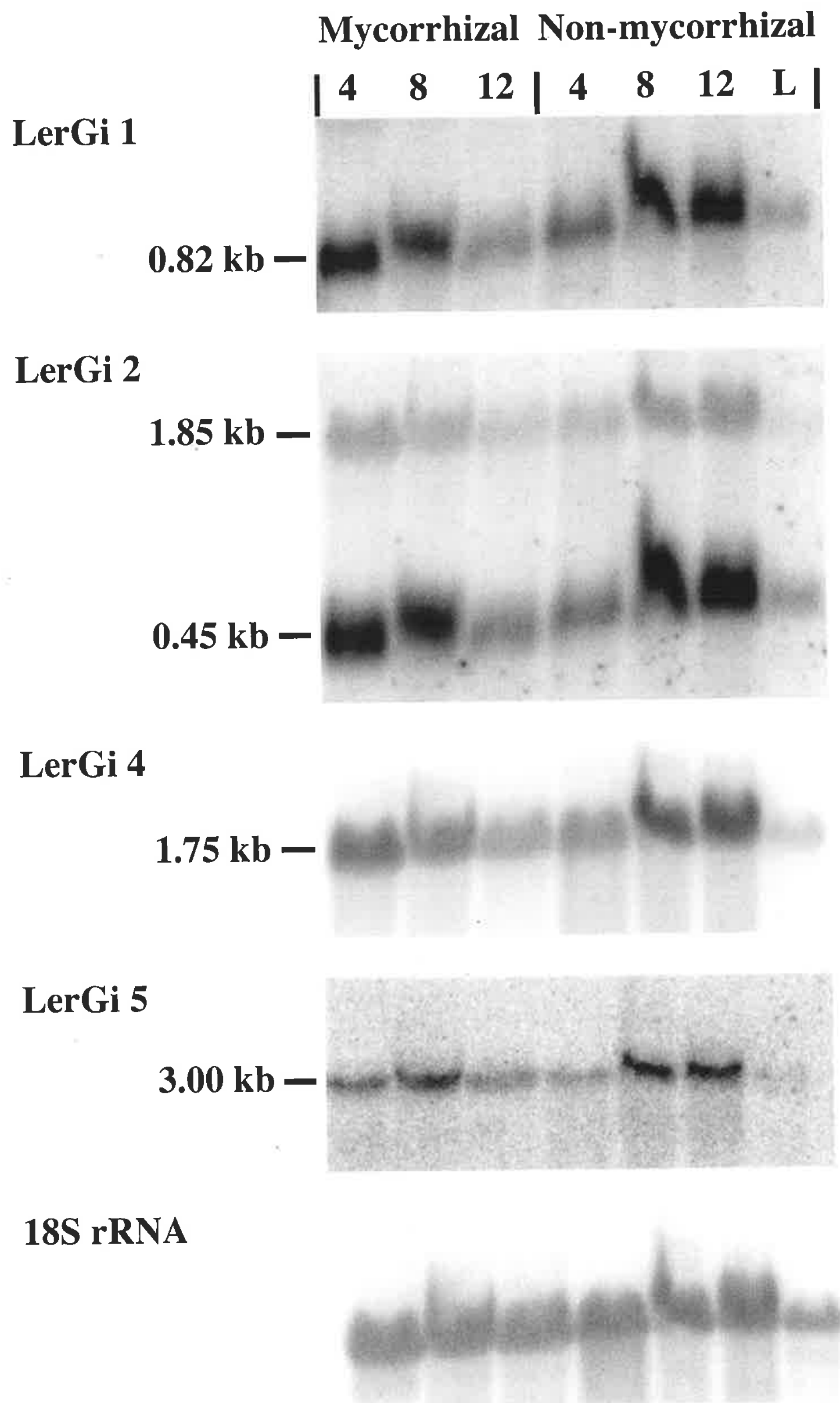


Figure 5.1 Sequencing of LerGi 1. LerGi 1 was sequenced in both direction using M13 forward and reverse primers. With this strategy, an overlap of 118 nucleotides was determined, with the total sequence length being 645 bp. Arrows represent direction of the sequencing runs.

Figure 5.2 RNA gel blot analysis of the LerGi clones. 10 µg of total RNA from mycorrhizal and non-mycorrhizal roots at four, eight and 12 days after transplantation and 4 µg from non-mycorrhizal leaf was run in a denaturing RNA gel and the gel blotted onto a nylon membrane. The membrane was sequentially probed with each of the LerGi clones (membrane stripped between hybridisations) and finally with a rRNA probe to quantify loading of each sample. Hybridisation signals were generated with A) LerGi 1 probe, B) LerGi 2 probe, C) LerGi 4 probe, D) LerGi 5 probe and E) ribosomal RNA probe.


Mycorrhizal
Non-mycorrhizal
4
8
12
4
8
12
L
LerGi 1
0.82 kb
LerGi 2
1.85 kb
0.45 kb
LerGi 4
1.75 kb
LerGi 5
3.00 kb
18S rRNA

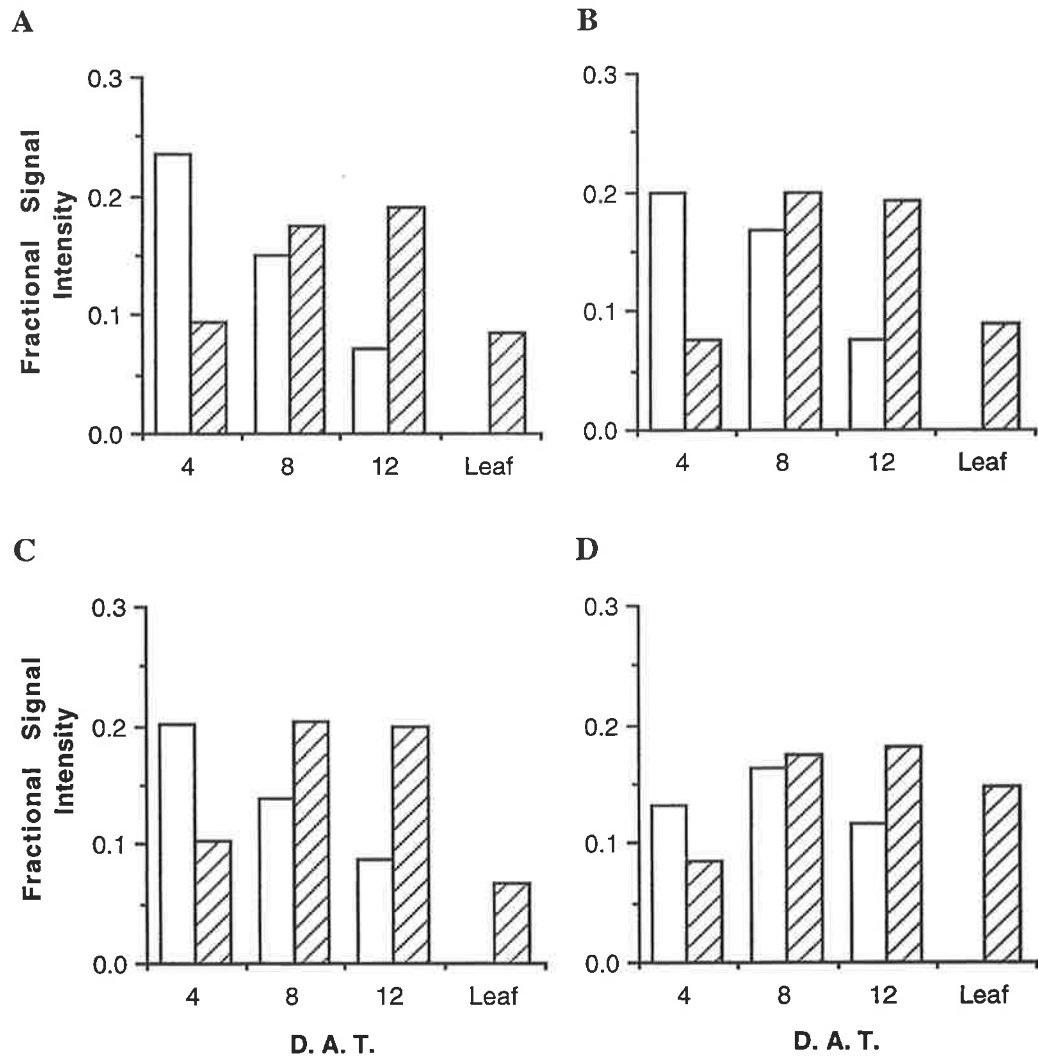


Figure 5.3 Graphical representation of the hybridisation signals generated from gel blots in Fig. 5.2. The individual bands in each blot were quantified and normalised with regard to the signal generated by the rRNA probe. Values represent a percetage of the total signal generated from the sum total of all bands for hybridisation of A)LerGi 1 probe, B) LerGi 2 probe, C) LerGi 4 probe, and D) LerGi 5 probe. RNA samples from mycorrhizal root tissue ☐ and non-mycorrhizal tissue ▨.

5.5 LerGi 2

5.5.1 Sequence Analysis of LerGi 2

The LerGi 2 clone was sequenced with the M13 forward and reverse primers (Fig. 5.4). The two sequences overlapped, with the complete clone being 448 bp in length (sequence details in Appendix 3). From the sequence several putative open reading frames were predicted (Fig. 5.4).

The LerGi 2 clone had no significant sequence similarities with any genes of known function in the data bases. However one cDNA sequence from *L. esculentum* showed very high levels of similarity to a portion of LerGi 2. This clone was identified as DB113 which had been cloned from giant cells induced in *L. esculentum* by the root knot nematode, *Meloidogyne incognita* (Bird & Wilson 1994). In the nematode work, a subtractive approach was used to clone 287 cDNAs that represented transcripts that were expressed in the giant cells. As both LerGi 2 and DB113 were cloned from oligo dT primed polyA RNA, the clones were expected to represent the three prime ends of the genes. DB113 was shorter than LerGi 2. No open reading frame could be predicted from the sequence available for DB113. A Blast protein database search was completed on all the predicted open reading frames from LerGi 2 but failed to show any significant amino acid sequence similarity to any characterised genes. These putative amino acid sequences had either not previously been characterised or were not real.

Although no function could be identified from the sequence analysis, the gene represented by this clone was of particular interest because it is potentially involved in both the pathogenic nematode interaction and the symbiotic mycorrhizal symbiosis. As part of the characterisation of LerGi 2, expression levels in mycorrhizal and non-mycorrhizal roots was investigated. The results and discussion are below.

5.5.2 RNA Gel Blot Analysis of LerGi 2

The LerGi 2 clone was radiolabelled and used to hybridise to RNA gel blots as outlined in Section 4.8. The signals generated with LerGi 2 showed that it bound to two bands in each RNA sample loaded. The approximate sizes of the RNAs were 1800 and 450 nucleotides (Fig. 5.2b). The larger band was much fainter, reflecting either lower transcript abundance, or a lower degree of sequence

similarity. The sequence data showed that LerGi 2 was 448 bp in length and this is likely to represent the full length of the transcribed sequence.

The relative expression of the two transcripts (1800 bp and 450 bp) was constant, regardless of the stage of development of mycorrhizal roots. The intensity of the stronger band (450 bp) was determined for RNA samples from each tissue and normalised with respect to loading as determined by an rRNA hybridisation (Fig. 5.2e). Expression was highest in the mycorrhizal roots at days four and eight after transplantation, but dropped by day 12. Conversely, the control tissue samples had low levels of expression at day four, but these levels increased threefold by days eight and 12.

Changes in gene expression occurring over time in uncolonised roots must relate to other biological changes in the root and are still unexplained. This expression pattern was common to all clones analysed, and is discussed in Section 5.8

5.5.3 DNA Gel Blot Analysis of LerGi 2

The method used for genomic DNA hybridisation has been outlined in Section 4.7 for the P11 clone. An identical procedure was used for the LerGi 2 clone. The probe bound to either one or two bands in the digests (Fig. 5.5a), suggesting that LerGi 2 is either a single copy gene or belongs to a small gene family. This is in agreement with data from RNA gel blot analysis, which also supported the idea that LerGi 2 belongs to a small gene family as two different sized transcripts bound to the probe.

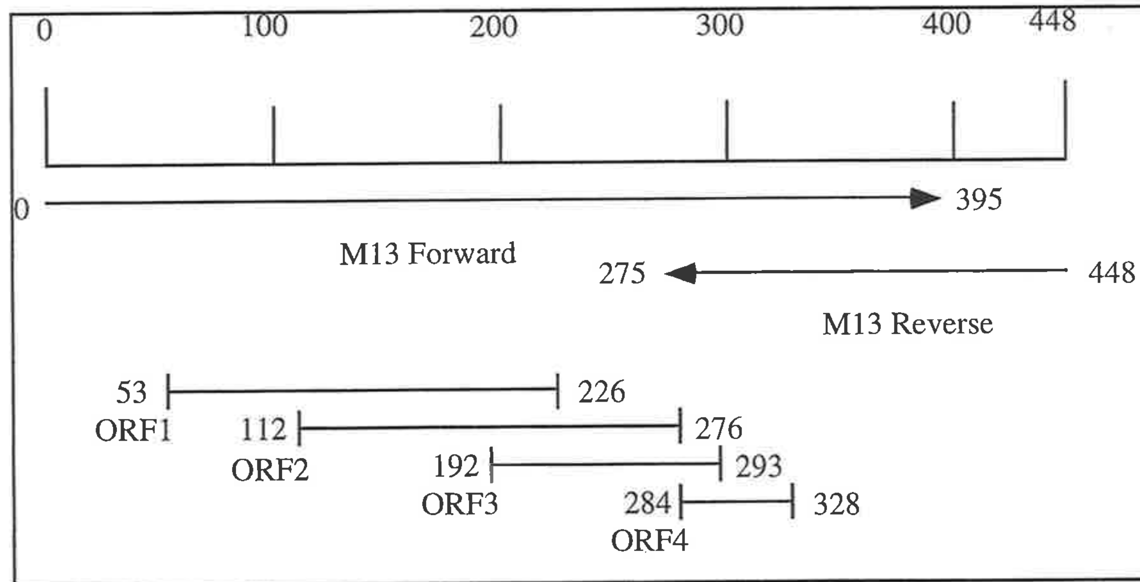


Figure 5.4 Sequencing and predicted ORFs of the LerGi 2 clone. LerGi 2 was sequenced in both direction using M13 forward and reverse primers. With this strategy, an overlap of 120 nucleotides was determined. Arrows represent direction of the sequencing run. Four putative Open Reading Frames (ORF) are indicated. The numbers represent the first nucleic acid of the start codon (to the left of the line) and the last nucleic acid of the stop codon (to the right of the line).

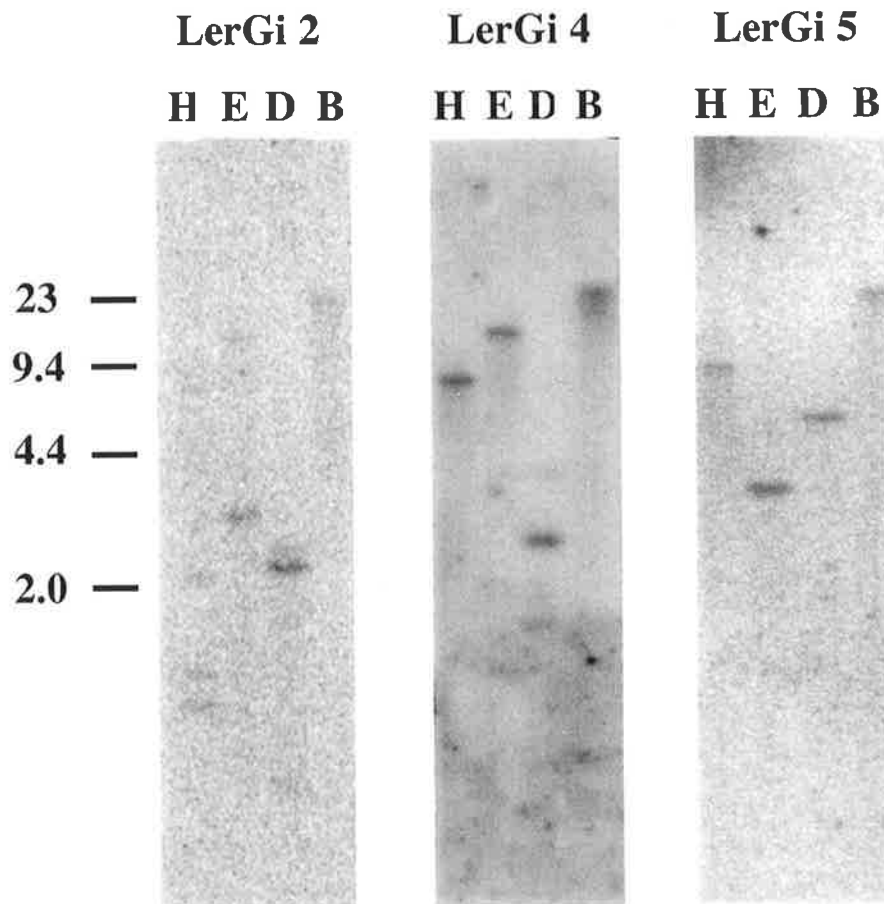


Figure 5.5 DNA gel blot of the LerGi clones. 5 µg of genomic *L. esculentum* DNA was run in a non-denaturing agarose gels and blotted onto nylon membranes. These were probed with A) LerGi 2, B) LerGi 4 and C) LerGi 5 and washed under high stringency conditions.

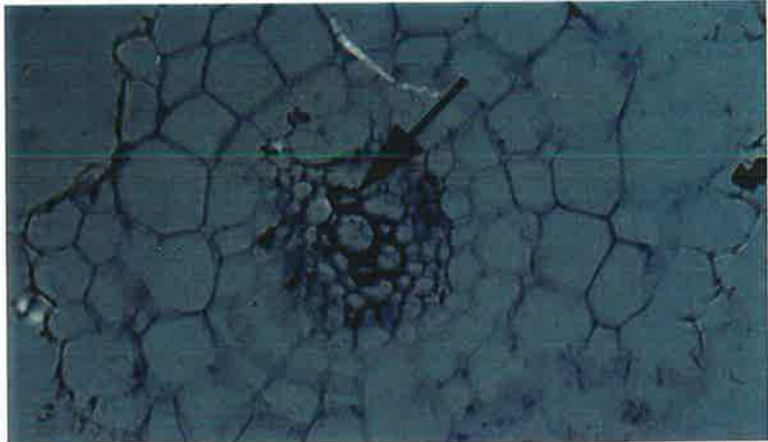
5.5.4 *In situ* hybridisation of LerGi 2

To further characterise the LerGi 2 clone was used for *in situ* hybridisation according to the procedure outline in Section 2.2.11. The anti-sense probe of LerGi 2 hybridised weakly to all root cortical cells and strongly to protoxylem tissue of both mycorrhizal and non-mycorrhizal roots. There was also a strong signal in arbuscule-containing cells in mycorrhizal roots (Fig. 5.6). Bird and Wilson (1994) showed that DB113 (the homologue of LerGi 2) was expressed in giant cells, which, during their early stages of formation, strongly resemble developing xylem (Grundler & Payne 1978). There are two possible processes that can be implicated with gene expression observed in arbuscule-containing cortical cells, protoxylem cells and giant cells. Firstly, all of these cells types represent a possible sink for carbon drain and LerGi 2 may be involved in the process of nutrient transport to these cells. Secondly, these cells are undergoing differentiation and this gene may play a common role in the development of all three cell types.

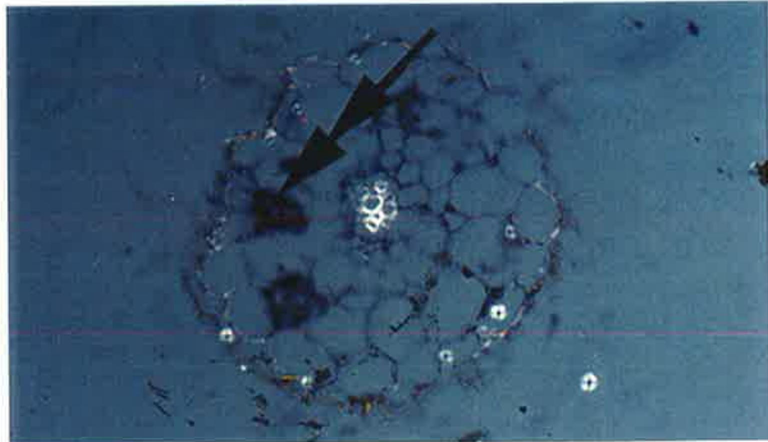
There are two problems in interpreting the data from *in situ* hybridisation of mycorrhizal roots in terms of changes in plant gene expression. Firstly, the clone LerGi 2 was not checked for cross hybridisation with fungal DNA in gel blot hybridisations. As VAM fungi are obligate symbionts, the availability of fungal DNA is limited. This is particularly so for *G. intraradices* as it has small spores that are difficult to isolate from soil. A PCR approach could be adopted if a small quantity of spore DNA could be obtained, rather than using DNA hybridisation. This would involve designing primers from the LerGi 2 sequence and amplifying DNA fragments from fungal and plant DNA. This approach would be relatively easy for large-spored members of the Glomales, but is difficult for *G. intraradices.* The second problem concerns the dual signal observed in the RNA gel blot hybridisations which may indicate the expression of two different genes. It is not known which homologue LerGi 2 hybridised to in the *in situ* hybridisation. To overcome this problem, a shorter probe could be made from the 3' untranslated region of the clone. This region is unlikely to be conserved between homologues in *L. esculentum* or the mycorrhizal fungus, and the elimination of cross-hybridisation to other gene family members could be confirmed with genomic DNA gel blot hybridisations.

Figure 5.6 *In situ* hybridisation of mycorrhizal (A and B) and non-mycorrhizal (C and D) root sections. Signal is generated with the anti-sense probe (A and C) compared to the sense probe (B and D). Single arrow indicates hybridisation to tissue within the vascular cylinder, double arrows to protoxylem tissue and triple arrows to arbuscule-containing cortical cells.

A)



B)



C)

5.5.5 Screening Additional Nematode Induced Giant Cell Clones

Following the finding that the sequences of DB113 and LerGi 2 were essentially identical, clones that represented genes expressed in giant cells induced by the root knot nematode (*Meloidogyne incognita*) in *L. esculentum* (Bird & Wilson 1994) were obtained in the form of colony blots bound to nylon membranes (colony blots supplied by D. Bird). All 287 giant cell-related cDNA clones were screened against RNA extracted from mycorrhizal and non-mycorrhizal *L. esculentum* roots. The aim was to determine if there were further similarities in genes involved in both the mycorrhizal and root knot nematode interactions. The membranes were hybridised to a probe made from reverse transcribed RNA from mycorrhizal roots. Probe synthesis and hybridisation is described in 2.2.3.4. After the first hybridisation, the membranes were stripped and hybridised to a probe from non-mycorrhizal roots.

The results of this screening were largely inconclusive, mainly due to the insensitivity of the procedure. The colony blots were large (approximately 5 mm diameter) and a signal from the probe (a mixture of labelled cDNA synthesised from a polyA RNA pool) would only be generated if the particular clone represented a high abundance gene. To increase the sensitivity of the hybridisation, RNA transcripts could be synthesised from the clones for blotting. This would provide more concentrated target DNA with less background that is associated with colony blots. The individual clones were not available for this study. However, one clone was observed to hybridise differentially to the two probes (DB297). The hybridisation signal from the control root probe was nearly three times that from the mycorrhizal root probe (Fig. 5.7). Unfortunately, DB297 did not show sequence similarity to any known gene in the databases (Bird & Wilson 1994). Although this finding is interesting, investigation of the DB297 clone has not been pursued further at this stage. RNA gel blots need to be completed to confirm the differential expression of the gene represented by DB297.

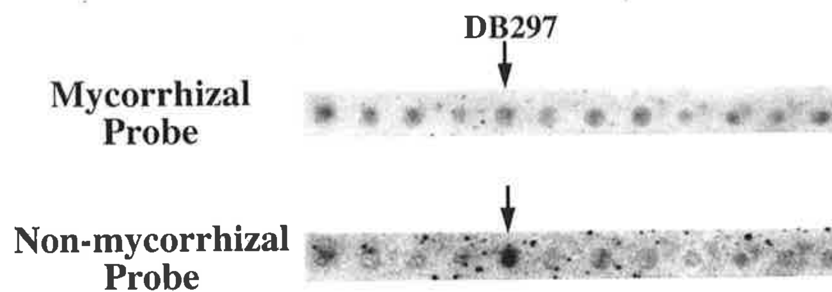


Figure 5.7 Representative differential screening of 12 of the 287 DB clones. ^{32}P dCTP was incorporated into were reverse transcribed polyA RNA from mycorrhizal (A) and non-mycorrhizal roots harvested at day 4 after transplantation and sequentially hybridised to the membrane (membrane stripped between hybridisations). Only clone DB 297 showed differential hybridisation to the two probes.

5.5.6 Discussion of LerGi 2

The independent cloning of a plant gene involved in both symbiotic and parasitic nematode interactions with roots was extremely interesting and to gain possible insights into what kind of gene would function in both biological systems, I will briefly summarise what is known of the plant-root knot nematode interaction.

Plant parasitic nematodes are very common in nature and it is likely that all vascular plants are hosts for at least one parasitic nematode species. The root knot nematodes (*Meloidogyne* ssp.) are endoparasites that cause much damage to many important crops and as a consequence are much studied (Bird 1997).

Meloidogyne ssp. share several characteristics with VAM fungi in that they both have very broad host ranges (Sasser 1980), are obligate on their hosts for reproduction, do not elicit a significant defence response from the host, and like all pathogenic or mutualistic interactions, are involved in nutrient transfer (Bird 1997). Given these factors one might expect there to be some similarities at the molecular level in the establishment and maintenance of these interactions.

Root knot nematodes hatch in the soil and emerge as motile, vermiform second-stage larvae that actively seek host tissue (Bird 1997). Invasion of the root occurs either immediately behind the root tip or at the rupture sites of lateral root emergence (Krusberg and Nielsen 1958). Following penetration of an epidermal cell, the nematode migrates apoplastically to the developing vascular cylinder (Wyss *et al.* 1992). Here the nematode stimulates the plant to produce giant cells and the nematode becomes sedentary for the remainder of its life cycle.

It is not know how giant cells are induced, but cells in the stele become enlarged, metabolically active and undergo rounds of nuclear division without cytokinesis, resulting in polyploidy. Extensive cell wall remodelling occurs with the development of wall ingrowths (Bird 1961). The giant cells are largely responsible for the characteristic gall that forms in infected roots.

The sedentary nematode relies on the giant cells as its only source of nutrition. The giant cell unloads photosynthate from the phloem. The nematode then gains access to the photosynthate via its feeding stylet. (Bird and Loveys 1975). At the onset of egglaying, the parasite increases its nutritive demand on the host.

As previously mentioned, Bird and Wilson (1994) used a subtractive approach to yield over 220 different clones, many of which showed different expression patterns in various plant tissues, but all were expressed in giant cells. Sequence analysis allowed function to be assigned to 56 of the clones, and Bird (1997) divided these into three classes based on their possible functions: 1) wound/defence response, 2) differentiated giant cell function, and 3) signal reception/transduction. These three categories of interaction could also apply to VAM fungal colonisation, with category 2 corresponding to symbiosis-specific genes or those genes coding for proteins associated with membranes, including nutrient transport proteins.

As no sequence homologies to known genes were found for LerGi 2, its role in plant microbe interactions is still inconclusive. However, one could speculate on the possible functions of this gene. It is most highly expressed in mycorrhizal roots at day 4. This time point was considered to be "pre-arbuscular" and as a consequence it seems unlikely that LerGi 2 is involved in phosphate transfer between the symbionts. However, the *in situ* hybridisation results also indicated that arbuscule-containing cortical cells expressed the LerGi 2 gene. The location of carbon transfer from plant to fungus is currently unknown. It has long been assumed that both carbon and phosphate transfer occurs at the arbuscular interface although this has been challenged (Gianinazzi-Pearson *et al.* 1991). If carbon transfer does occur at the arbuscular interface, then expression of LerGi 2 in arbuscule-containing cells may indicate an involvement of LerGi 2 in carbon uptake. As with LePT 1 there is a conflict between the *in situ* hybridisation and whole organ expression (RNA gel blots), where overall expression fell as arbuscules developed. However a transport related protein may function more effectively in the presence of higher concentrations of the transported solute, leading to a smaller requirement for the protein molecules and possibly, gene transcripts in arbuscular roots. LerGi 2 also hybridised to protoxylem. These cells may be carbon sinks which require the import of photo-assimilates throughout their development.

These data suggest an involvement in carbon transfer to the invading organisms, but other functions cannot be ruled out. The expression pattern could indicate an involvement in signal transduction in developing xylem and in response to colonisation by VAM fungi. Further work could involve immunolocalisation of the translated protein of LerGi 2 with sub-cellular localisation of the protein giving further clues to its function. It would be interesting to examine the phenotype of plants expressing the antisense construct of LerGi 2 in both

mycorrhizal and *M. incognita* infected plants, to see if this gene is essential for colonisation by these organisms.

5.6 LerGi 4

5.6.1 Sequence Analysis of LerGi 4

LerGi 4 was sequenced from either end of the clone and the initial sequence was used to design primers for continued sequencing (Fig. 5.8). Four internal primers were designed (Appendix 1; LerGi4F1, LerGi4F2, LerGi4R1 and LerGi4R2) and the resulting sequences overlapped such that every base had been determined in at least one direction. The insert was 1721 bp with a 1455 bp open reading frame (Appendix 3). The predicted protein consisted of 485 amino acids, producing a protein with a molecular weight of 53,371.

The predicted amino acid sequence of LerGi 4 revealed high levels of sequence similarity to genes coding for the enzyme S-adenosyl-L-homocysteine hydrolase (SAHH) from a number of organisms including plants (*Nicotiana tabacum, Petroselinum crispum, Catharantus roseus, Medicago sativa* and *Phalaenopsis* sp.), animals (*Homo sapiens, Rattus rattus, Caenorhabditis elegans, Xenopus laevis*) and the photosynthetic bacterium *Rhodobacter capsulatus*. The high degree of homology occurring across a variety of organisms reflects a high level of functional constraint on the protein (Seery *et al.* 1994). It is highly likely that LerGi 4 does code for a SAHH gene. A predicted amino acid sequence alignment of LerGi 4 with plant SAHH proteins is shown in Fig. 5.9.

The SAHH protein catalyses the reversible hydrolysis of S-adenosyl-L-homocysteine (SAH) to adenosine and L-homocysteine, through an oxidation/reduction reaction involving NAD as a cofactor (de la Haba & Cantoni 1959). SAH forms part of the activated methyl cycle (Stryer 1988) where S-adenosylmethionine (SAM) is converted to SAH in the process of methylating a variety of substrates including nucleic acids, proteins and lipids. SAHH is a major adenosine- and cAMP-binding protein in many tissue types, being pivotal in normal cellular metabolism (Hershfield & Kredich 1978). Methyl transferases that use SAM as a methyl donor are inhibited by SAH. The SAH:SAM ratio is controlled by SAHH, thereby regulating SAM-dependant transmethylation reactions in most eukaryotic organisms (Shimizu *et al.* 1984). SAHH forms tetramers in *Rattus rattus* (Gomi *et al.* 1986) and either diamers or tetramers in

plants, with each subunit binding one NAD molecule (Seery *et al.* 1994 and references therein).

Sequence analysis reveals several highly conserved characteristics of the gene. A consensus site for NAD binding was observed and contains the amino acid sequence, GXGXXG, located at amino acid 269-274. An ADP moiety is thought to bind in this position, held in place by an α-helix, β-sheet and α-helix motif. Two cysteine residues, Cys^{86} and Cys^{120} are also highly conserved and likely to be involved in the secondary structure of the protein. Cys^{86} corresponds to Cys^{78} in the rat liver SAHH. There was greatly reduced activity of the rat liver SAHH when site specific mutations of Cys^{78} - Ser^{78} and Cys^{78} - Ala^{78} were completed. The corresponding mutations on Cys^{112} (Cys^{120} in LerGi 4) resulted in small kinetic changes, indicating this cysteine is not as important in function (Aksamit *et al.* 1994). The Cys^{112} is conserved in all except the *R. capsulatus* SAHH sequences, supporting the findings of the mutagenesis experiments.

Table 5.2 compares LerGi 4 with other cloned SAHH genes. The sequence length of SAHH genes cloned from plants and from animals is different. All SAHH genes from higher animals lack a 41 amino acid sequence at approximately 150 amino acids from the amino terminus. It has been proposed that a single deletion gave rise to a modified SAHH gene prior to or during the protozoan radiation (Seery *et al.* 1994).

Table 5.2 Predicted protein length and size of SAHH proteins from a variety of organisms.

Origin	Predicted Protein Length (aa)	Molecular Mass (kDa)
L. esculentum (LerGi 4)	485	53.4
N. tabacum	485	-
N. sylvestris (CBP57A)	485	57
P. crispum	485	53.2
Xenopus laevis	433	47

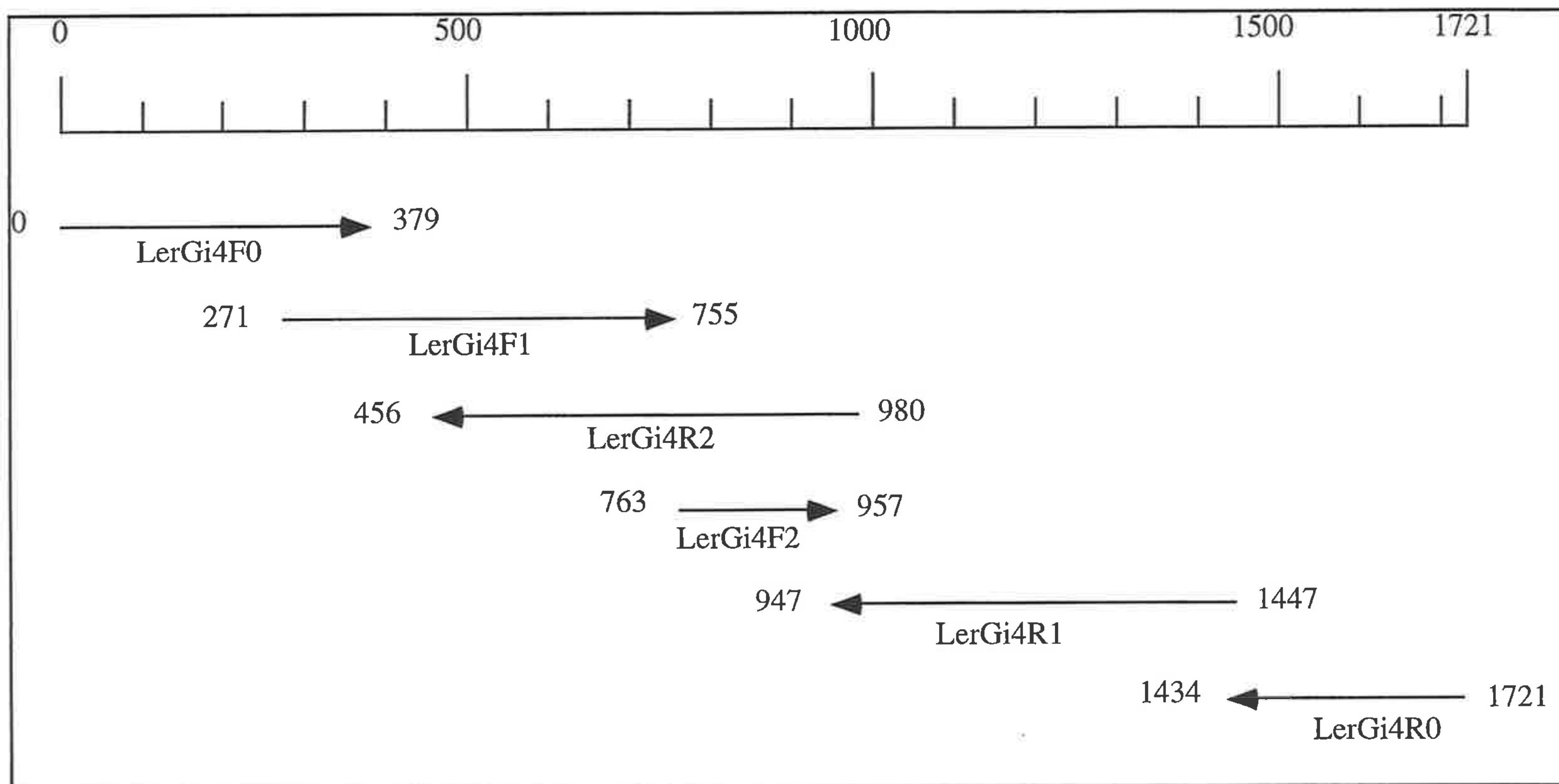


Figure 5.8 Sequencing of the LerGi 4 clone. The full sequence of LerGi 4 was determined in at least one direction. Arrows represent the length and direction of the sequencing runs. The LerGi 4 clone was initially sequenced using M13 forward and reverse primers. These sequences were used to design additional primers to complete sequencing of the LerGi4 clone.

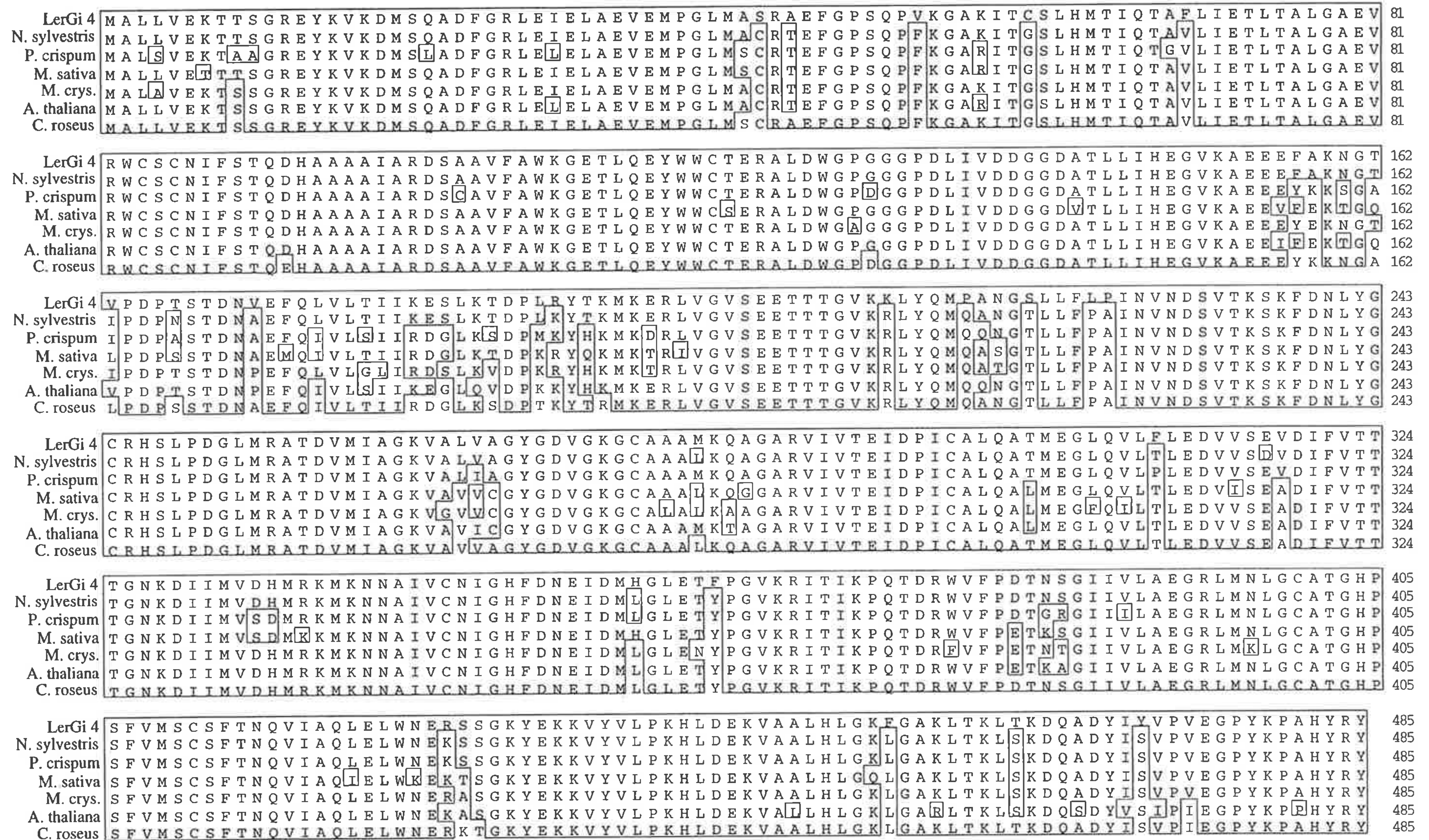


Figure 5.9 Sequence alignment of the predicted amino acid sequence of LerGi 4 with other plant S-adenosyl-L-homocysteine hydrolase amino acid sequences.Conserved sequences are boxed.

5.6.2 RNA Gel Blot Analysis of LerGi 4

The LerGi 4 clone hybridised to RNA transcripts of approximately 1.75 kb in size (Fig. 5.2c). This corresponds closely with the cDNA size of 1.721 kb, indicating that the clone is almost the full length of the polyA RNA transcript, with the cDNA of LerGi 4 covering the entire open reading frame. Other plant SAHH transcripts are close to this size, with those from *Petroselinum crispum* being 1.9 kb (Kallaweck *et al.* 1992) and *Medicago sativa* being 2.3 kb in length (Abrahams *et al.* 1995). LerGi 4 was expressed more highly in roots than in leaves (Fig. 5.3c) These results agree with observations of Mitsui *et al.* (1993) and Tanaka *et al.* (1996).

Analysis of the expression pattern of LerGi 4 shows that the gene is highly expressed in the early stages of the mycorrhizal symbiosis (day four after transplantation) but that expression declines to approximately half of the day four level by day 12. Expression of an SAHH gene in *P. crispum* cells increased after treatment with a fungal elicitor (Kallaweck *et al.* 1992), and the high levels of expression observed early in the symbiosis could be related to a response of the plant to the invasion of the root system by the mycorrhizal fungus. Kallaweck *et al.* (1992) suggested that the increase in expression was in response to a greater requirement for methylation for the production of plant phytoalexins. As stated in Chapter 1, there appears to be a small transient defence response early in the mycorrhizal symbiosis, and the high levels of expression observed here could be a reflection of this response. Attenuation of the plant defence response would then also be reflected in the lower expression levels of this gene at later stages of the symbiosis, as observed.

Another reason for the decline in gene expression observed in mycorrhizal roots could be related to root growth. By day 12 the mycorrhizal roots had stopped increasing in fresh weight (Fig. 3.5). Richards and Gardner (1994) treated wheat roots with toxic levels of aluminium, and found that root growth was inhibited after 4 days, and that this correlated with decreased levels of expression of SAHH. They surmised that this expression pattern was a reflection of a decreased requirement for methylation due to decreases in synthesis of DNA and/or lignin.

Expression of LerGi 4 in non-mycorrhizal roots increased between the first two harvests (days four and eight after transplantation), remaining high through to the final harvest (12 days after transplantation). The link between SAHH expression

and lignin requirements may explain the observed trends of higher expression in the day eight and 12 harvests. These roots probably have greater secondary thickening and therefore a greater requirement for lignification, although this was not tested.

5.6.3 DNA Gel Blot Analysis of LerGi 4

LerGi 4 hybridised to a single band in the DNA gel blot. This indicates that it may be a single copy gene. Other researchers have found that SAHH genes belong to small gene families in other plant species. Mitsui (1993) cloned two similar SAHH genes from *Nicotiana sylvestris* that had approximately 80% amino acid sequence similarity with each other. DNA gel blot analysis in Mitsui's study showed between one and three restriction fragments hybridised to DNA digested with various restriction enzymes, indicating that more than one gene is present. DNA gel blot analysis of *N. tabacum* (Tanaka *et al.* 1996) and *Petroselinum crispum* (Kawalleck *et al.* 1992) indicated that more than one gene also occurred in these plants. Similar results were obtained with *Medicago sativa*, indicating gene duplication. However, as *M. sativa* is autotetraploid and out-crossing, a situation which tends to generate polymorphisms, the results could indicate heterozygosity. It seems likely that LerGi 4 belongs to a small gene family in *L. esculentum*. The DNA blot analyses mentioned above used relatively low final stringency washes, and a lower stringency wash would probably reveal homologues of this clone in *L. esculentum*.

5.6.4 Discussion of LerGi 4

The implication of SAHH with the mycorrhizal symbiosis (as identified in this study) indicates that a targeted approach to gene cloning would be worthwhile to investigate related genes and their expression and roles in the symbiosis. Other genes likely to be differentially regulated are those involved in the activated methyl cycle, such as homocysteine methyltransferase, ATP:L-methionine-S-adenosyltransferase and the enzymes involved in the transmethylation reactions of the large variety of compounds methylated by SAM.

SAHH has been shown to have increased rates of expression in cultured cells and intact leaves of *P. crispum* after treatment by a fungal elicitor (Kawalleck *et al.* 1992). As methylation reactions are required in the production of various phytoalexins in the phenylpropanoid pathway (Hahlbrook & Scheel 1987), Kawalleck *et al.* (1992) concluded that the activated methyl cycle is closely linked

metabolically with phytoalexin production. Assuming a link between SAHH and phytoalexin production, expression of both the SAHH gene and genes associated with phytoalexin production could follow similar expression patterns. As stated in chapter one, a mild defence response by roots upon colonisation by VAM fungi has been observed, which, on occasion, was repressed as the colonisation developed. A similar pattern was observed with the SAHH gene in the mycorrhizal roots of this study, where SAHH expression was high in roots at an early stage of colonisation and expression decreased as the colonisation developed. SAHH may be part of a signal transduction pathway that produces defence related transcripts, but the question remains, how is SAHH regulated?

Mitsui (1993) identified an SAHH protein through its capacity to bind cytokinin and postulated that cytokinins may modulate SAHH activity by binding to this enzyme in such a way that function is inhibited. Masuta *et al.* (1995) showed increased levels of cytokinin activity in transgenic tobacco plants expressing SAHH antisense RNA, while Tanaka *et al.* (1996) used the SAHH promoter region fused with a GUS reporter gene to show that kinetin activated the expression of GUS. These data indicate that SAHH is involved in a tightly regulated cycle involving cytokinin, in which the SAHH enzyme can control the level of cellular cytokinin, while cytokinin activates the expression of SAHH. Investigations into how cytokinins may interact to regulate SAHH transcription in the mycorrhizal symbiosis may provide insights as how colonisation is regulated by the plant. Cytokinin levels are known to be increased in VAM roots (van Rhijn *et al.* 1997), supporting that a possible interaction occurs.

5.7 LerGi 5

5.7.1 Sequence Analysis of LerGi 5

The LerGi 5 clone was sequenced fully in both directions and was 345 bp in length (Fig. 5.10) with a predicted partial open reading frame of 66 amino acids, followed by an untranslated region of 145 bp (sequence details in Appendix 3). The predicted amino acid sequence has high levels of similarity to the carboxy terminal of phospholipase D (PLD) proteins isolated from *Ricinus communis* L. (Wang *et al.* 1994), *Zea mays* L. and *Oryza sativa* L. (Ueki *et al.* 1995). As other characterised plant phospholipase D proteins are over 808 amino acids in length (Table 5.3), LerGi 5 probably represents the partial sequence of the full-length gene.

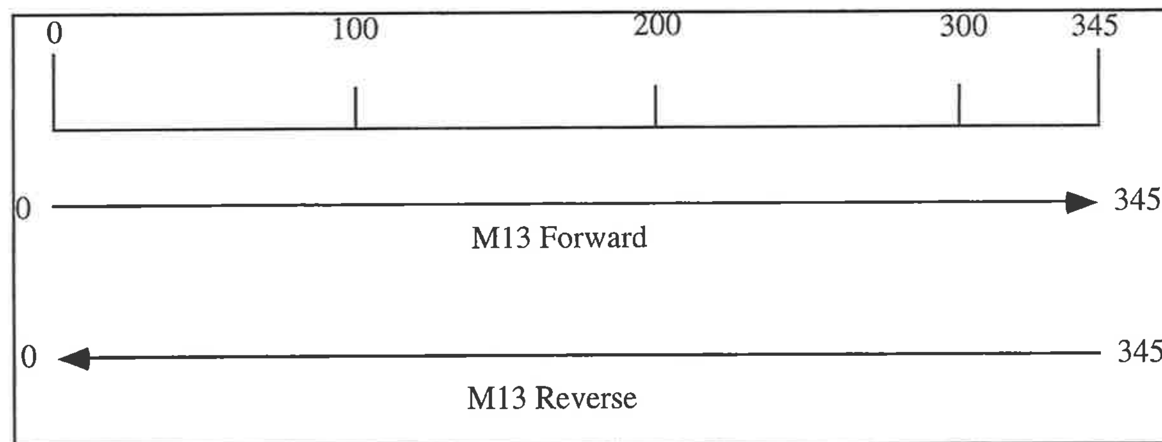


Figure 5.10 Sequencing of LerGi 5. LerGi 5 was sequenced in both direction using M13 forward and reverse primers, emabling the complete sequencing of the clone in both directions. Arrows represent direction of the sequencing run.

Table 5.3 Percentage amino acid identity and similarity of a LerGi 5 predicted partial open reading frame with phospholipase D proteins from a variety of plants (accession no. bracketed).

Plant	% Amino Acid Identity	% Amino Acid Similarity
Arabidopsis thaliana(Q38882)	93	95
Ricinus communis (U72693)	91	93
Nicotiana tabacum (Z84822)	91	93
Vigna unguiculata (U92656)	85	93
Brassica oleracea (U85482)	79	87
Oryza sativa (Q43007)	77	89
Zea mays (Q43270)	77	85

The functioning of PLD enzymes has been extensively investigated in some animal species. Billah (1993) summarised the strong evidence that intracellular phospholipase D is involved with the receptor-linked hydrolysis of phospholipids and the important role played by the enzyme in cellular signal transduction. Through the hydrolysis of phospholipids, phosphatidic acid (PA) is formed and is thought to function as a secondary messenger in signal transduction (Dennis *et al.* 1991). PA is also dephosphorylated to form 1,2-diacylglycerol, a molecule also implicated in signal transduction pathways via its ability to activate protein kinase C (Shukla & Halenda 1991). Working with green algae and carnation petals, Munnik *et al.* (1995) showed that G-protein activators stimulated PLD, implying a role for PLD in signal transduction in plant cells. The mobilisation of storage lipids in seeds after germination is also likely to involve PLD (Wang *et al.* 1994), as is membrane deterioration in ageing seeds (Samama & Pearce 1993). Involvement of PLD in resistance of *Oryza sativa* L. to *Xanthomonas oryzae* pv. *oryzae* has also been proposed (Young *et al.* 1996). These studies indicate that PLD is involved in various functions within the plant.

5.7.2 RNA Gel Blot Analysis of LerGi 5

The LerGi 5 clone was hybridised to RNA gel blots as shown in Fig. 5.2d and the signal intensities were quantified and represented graphically (Fig. 5.3d). At day four, mycorrhizal roots had 50% higher levels of LerGi 5 expression than non-mycorrhizal roots. The expression levels in both mycorrhizal and non-mycorrhizal roots increased to similar amounts by day eight, and then declined in the

mycorrhizal roots by day 12. The higher steady state levels of LerGi 5 RNA in mycorrhizal roots over non-mycorrhizal roots at the day four harvest indicates that PLD may be involved with the early establishment of the symbiosis and this could be related to a mild defence response, signalling or changes in cell membranes. Only one study has examined the role that PLD plays in a plant defence response (Young *et al.* 1996). They examined expression levels of PLD over a five day period in varieties of *O. sativa* that were resistant or susceptible to the bacterial disease cause by *Xanthomonas oryzae* pv *oryzae*. In the resistant plant variety, PLD transcripts increased to a maximum by day two and dropping to control levels by day four. This contrasted to susceptible varieties where transcript levels increased slowly over the first four days after inoculation. Immunolocalisation studies revealed that in the resistant varieties, PLD protein was concentrated in cell membranes adjacent to invading bacteria. In contrast, in the susceptible varieties the PLD protein distribution was dispersed throughout the cell membrane. It would be useful to localise PLD protein in mycorrhizal roots to see if, as was the case with *O. sativa/X. oryzae*, the protein was localised to plant membranes associated with the invading organism (Young *et al.* 1996). Similarities in PLD distribution between resistant plants being invaded by a pathogen and plants in association with a mutualistic symbiotic partner would indicate a degree of commonalty in the recognition and/or signalling pathways used by the host in response to these two very different micro-organisms.

Although both mycorrhizal and non-mycorrhizal RNA samples had a similar level of steady state RNA at the day eight harvest, the increase in expression in the mycorrhizal roots was of interest as none of the other clones analysed (LerGi 1, 2 and 4) showed increased expression from the day four to day eight harvests. This increase occurred at the same time as the peak in arbuscule development in the day eight root material (Fig. 3.5c). As phospholipids are the primary structural component of plant cell membranes, and also are PLD substrates, PLD may play a role in the formation and alterations of the plant plasma membrane surrounding the arbuscule. The peak in PLD expression observed at day eight could either be linked directly to processes operating in plant cells that are undergoing arbuscule formation, for example membrane synthesis, or indirectly in some form of signal transduction pathway, involved in the co-ordination of cellular responses by the plant to the colonising fungus.

Uncolonised roots initially had very low levels of expression that increased 2.5 fold by day eight. Thereafter the expression levels dropped slightly. This is a common pattern observed with genes cloned in this study and is discussed later.

5.7.3 DNA Gel Blot Analysis of LerGi 5

The DNA gel blot hybridisation of LerGi 5 revealed that only one fragment hybridised to the DNA when it was digested with each of the four restriction enzymes used (Fig. 5.5c). This suggests that one copy of the gene exists in *L. esculentum*. Similarly, Ueki *et al.* (1995) noted that *O. sativa* contained a single copy of the PLD gene. Young *et al.* (1996) observed one strong band and two weak bands when probing *O. sativa* DNA gel blots with an *R. communis* PLD clone, suggesting PLD is a member of a small gene family. Similarly, Wang *et al.* (1994) estimated that *R. communis* had one to two copies of the PLD gene. The PLD gene is a member of a small gene family in both *R. communis* and *O. sativa*, however only a single gene was observed in *L. esculentum*.

5.7.4 Discussion of LerGi 5

Sequence analysis of LerGi 5 clearly showed that this clone represented a partial sequence of a PLD gene. PLD hydrolyses the terminal phosphate ester bond of a variety of phospholipids with phosphatidylcholine (PC) being the predominant form hydrolysed (Shukla & Halenda 1991). Plant PLD proteins are involved in a variety of processes including the germination (Wang *et al.* 1994), growth of seedlings (Herman & Chrispeels 1980), changes in membrane phospholipid composition (Yapa *et al.* 1986) and possibly in defence responses to pathogens (Young *et al.* 1996).

Due to the limited characterisation of *L. esculentum* PLD completed in this study, assignment of function to this gene is not possible. However, it can be speculated that PLD expression in VAM roots is associated with either a defence response or changes in membrane composition. As the RNA gel blot indicated that the highest steady state RNA level occurred at the day eight harvest, the involvement of PLD with a defence response seems unlikely as mycorrhizal roots display only a weak response during the early stages of colonisation (Chapter 1). If PLD was involved in defence, it might be expected that the highest levels of steady state RNA would have occurred in the earliest harvest. The involvement of PLD in changes to membrane structure seem more plausible, given the colonisation stage of the material at the day eight harvest. Roots at this harvest contained the maximum level of developing arbuscules, as compared to the day four harvest (where there were very few arbuscules) and the day 12 harvest (where the fraction of the root colonised by arbuscules had reached their maximum) (Fig. 3.4c). During the

development of arbuscules, fungal hyphae penetrate the cortical cell wall and branch profusely. The plant plasma membrane extends around these branches, dramatically increasing the surface area of the membrane. Not only is new plasma membrane synthesised by the plant, but evidence for a different composition of the peri-arbuscular membrane includes high ATPase activity localised to membranes surrounding the arbuscular branches (Gianinazzi-Pearson *et al.* 1991b). As PLD is involved in changes to membrane structure, it is possible that it plays a role in the development of the peri-arbuscular membrane. Further evidence is required to confirm this. *In situ* hybridisation and antibody immunolocalisation would enable the identification of cells and subcellular compartments where the gene is expressed and protein targeted within the cells.

LerGi 5 was assumed to be a partial sequence of a PLD gene. This could be confirmed by expressing the complete open reading frame in *E. coli* and using a bioassay to check for PLD activity. Furthermore, antibodies to known PLD genes could be used to check for cross-reactivity to expressed *L. esculentum* PLD.

5.8 Discussion of the LerGi Clones

In this study, a non-targeted approach was used to identify four clones that represented genes that were differentially expressed in *L. esculentum* roots during the early stages of colonisation by the mycorrhizal fungus *Glomus intraradices*. Sequence analysis identified the likely function of proteins encoded by three clones LerGi 1, LerGi 4 and LerGi 5. The function of a fourth clone, LerGi 2, could not be identified. However, it was particularly interesting as it had previously been cloned from *L. esculentum* in association with a pathogenic nematode.

RNA gel blot analysis confirmed that all four clones represented genes that were upregulated during the early stages of colonisation. With all clones except LerGi 5, expression decreased as colonisation progressed. This may mean that;

1) the clones represent genes which are involved in very early stages of colonisation,

2) the changes in expression could be linked to the growth rate of the mycorrhizal roots that had clearly slowed by the day 12 harvest or,

3) the contribution of fungal RNA to the total RNA diluted specific plant polyA RNA. Expression of LerGi 5 increased at the day eight harvest in both mycorrhizal and non-mycorrhizal roots. This clone coded for the phospholipase D gene and has been implicated in changes to plant membranes. From the biological

data, the roots harvested at day eight contained many developing arbuscules and these are surrounded by the plant plasma membrane. Presumably there was high production of the plant membrane at this stage and LerGi 5 may have been associated with this.

The temporal expression pattern in non-mycorrhizal roots was similar for all of cloned genes. The day four roots had low levels of expression that rose and reached a plateau by days eight and 12. The reasons for these expression patterns have not been determined, but could be related to changes in the ratio of polyA RNA to rRNA. Biological reasons for such changes could relate to either increased phosphate stress or an increase in metabolically active tissue such as root tips. To overcome this possibility, either purified polyA RNA could be used for the gel blots, or the blots could be quantified by probing with genes that are constitutively expressed. There are problems with both of these options. The hybridisation of probes to polyA RNA may show that a particular gene is differentially regulated within the polyA RNA pool. However, an exception to this could occur if many genes within the polyA RNA pool have increased expression. The relative expression of any one of these genes compared to the RNA pool will change little. Alternatively, a gene in such a pool that has a steady rate of expression will appear as being less abundant. The quantification of RNA loading with a constitutively expressed gene also has problems. "House-keeping" genes, those involved in normal cellular metabolism, are often proposed as being constitutively expressed, however in metabolically active tissue, these too are likely to have increased levels of expression. Another way to overcome this problem would be to probe the blots with both a ribosomal gene and with an oligo dT probe. As rRNA and polyA RNA make up approximately 90% and 0.7% (Cox & Goldberg 1988) respectively of the total RNA in *N. tabacum* roots, an oligo dT probe would indicate changes in the polyA RNA in relation to rRNA. Steady state RNA levels of any particular gene could then be compared to both RNA pools.

A further contributing factor that needs to be assessed in mycorrhizal roots, is the contribution of fungal RNA to the total RNA pool. As previously mentioned, the proportion of fungal RNA is likely to increase as colonisation increases. Therefore further analysis could be undertaken by probing blots with a fungus-specific probe to determine the levels of fungal RNA. Careful consideration is also required in the choice of probe, as particular genes may be differentially regulated within the fungal RNA pool.

The identification of a gene expressed in both mutualistic and pathogenic interactions (LerGi 2) allows the development of some interesting ideas. Although the function of LerGi 2 could not be identified, the possibility that this clone and is involved in both interactions suggests common molecular pathways are in use by both invading organisms. The VAM symbiosis has evolved over an extremely long period of time, with the fungus being able to invade the root cortex without eliciting a significant defence response. The nematode may have duplicated some of the molecular mechanisms used by VAM fungi to bypass host defence mechanisms. If this is the case, future work in either symbiosis may provide insights to how the other interaction functions.

The proteins encoded by LerGi 4 and 5 have roles consistent with signal transduction pathways. This is interesting, particularly as both appear to be most highly expressed during the early stages of colonisation. To gain a greater understanding of the molecular communication involved between the two symbionts, future work investigating transcriptional regulators involved in the activation of the LerGi genes may provide details of the mechanisms activated in pathways upstream of the LerGi genes.

In conclusion, this research has contributed to the understanding of some of the molecular mechanisms involved in the early stages of the mycorrhizal symbiosis. This initial work can be used as a base from which to investigate in greater detail the role each of these genes play in the mycorrhizal symbiosis.

Chapter 6

General Discussion

This research describes the cloning and characterisation of five plant genes that are associated with the VAM symbiosis. To facilitate the identification and characterisation of these clones, emphasis was placed on the choice of organisms and the development of a model biological system with which to establish colonisation of roots. The plant of choice was *Lycopersicon esculentum* Mill. as it is one of the best understood plants in terms of molecular/genetic characterisation. *Arabidopsis thaliana* is superior to *L. esculentum* in this regard,. However *A. thaliana* is one of the relatively few plants that are non-mycorrhizal. Another plant that is commonly used as a model for molecular research into the VAM symbiosis is the legume, *Medicago truncatula.* This has the advantage of being able to form a symbiosis with *Rhizobium* spp., enabling the comparison of the two symbioses in the one system. As knowledge of the rhizobial symbiosis is more advanced than the VAM symbiosis, opportunities exist to allow rapid progress in VAM molecular research, as has been shown with mutant studies (1.5.2). However, the molecular/genetic tools and materials for *L. esculentum* are considerably more advanced than for *M. truncatula*. Furthermore, as the VAM and nodule symbioses overlap, working with a legume would mean either potential for difficulty in distinguishing molecular responses or a need to grow *M. truncatula* at artificially high nitrogen to inhibit nodulation and hence a possibility that gene expression observed is physiologically altered. Characteristics which make *L. esculentum* amenable to molecular/genetic research include it being self-fertile, diploid and having a relatively small genome with little repetitive DNA (DeVerna *et al.* 1987). It has well developed genetic maps (Tanksley *et al.* 1987) and is easily manipulated in tissue culture, with transformation and regeneration techniques standard (McCormick *et al.* 1986). Furthermore, a mutant population is available, the screening of which has revealed families with the *myc*$^-$ phenotype (Barker, pers. comm.). The fungus chosen was *Glomus intraradices*. This fungus is commonly used in mycorrhizal research as it is considered to colonise roots well and have rapid hyphal growth (Smith pers. comm.). Other molecular work has also been completed using *G. intraradices* as the fungal symbiont (Murphy *et al.* 1997).

Much emphasis was placed on developing a model inoculation system with which to develop root material for molecular analysis. To identify genes important in the symbiosis, an understanding of the biological nature of the roots from which the

genes are isolated is useful in elucidating their function. Several requirements needed to be fulfilled in generating the colonised roots. Firstly, colonisation had to be abundant so as to enhance the molecular responses of the plant. To achieve this, plants are usually grown for several weeks. However, this leads to the formation of colonisation units at different stages of development, an undesirable trait in material to be used for molecular analysis. To overcome this problem, a method of Brundrett *et al.* (1985), where a one year old mycorrhizal leek was used as a source of live inoculum to colonise young leek seedlings, was modified. Their method achieved rapid synchronous colonisation but it was unsuitable for molecular work as the seedlings were too small, and the one year old host was likely to harbour diseases. Colonisation was achieved in *L. esculentum* roots by transplanting into pots containing three to six week old mycorrhizal leeks. The leeks were extensively colonised and gave rise to external hyphae which acted to effect numerous entry points in *L. esculentum* almost simultaneously. A reasonable mass of *L. esculentum* roots was achieved by first growing the tomatoes in uninoculated soil for two weeks. This procedure resulted in extremely efficient colonisation, with a maximal level being reached after only eight days. Furthermore the synchronous nature of colonisation was exemplified by a distinct peak in the transient arbuscular structure. The chief advantages of rapid, abundant and synchronous colonisation achieved by this method maximises any changes in gene transcription rates of both plant and fungal genes occurring during the interaction. Lengthy colonisation times have been traditionally used to maximise the plant/fungal interactions, but this is undesirable for molecular studies aimed at understanding the distinct stages of establishing the symbiosis, especially during the early stages. With synchronous colonisation, the VAM symbiosis can be examined in a developmental context rather than a steady state/nutritional one. Also, the "nurse" pots can be considered as disposable, facilitating rapid harvesting of roots and avoiding gene expression stimulated by root damage, a factor that could mask changes in gene expression caused by the fungal interaction. This method has been trailed on barley (Delp pers. comm.) with similar results, and is now in common use in our laboratory.

A targeted approach of gene cloning was used to identify specific transport mechanisms involved in the symbiosis. Specifically, phosphate transport was targeted as phosphorus is an essential element transferred from the fungus to the plant. An *L. esculentum* gene that showed extremely high sequence similarity to a characterised proton-coupled phosphate transport proteins (Leggewie *et al.* 1997; Muuchal *et al.* 1996) was cloned and designated LePT 1. RNA gel blot analysis showed expression of this gene increased over time in non-mycorrhizal plants

while expression remained constant in the roots of mycorrhizal plants. These results may indicate that expression of the gene was not directly related to mycorrhizal colonisation but to the P status of the plant. This suggestion was further supported by the examination of expression in P sufficient and deficient plants, where lower transcript levels were observed in the P sufficient plants. However, *in situ* hybridisation indicated localised gene expression to arbuscule-containing cortical cells. This implicates LePT 1 with possible involvement in uptake of P from the apoplast surrounding the fungus in arbuscule containing cells. While this research was in progress, an identical LePT 1 gene was isolated from a root hair library, indicating the gene is expressed in root hairs. This was not shown on the *in situ* hybridisation experiments as the root hair structure is too fine for adequate preservation and sectioning. From these data, a model of phosphate uptake in mycorrhizal plants is proposed (Fig. 6.1) and outlined below.

In non-mycorrhizal roots, P uptake is likely to occur in root hairs, being facilitated by LePT 1 (Fig. 6.1, site 1). The level of expression of LePT 1 responds inversely to internal root P concentration (Fig. 4.6). In mycorrhizal plants, phosphate uptake by the roots can be almost completely taken over by fungal hyphae (Pearson & Jakobsen 1993). This would be consistent with a much reduced requirement of LePT 1 in root hairs, with P uptake by the fungus being facilitated through the GvPT phosphate-transporter protein (Fig. 6.1, site 2). GvPT was isolated from *G. versiforme*, (Harrison & van Buuren 1995). As the fungus is more efficient at P uptake than the plant, overall plant uptake of P increases, resulting in decreased expression of LePT 1 in the root hairs. The plant needs a mechanism for P uptake from the fungus, often predicted to be at the arbuscular interface (Gianinazzi-Pearson *et al.* 1991b). Mycorrhizal plants have increased ATPase gene expression (Murphy *et al.* 1997) and the plant peri-arbuscular membrane has ATPase activity (Gianinazzi-Pearson *et. al.* 1991b), indicating a role for proton coupled nutrient transport at the arbuscular interface. LePT 1 is a member of a proton coupled P uptake protein family (Leggewie *et al.* 1997; Harrison & van Buuren 1995), and the *in situ* data showing expression of LePT 1 in arbuscule-containing cortical cells is consistent with its involvement in uptake of P at the arbuscular interface (Fig. 6.1, site 3). The anomaly that whole tissue LePT 1 expression is less in mycorrhizal roots than in non-mycorrhizal roots could be caused by the LePT 1 protein being able to transport P more efficiently at the interface than from the soil solution. The apoplastic interface between plant and fungal membranes in the arbuscule is reported to have a very small volume (Bonfante & Perotto 1995). Therefore, relatively few protons and P molecules would be required to increase the concentration of these substances in this space, allowing LePT 1 to function

very efficiently. This compares to the situation occurring on the surface of the root hair, where P would be scarce (particularly in the low P soil) and the proton motive force able to dissipate into the soil solution. Thus the overall picture is one in which :

1) P is scavenged by the external mycorrhizal hyphae (involvement of GvPT) and translocated to the arbuscules, bypassing root uptake processes.

2) P effluxes (presumable passively) by an unknown mechanism to the peri-arbuscular apoplast and is there absorbed through the peri-arbuscular membrane (involvement of LePT 1: PMF provided by H^+ATPase localised on this membrane).

3) Reabsorption of P from the apoplast by the fungus is not likely as the fungal transporter GvPT is not expressed within roots (Harrison & van Buuren 1995). This would promote net transport of P from fungus to plant, the rates of which have been shown to be relatively high, considering the involvement of an efflux step in the process (see Smith & Read 1997).

There have been few non-targeted approaches to gene cloning associated with the VAM symbiosis. Murphy *et al.* (1997) identified two genes that have increased expression in roots upon mycorrhizal colonisation (BMR6 and BMR78) and a further two that have decreased expression in mycorrhizal roots, although no further information was presented for the last two clones. BMR78 represented a proton ATPase gene and expression of this gene was slightly increased in plants grown in a P supplemented medium. Sequence analysis could not positively identify BMR6, and its expression was not altered in roots by P addition. Burleigh and Harrison (1997) have identified a gene inducible by phosphate starvation from *M. truncatula* (Mt4) that has decreased expression in mycorrhizal roots. Interestingly, this gene also has decreased expression in colonised roots of a *myc*⁻ mutant of *M. sativa.* In this mutant, *G. versiforme* forms many appressoria and has extensive external hyphal growth, but there is little or no penetration of the root cortex. The decreased expression of Mt4 in mycorrhizal challenged roots of the *myc-* plant indicate that VAM fungi can alter expression of phosphate-repressible genes presumably without a requirement of increased P nutrition. This indicates a second signalling pathway by which VAM fungi elicit a phosphate related response other than that occurring by P nutrition alone.

The LerGi clones described here significantly contribute to the number of genes isolated through non-targeted approaches, that have differential expression in response to VAM symbiosis establishment. All four cloned genes reported in this study have increased expression during the early stages of colonisation. For three

of these clones, clear functions were able to be assigned from the sequence data. LerGi 4 and 5 both have, among other roles, previously been associated with signal transduction pathways (Mitsui 1993; Masuta *et al.* 1995; Young *et al.* 1996) and Barker *et al.* (1998) details predicted regulatory points in the symbiosis. Most of these regulatory points are yet to be investigated, and genes represented by LerGi 4 and 5 may play roles at some of these points. As the differential expression was noted in early, pre-arbuscular stages of colonisation, there is increased likelihood that the translated proteins are associated with signal transduction directly related to the colonisation, rather than nutrition.

The LerGi 2 clone had previously been identified as being expressed in giant cells of *L. esculentum,* which are induced upon parasitism by the pathogenic root-knot nematode *Meloidogyne incognita* (Bird & Wilson 1994). The identification of induced gene expression common to plant responses to very different biotrophic organisms is not novel to the case mentioned here. There is increasing evidence for similar, parallel, mechanisms of colonisation occurring in both the VAM symbiosis and the legume/*Rhizobium* symbiosis (reviewed by Gianinazzi-Pearson & Dénarié 1997). These commonalties are of evolutionary significance. The VAM symbiosis has ancient origins and predates both the nodulation symbiosis and nematode pathogenesis (Kidston & Lang 1926; Nicolson 1975; Remy *et al.* 1994). None of the three interactions elicit significant defence responses from the host plant. It is unclear how the invading organisms avoid detection by the host, but the common links currently being unravelled at the molecular level suggest a the successful infection process developed by VAM fungi may have subsequently been duplicated or "pirated" by the other biotrophs.

Key experiments in the interpretation of the functioning of LePT 1 and LerGi 2 were the *in situ* hybridisations. Both gave similar patterns in that expression was localised to arbuscule containing cortical cells. Similarly, a ribosomal RNA probe used as a control also indicated there were high levels of those transcripts in colonised cells (data not shown). These cells are metabolically active and have been identified as having an increase in cytoplasmic volume as compared to uncolonised cortical cells (Alexander *et al.* 1988). The possibility does exist that the dense cytoplasm produces an artefact in arbuscular cells. Sections through root tips, cells that are also metabolically active and have a high cytoplasmic volume, also appeared to generate a signal with both the P11 and LerGi 2 antisense probes (data not shown). The signals are a true reflection of genes expression and not an artefact of the DIG labelling procedure as the control sense probes did not hybridise to these cells. However, many gene may have increased expression in

these metabolically active cells. This is an important question that needs to be resolved if interpretation of *in situs* is to be straightforward in future. An interesting control would be to include an isoflavone reductase gene (IRF). This gene was not detected in arbuscule-containing cortical cells in a radioactive *in situ* hybridisation (Harrison & Dixon 1994) and is one of the very few genes not found to have increased expression in those cells. To further confirm the findings, the immunolocalisation of the LePT 1 protein with specific antibodies would show unequivocally where these proteins are located within the cell.

This research has set the scene for molecular developmental studies and molecular overlap studies. Due to the ancient nature of the VAM symbiosis, characterisation of the molecular processes enables the elucidation of functions that are potentially fundamental to the regulation of land plant growth, development and responses to the environment. In this study, a number of genes that were differentially regulated in mycorrhizal roots were cloned and analysed. Analysis included sequence data, which gave putative functions to four clones and an interesting correlation with another biotrophic interaction in the fifth clone. A time course of expression was investigated covering three specific stages in mycorrhizal development. Further studies in expression were completed, in which transcripts were cellularly localised through *in situ* hybridisation. The success of the molecular work in this project was largely influenced and facilitated by the biological work undertaken in the early stages of this research, providing a method whereby plant material could be produced with staged mycorrhizal development.

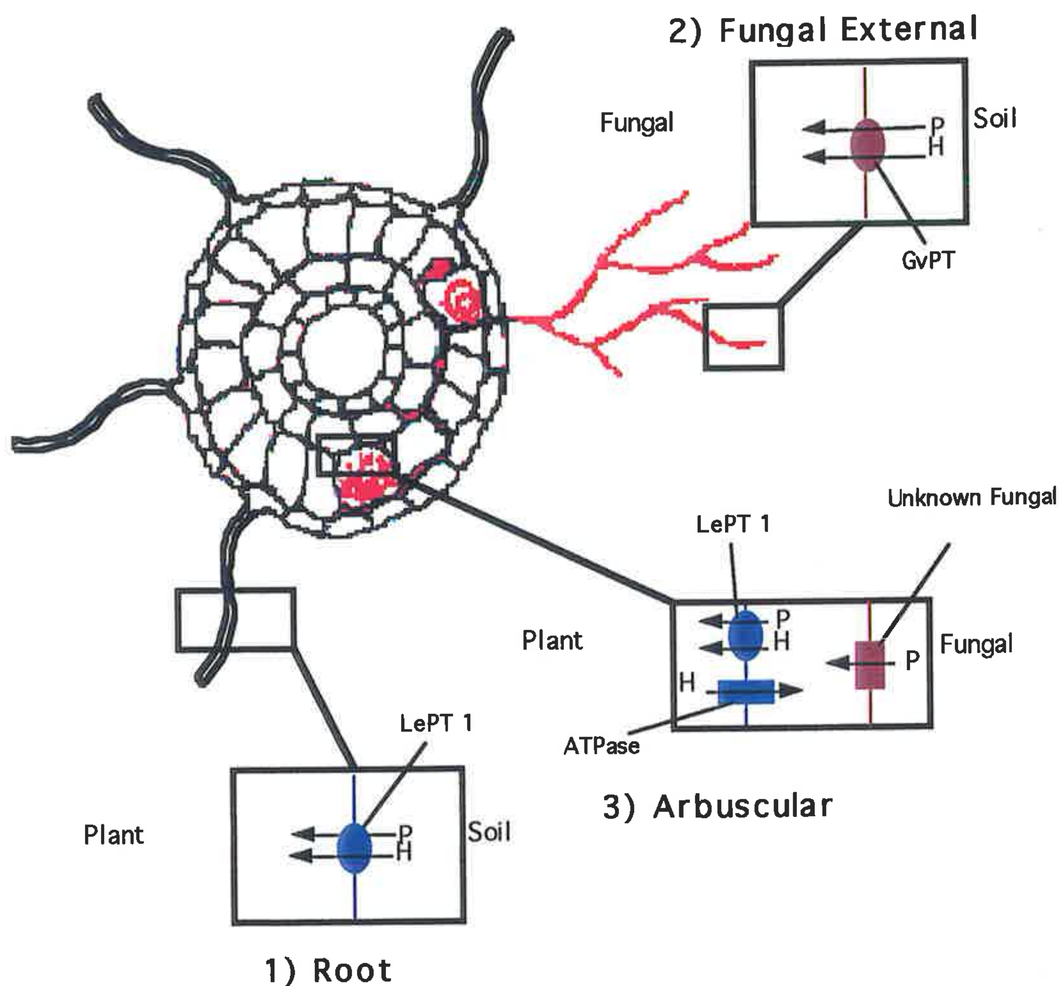


Fig. 6.1 A stylised diagram of a transverse root section colonised by a mycorrhizal fungus to illustrate a proposed model of P uptake in plant roots. At site 1, soil P is taken up from the soil solution by LePT 1 and enters the plant cytoplasm. The expression of this gene is regulated by the plant P status. In mycorrhizal roots, an additional P uptake protein is provided by the fungus (GvPT at site 2). The P obtained through GvPT is translocated to the arbuscular interface (site 3). Here, an unknown fungal P transporter releases the P to the peri-arbuscular space, where proton-coupled P transport into the plant cytoplasm is facilitated by ATPase activity and LePT 1. The resulting increase in plant P reduces expression of LePT 1 in the root hairs.

Appendix 1

Media

LB Media

1% bacto-tryptone
0.5% yeast extract
0.5% NaCl

LB Agar

As for LB Media with the addition of 1.5% agar

SOB

2% bacto-tryptone
0.5% yeast extract
0.05% NaCl
2.5mM KCl
10mM $MgCl_2$

TB Top Agarose

1% bacto-tryptone
0.5% NaCl
0.6% agarose
10mM $MgSO_4$ (added after autoclaving)

Solutions

Buffer A

1% BSA
0.3 % triton x100
100 mM Tris pH 7.5
150 mM NaCl .

CIP Stop Buffer

10mM Tris
1mM EDTA
0.2M NaCl
50mM SDS

Denaturing Gel Loading Buffer

50% glycerol
0.1mg/ml bromophenol blue

Denhardts Reagent

2% poly-vinyl pyrolidone
2% bovine serum albumin
2% Ficoll type 400

DIG Buffer 1

0.1M maleic acid
0.15M NaCl pH 7.5

DIG Buffer 2

DIG Buffer 1 containing 10% (w/v) blocking reagent (Boehringer Mannheim)

DIG Buffer 3
100mM Tris-HCl
100mM NaCl
50mM $MgCl_2$ pH 9.5

DNA Extraction Buffer
1% sarkosyl
100 mM Tris HCl
100 mM NaCl
10 mM EDTA di sodium salt
pH 8.5

DNA Prehybridisation Solution
1 ml water
3 ml 5X HSB buffer
2 ml denhardts reagent
3 ml 25% dextran sulphate
2 ml 10% SDS
0.25 ml denatured salmon sperm

Fixative Solution
0.05 M Na cacodylate pH 7.17
1 % glutaraldehyde

5X HSB Buffer
3M NaCl
100 mM PIPES
25 mM EDTA di sodium salt
pH 6.8

in situ hybridisation solution
50% formamide
300 mM NaCl
10 mM Tris pH 7.5
1 mM EDTA di sodium salt
1x denhardts solution
10% dextran sulphate
70 mM DTT
150 μg / ml tRNA
2-3 μg / ml probe

10X Gel Loading Buffer
0.25% bromophenol blue
0.25% xylene cyanol FF
15% ficoll type 400

Klenow Buffer
50mM Tris HCl pH 7.2
10mM MgCl2
1mM DTT
40μM dNTP
20 μg/μl BSA

10X MOPS
0.2 M 3-[N-Morpholino]propane-sulphonic acid
0.5 M sodium acetate pH 7.0
0.01 M EDTA di sodium salt

PBS

0.14 M NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
pH 7.4 with HCl

Probe Hydrolysis Stock Solutions

a) 200 mM Na_2CO_3
b) 200 mM $NaHCO_3$

RNA Loading Buffer

50% glycerol
0.1 mg/ml bromophenol blue

RNA Prehybridisation Solution

1.5ml denhardts solution
0.5ml 10% SDS
2.5ml 20X SSPE
4.5ml formamide
1.0ml denatured salmon sperm DNA (10mg/ml)

RNase Buffer

500 mM NaCl
10 mM Tris pH 7.5
1 mM EDTA di sodium salt

SM Buffer

0.01% gelatine
50mM Tris-HCl pH 7.5
100mM NaCl
8mM $MgSO_4$

20X SSC

3M NaCl
0.3M Na-citrate pH 7.0

50X TAE buffer

2M Tris HCl
0.06M EDTA di sodium salt
Adjusted to pH 8.3 with glacial acetic acid

TE

10 mM Tris HCl (pH 8.0)
1 mM EDTA di sodium salt

Appendix 2

Primers

LePTF1
5' 3'
GCT CTA GAC TTT AAG GAA GTT TAG T

LePTF2
5' 3'
CCC TGC TGG ACT TAC TTA

LePTF3
5' 3'
AAT TGG ATG GAT TCC ACC

LePTF4
5' 3'
CGG GAT CCA AGT TAT CAG TTA GTA T

LePTR1
5' 3'
GGA ATT CAG AAG TAA GGT AAT AA

LePTR2
5' 3'
CCC AAA CAA ACA AAC AAA AA

LePTR3
5' 3'
TAC AGT TTC CTC TTC CCC

LePTR4
5' 3'
CAG TTT CAG GCA TCT TCA TA

GSP1
5' 3'
GCC CTT GCA ATC TTG TAA

GSP2
5' 3'
GGC CCC ATC CAA TTG CAC TGA AAA

GSP 2.5
5' 3'
AGC CAG AAT TCC GAA ACC TT

GSP 3
5' 3'
CGC TCC ACG GGT TTT TTT GT

GSP 4
5' 3'
GGT GGC AGA AAG GGG ATA AT

Abridged Anchor Primer (AAP)
5' 3'
GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG

Abridged Universal Anchor Primer (AUAP)
5' 3'
GGC CAC GCG TCG ACT AGT AC

LerGi4F1
5' 3'
CTC TGC ATA TGA CTA TCC AAA

LerGi4F2
5' 3'
GCT TTT CCT GCC TAT CAA TG

LerGi4R1
5' 3'
AGT GAG CAG GCT TGT AAG GA

LerGi4R2
5' 3'
CGG TGG TGG TCA CAA AGA TA

λgt10F
5' 3'
GCA AGT TCA GCC TGG TTA AG

λgt10R
5' 3'
TGA GTA TTT CTT CCA GGG TA

Appendix 3

DNA Sequences

P11 and 5' RACE Product

CTAGGATTTG GCATTGGTGG TAATTATCCC CTTTCTGCCA 40
CCATCATGTC TGATTATGCT AACAAAAAAA CCCGTGGAGC 80
GTTCATTGCT GCTGTGTTTG CTATGCAAGG TTTCGGAATT 120
CTGGCTGGTG GAATGGTGGC AATCATTGTT TCTGCAGCAT 160
TCAAGGGAGC ATTCCCTGCA CCAACATATG AGGTTAATGC 200
TATTGGTTCA ACACTCCCCC AGGCTGATTT CGTTTGGCGT 240
ATAATTCTCA TTTTTGGTGC CAATCCCTGC TGGACTTACT 280
TATTACTGGC TATGAAGATG CCTGAAACTG CCCGTTACAC 320
TGCCTTGGTC GCCAAGAACT TGAAACAGGC AGCTAACGAC 360
ATGTCCAAGG TGTTGCAAGT CGAAATTGAA GCAGAGCCAG 400
AGAAAGTTAC AGCTATTTCT GAAGCAAAAG GAGCCAATGA 440
CTTTGGTTTG TTCACTAAGG AGTTCCTCCG TCGCCATGGA 480
CTTCACTTGC TTGGAACTGC TAGCACATGG TTCTTGTTGG 520
ACATTGCTTT CTACAGTCAA AACCTTTTCC AGAAGGACAT 560
TTTCAGTGCA ATTGGATGGA TTCCACCAGC ACAAACCATG 600
AACGCGTTGG AAGAAGTTAA CAAGATTGCA AGGGCACAAA 640
CACTTATTGC TCTTTGTAGT ACTGTTCCTG GTTACTGGTT 680
CACAGTTGCA TTCATCGATA AGATTGGTCG ATTTGCAATT 720
CAGTTGATGG GATTCTTCTT CATGACAGTC TTCATGTTTG 760
CCTTAGCCAT TCCATACCAT CACTGGACTC TCAAGGATCA 800
CAGAATTGGC TTCGTGGTCA TGTACTCATT CACCTTTTTC 840
TTCGCCAATT TTGGTCCAAA CGCCACAACA TTCGTCGTCC 880
CTGCTGAGAT TTTCCCAGCC AGGCTTAGGT CCACATGCCA 920
TGGAATATCA GCAGCAGCAG GTAAAGCAGG AGCTATGGTT 960
GGTGCATTTG GATTCTTATA CGCTGCTCAG CCCACGGATC 1000
CAACAAAGAC TGACGCCGGT TACCCTCCTG GCATTGGTGT 1040
GAGGAACTCG TTGATCGTCC TTGGTTGTGT TAACTTCCTC 1080
GGTATGCTGT TCACATTCTT GGTTCCAGAA TCCAATGGGA 1120
AGTCATTGGA AGATTTGTCG AAGGAAAACG AAGGGGAAGA 1160
GGAAACTGTA GCTGAAATAA GAGCAACAAG TGGAAGGACA 1200
GTTCCTGTGT GAGTTTTAGA CAAGTTATCA GTTAGTATAC 1240
ACTACAATGC AGTTTGAGTT AATTTGTGGT ATTTGGGATT 1280
AGAAAGAGAT TGTTTGTTGG TTTGTTATAA GAAGATGGAA 1320
TAAGCTCTTA TCTTTTTGTT TGTTTGTTTG GGTAATTAAA 1360
CATTATTACC TTACTTCTGC AAATCTCAGA AATTCTGAGA 1400
TTATATAAAG TAACCAAAGG AGGTTCTTTG GTTGTCTAAA 1440
AA 1442

LerGi 1

AACTTTCTGC TGCTTATCTC TGTCTCGTCT AAGGAAATTC 40
TGCAAGGATG GGTCGTGTGC GCACCAAGAC CGTGAAGAAG 80
TCATCTCGAC AGGTAATTGA GAGGTACTAC TCCAAAATGA 120
CATTGGATTT CCACACCAAC AAGAAGATTT TGGAGGAAGT 160
TGCTATAATT CCTTCCAAGC GTCTCCGCAA CAAGATTGCT 200
GGATTCTCCA CTCACCTCAT GANNCGTATC CAGAANGGAA 240
CAGTCCGTGG TATCTCCCTG AAACTGCAAG AGGAGGAACG 280
TGAGAGACGC ATGGACTTTG TTCCTGATGA ATTCTGCCAT 320
CAAGACTGAT CTCATTGAGG TTGACAAGAA CCCTTGACAT 360
GCtTTCAGCC CTCGGCATGT TCTGACCTTC CAGGCGTTGT 400

TCAAGCAGGC	CGTTGAGCCA	CAAGCAGTGG	CAGTTTTCCC	440
ATTCATATGG	CCGCGGTGGA	GGTGGTTTTG	GACGGAAATA	480
NTAATAAGAG	AACAGTTACA	ATGNTTNNTT	ATGANNCTAT	520
NGAAGAAACT	TTTNGGTAGA	TNATATGTTT	AGTTTNCCCC	560
TAATTTTTCT	TTTAAAGATN	CTTTGAAATT	CATTCCCAGT	600
GTTTTTGAAA	CATAAATGAT	GAAACANCCC	CTTTTGGTGT	640
TAAAA				645

LerGi 2

AAAATCTAAA	AATTAGGAAT	GTATAGAACA	AAATATAAAA	40
TCTTAAAAGC	TAGTCTTTCT	TAGGTTCACA	TAGCAGTTAC	80
TTCCAAAAGT	TAAAACATAA	ATCAAAATTA	TCCCATTACA	120
TCATTTAACT	GAAGGGAGAT	GCAAATCAAA	AAGCTCTAAT	160
GCCATATGTC	TTCTACCAGC	ACCACATGTA	GAGGTGCTCG	200
TCGAAACTCG	TATTCAAGAT	TTTTAACGGT	ACTTGATGAA	240
GCCAATTTTC	TTGGCATTGC	TGCGGTGGGA	CTGTCTGCAA	280
CACATGAGTC	CAGTACTTTC	TAATAATTGC	ATGAGGAGTG	320
TACCACACAC	ACGGCATGTG	CGAGAGCCAG	GGCCGTAGTT	360
CTTTGGGTGA	gCGTTCCAGA	TGTTAGAGTG	ACCCATCTTC	400
ACAGAAAAGC	GAGCTGCGCG	ATGTGTTTGC	AAGAAAACCC	440
TACTTTTG				448

LerGi 4

CTTAAACCCT	TTTTTTTCTC	TTATACTCGC	CTTACCCATG	40
GCTCTACTCG	TTGAGAAGAC	CACCTCTGGC	CGCGAGTACA	80
AGGTGAAGGA	CATGTCTCAG	GCTGACTTCG	GCAGGCTCGA	120
AATCGAGCTT	GCTGAAGTTG	AAATGCCTGG	TCTCATGGCT	160
TCACGGGCTG	AATTTGGGCC	TTCACAGCCC	GTTAAAGGTG	200
CAAAGATCAC	TTGTTCTTTG	CATATGACTA	TCCAAACTGC	240
TTTCCTGATT	GAAACCCTAA	CTGCTTTGGG	TGCTGAAGTT	280
AGATGGTGTT	CTTGCAACAT	CTTCTCAACT	CAGGACCATG	320
CTGCAGCAGC	CATTGCACGT	GACAGTGCTG	CTGTCTTTGC	360
CTGGAAAGGT	GAGACTTTGC	AGGAGTACTG	GTGGTGTACT	400
GAGAGGGCAC	TTGACTGGGG	TCCAGGTGGT	GGTCCTGATC	440
TGATTGTTGA	TGATGGAGGT	GATGCTACTC	TGTTGATTCA	480
TGAGGGAGTT	AAGGCTGAAG	AGGAGTTTGC	TAAGAATGGA	520
ACAGTCCCAG	ATCCCACTTC	TACTGACAAT	GTTGAGTTTC	560
AACTTGTGCT	TACTATTATT	AAGGAGAGCT	TAAAGACTGA	580
TCCATTAAGG	TACACTAAGA	TGAAGGAGAG	ACTTGTTGGT	620
GTTTCTGAGG	AAACTACCAC	TGGTGTTAAG	AAGCTTTACC	660
AAATGCCAGC	TAATGGATCT	TTGCTTTTCC	TGCCTATCAA	700
TGTTAATGAC	TCTGTTACCA	AGAGCAAGTT	TGACAACTTG	740
TATGGATGCC	GCCACTCACT	TCCCGATGGT	CTCATGAGGG	780
CTACTGATGT	TATGATTGCT	GGAAAGGTTG	CTCTTGTTGC	820
TGGTTATGGA	GATGTCGGCA	AGGGATGTGC	TGCTGCCATG	860
AAACAAGCTG	GTGCCCGTGT	GATTGTGACT	GAGATTGACC	900
CAATCTGTGC	TCTCCAGGCT	ACCATGGAAG	GCCTTCAGGT	940
TTTGTTCTTG	GAGGATGTTG	TTTCTGAGGT	TGATATCTTT	980
GTGACCACCA	CCGGTAACAA	GGACATCATC	ATGGTTGACC	1020
ACATGAGGAA	GATGAAGAAC	AATGCCATTG	TCTGCAACAT	1060
TGGTCACTTT	GACAACGAAA	TCGACATGCA	TGGTCTTGAA	1100
ACCTTCCCTG	GTGTGAAGAG	GATCACAATC	AAGCCACAAA	1140
CCGACAGATG	GGTCTTTCCC	GACACCAACA	GTGGCATCAT	1180
TGTGTTGGCC	GAGGGTCGTC	TCATGAACTT	GGGATGTGCC	1220
ACTGGACACC	CCAGTTTTGT	GATGTCTTGC	TCTTTCACTA	1260
ACCAAGTCAT	TGCCCAACTC	GAGTTGTGGA	ATGAGAGGAG	1300

CAGTGGCAAA TACGAGAAGA AGGTGTACGT CTTGCCAAAG 1340
CACCTTGACG AGAAGGTTGC TGCCCTTCAT CTTGGAAAGT 1380
TCGGAGCCAA GCTTACCAAA CTCACCAAGG ATCAAGCTGA 1420
CTACATTTAC GTACCTGTTG AGGGTCCTTA CAAGCCTGCT 1460
CACTACAGGT ACTGAGGAAG AGACGCTCAC AGTGGAACAA 1500
CGATACGGCG GCATGATTGT TTTGTTTTAA ACTTTTATTT 1540
TGTTTAGGTA GTGTGTTTTT ATTTTGTTGG GGGATATTTT 1580
GCTGGAAAGT TGACCTAAAT GTGTTTGAAT AATATTTGAA 1620
TTATGGTTGG GGTGGTGTCA TATGATATTG TACCAAGTTA 1660
GATTCATTTG CTTTCTTGTT TCTATAAAAT TTGCTTCAAG 1700
GAAACAAAGC ATCATGTTTT T 1721

LerGi 5

ATCTGCCTGG TCACTTGCTC CGCTACCCTA TTGGAGTGGC 40
TAGCGAAGGG GATATCACAG AGCTACCAGG CCATGAATTT 80
TTCCCCGACA CCAAGGCCCG GGTTCTTGGT ACTAAATCTG 120
ACTACCTTCC TCCCAACCTC ACTACATAAG TGGGTTAATT 160
CGCGTATTAC TTGTTATGCA AAGAGGTAAC AAACTCGTGT 200
GTTAGTTGAA GGGTGCAGTT TGTTGTGGAA TAATAATACA 240
TGATAGTAGT TGTTTTTTAA GATTTCTGTT TTTCTTTTTG 280
TTTGGTTGGA TAGTGCACTT GAGTTTGTAT GAGATGCTTT 320
ATTGACTGAA AGTCTGTTG 339

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