PLANT-FUNGAL INTERACTIONS DURING VESICULAR-ARBUSCULAR MYCORRHIZA DEVELOPMENT: A MOLECULAR APPROACH

Phillip James Murphy

B.Ag.Sc. (Honours), Adelaide

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY
in
Department of Plant Science
Faculty of Agricultural and Natural Resource Sciences
The University of Adelaide

October, 1995

DECLARATION

The work presented in this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or 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 is made in the text.

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

P.J. Murphy

August 1995

Acknowledgement

Special thanks must go to my supervisors, Dr. Sally Smith and Dr. Peter Langridge, not only for their academic direction and help, but also their enthusiasm, encouragement and humour. It has been a joy to learn from them. Thank you to all my colleagues at the Waite Institute and to Jill, Sam, Brett and Suzie whose friendships I treasure dearly.

Finally, I must acknowledge the very generous help of Angelo Karakousis who supplied many of the hybridisation membranes used for the mapping experiments and also assisted in the preparation of the linkage maps.

Table of Contents

Chapter 1
Introduction

Chapter 2
Materials and Methods

Chapter 3

Growth Response and Infection Studies of the Hordeum vulgare - Glomus intrardices Mycorrhizal Symbiosis

Chapter 4

Isolation of Putative Mycorrhiza-deficient (*myc*-) Barley Phenotypes

Chapter 5
Molecular Cloning and Isolation of Barley-mycorrhiza Related Plant cDNA's

Chapter 6

Molecular Characterisation of the Barley-mycorrhiza Related cDNA's: BMR6 and BMR78

Chapter 7

General Discussion

Appendices

Plant-fungal Interactions during Vesicular-Arbuscular Mycorrhiza Development: A Molecular Approach.

Phillip James Murphy

Vesicular-arbuscular (VA) mycorrhiza formation is a complex process which is under the genetic control of both plant and fungus. It has proved difficult to elucidate the mechanisms which mediate the formation and maintenance of these long term compatible symbioses. Experimental evidence suggests that host plant cells are cytologically modified in direct response to infection by VA mycorrhizal fungi. It is reasonable to suggest that this is a result of altered gene expression within host-plant cells.

The aims of the project were; to develop a model infection system in *Hordeum vulgare* L. (barley) suitable for undertaking a molecular analysis of the symbiosis; to identify host plant genes differentially expressed during the early stages of the infection process; and to produce and screen a mutant barley population for phenotypes which form abnormal mycorrhizas.

A model infection system was developed using *H. vulgare* cv. 'Galleon' and the VA mycorrhizal fungus *Glomus intraradices* Schenk and Smith. High levels of early-infection stage mycorrhizas (20-35% of the total root length) were routinely obtained in the roots of 12-14 day old barley seedlings. Detailed infection studies showed that the majority of the infection occurred in the first 5-10 lateral roots of each primary root. The level of infection within these roots was as high as 75% of the total root length.

Complementary DNA (cDNA) libraries were prepared in the bacteriophage vector λgt10 from reverse transcribed poly(A) tailed RNA's extracted from 12 day old mycorrhizal barley roots. Differential screening of the phage library with radio-labeled single stranded cDNA's (reverse transcribed from nonmycorrhizal and mycorrhizal lateral root RNA), identified over 500 differentially expressed clones.

Further hybridisation studies, using total RNA extracted sequentially during VA-mycorrhiza development, confirmed that several of the barley mycorrhiza-related (BMR) clones represent RNA species which increase or decrease in abundance during mycorrhiza formation.

Two clones, BMR6 and BMR78, were characterised in greater detail. These clones detect RNA transcripts that accumulate to much higher levels in mycorrhizal roots than in nonmycorrhizal root tissues. The levels of these transcripts within the roots did not appear to change in response to infection by virulent or avirulent strains of the root pathogen *Geaumanomyces graminis* or variable phosphate nutrition but may have been slightly increased by droughting the plants. DNA hybridisation analysis confirmed that both of the clones were of plant rather than fungal origin.

Hybridisation studies and sequence analysis strongly suggest that BMR78 represents a low copy proton-ATPase gene which is located on the long arm of barley chromosome 2. BM6 detects a low copy gene located on the short arm of barley chromosome 6; its function is less clear. The DNA sequence of BMR6 has regions, which have homologies to 2 stress induced genes isolated from *Escherichia coli* and a senescence-associated gene isolated from *Raphanus sativus* L.. The implications of these results are discussed with respect to available cytological evidence.

Well characterised mutant plant phenotypes have proven essential to the understanding of many physiological, biochemical and developmental processes. To complement this investigation of plant gene expression during VA mycorrhiza development, two populations of mutagenised Galleon barley were generated by ^{60}-Co irradiation treatment. The screening of approximately 1850 M2 plants identified several lines which appeared to be VA mycorrhizal (*myc*) mutants. Rescreening of the putative *myc* phenotypes identified two lines (MF25-10 and MF4-10a), that were less susceptible to mycorrhizal infection than the parental Galleon genotype.

Plants of the MF25-10 line developed normally except for slightly lower levels of otherwise-normal VA mycorrhizas when compared with the parental Galleon. The other putative *myc* mutant, MF4-10a, displayed unusual shoot and root development and formed very few VA mycorrhizas. All plants from line MF4-10a died from what appeared to be nutrient deficiency within 25 days of germination; no genetic analyses were carried out, but the segregation data available indicates that this lethal-*myc* phenotype is inherited as a recessive trait. It is proposed that an induced mutation affecting the visco-elastic properties of line MF4-10a cell walls (as evidenced by thickened root morphology) could be responsible for rendering the root cells not only impermeable to soil solutes but almost inpenetrable to *G. intraradices.*

Chapter 1

Introduction

1.1 General Introduction

The term mycorrhiza describes tissue that arises from several distinct symbiotic associations which occur between fungi of the Glomales (Zygomycetes) and the roots of most higher plants. These associations are mutualistic (Lewis, 1973) rather than parasitic; no disease symptoms are observable, and indeed, the growth of the plant is very often enhanced by the interaction. In essence, these root structures represent the formation of an extensive and intimate interface between a fungus and a higher plant which is then maintained for the life of the plant.

The classification of mycorrhizas is largely based on the general morphology of the tissues. Traditionally, they have been classified into two groups; ectomycorrhizas and endomycorrhizas. In ectomycorrhizas the interface arises from the establishment of a fungal sheath which completely surrounds the root epidermis and an intercellular net of fungal hyphae growing between the cells of the plant-root cortex. No intracellular penetration of plant-root cells is known to occur. On the other hand, in the endomycorrhizas there is intracellular penetration into plant-root cortical cells by the fungal hyphae. The resultant intracellular interface being formed between modified hyphae (coils or much-branched arbuscules) and the invaginated cortical cell plasmalemma. An intercellular network of fungal hyphae may also arise. Ecto- and endomycorrhizas both form an extensive web of external hyphae.

Five main groups have been proposed and generally adopted to describe the diversity of mycorrhizal associations (Smith, 1980). Ectomycorrhizas remain as previously

classified whilst the endo- (and the sub-group ectendo-) mycorrhizas have been reclassified as vesicular-arbuscular, ericoid, orchidaceous and arbutoid mycorrhizas. The delineation between types of mycorrhizal associations is by no means distinct but it has been argued that this system more fully emphasises structural and physiological similarities (and differences) between the groups. For a more complete discussion on the basis and details of mycorrhizal classification see the reviews by Lewis (1973, 1975), Smith (1980) and Harley and Smith (1983).

1.2 Vesicular-Arbuscular Mycorrhizas

Fungi capable of entering into mycorrhizal associations with higher plants are found in all the taxonomic groups. The genera from the family Glomales (Morton and Benny, 1990) form morphologically distinctive tissues termed vesicular-arbuscular (VA) mycorrhizas. These appear to be the commonest and most widespread of the mycorrhizal fungi. As the experimental work presented in this thesis only involved VA mycorrhizal fungi, my discussion and comments shall only pertain to these types of mycorrhizas unless otherwise stated.

The great majority of terrestial plants, including most agricultural and horticultural species, can enter into symbiotic associations with VA mycorrhizal fungi. As a result of infection, the plant may show greater stress tolerence or disease resistance and enhanced growth, particularly in soils of moderate or low nutrient status. Indeed, some horticulturally important tree species seem to have an almost obligate requirement for VA mycorrhizal associations in order to attain satisfactory growth (see Harley and Smith, 1983).

The beneficial consequences associated with VA mycorrhiza formation have stimulated considerable research to determine if these fungi can be manipulated on an agronomic scale. It may be possible to improve plant growth and soil status whilst reducing the use of expensive and environmentally damaging chemical inputs.

A major stumbling block in the effective management of these beneficial micro-organisms, at both the experimental and field level, is the inability to culture VA mycorrhizal fungi axenically. The organisms are obligate biotrophs which suggests that specific plant gene products, possibly resulting from chemical signalling between the symbiotic partners, are required to mediate and maintain fungal growth and differentiation. Identifying the types of molecules involved may facilitate effective manipulation of these fungi. Investigation of plant gene expression in mycorrhizal roots is one approach to this particular problem. This introductory chapter aims to outline the salient features of VA mycorrhizas (predominantly from a host-plant perspective) with a view to using a molecular genetic approach to isolate and identify plant genes controlling the development of the symbiosis.

1.3. VA Mycorrhizas: Structure and Development

Investigating plant-gene expression in mycorrhizal roots requires a clear understanding of the developmental events which give rise to an effective VA mycorrhiza. Consideration of the infection process also provides the framework for predicting the types and expression patterns of plant genes which may be of importance during VA mycorrhizal formation.

The infection process is a well defined series of events. In order to reflect the natural course of events, it seems logical to discuss mycorrhiza development in terms of three separate regions; the rhizosphere where there is no direct contact between the fungus and plant cell walls; the root surface where initial contact is made; and within the plant root where contact is intimate and appears continuous.

(i) The rhizosphere

Infection of plant roots by VA mycorrhizal fungi is initiated by hyphae growing from neighbouring mycorrhizal roots or soil-borne propagules such as spores or root segments containing live hyphae (Tommerup and Abbott, 1981; Friese and Allen, 1991). Attempts to culture VA mycorrhizal fungi axenically have so far proved

fruitless although viable spores of VA mycorrhizal fungi will readily germinate in moist sterile soil or on water agar plates. Intact plant roots do not appear to be necessary for initiation of hyphal growth but enhanced germination in the presence of plant-roots or their exudates has been observed for VA mycorrhizal fungi (Powell, 1976; Gianninazi-Pearson *et al.,* 1989; Becard *et al.*, 1992).

One or more germ-tubes may emerge from each spore but hyphal growth is restricted to only a few centimetres. Germ-tube growth *in vitro* can be altered by the addition of a variety of nutrient substrates to the medium. Many organic substrates, vitamins, and sulphur compounds have been found to promote growth while others such as magnesium and zinc can inhibit growth (Hepper, 1979, 1984a and 1984b). The observation by Mosse (1959) that spore reserves are unlikely to be limiting during early hyphal growth suggests that lack of nutritional sources in the rhizosphere (or *in vitro*) are of minor importance to the continued hyphal growth of the free living mycelium. It appears likely then, that specific growth factors are required to ensure continued hyphal growth in freshly germinated spores.

The existence of these proposed growth factors is yet to be established but it seems reasonable to speculate that they are probably of plant origin. This is supported by the findings that roots (Mosse, 1988; Becard and Piche, 1989 and 1990), root exudates (Graham, 1982; Elias and Safir, 1987), cell suspension cultures (Carr *et al*, 1985) and cell products (Paula and Sequeira, 1990) all stimulate hyphal growth. Fungal morphogenesis in the rhizosphere prior to root-contact is induced by the roots of host plants but not non-host plants (Giovannetti *et al.*, 1993; Giovannetti *et al.*, 1994a and 1994b). Biologically active soil extracts, dialysates or filtrates also influence germination and early hyphal growth (Mosse,1959; Daniels and Graham, 1976; see Hepper 1984b) so microbial sources cannot be ruled out. More recently, CO_2 and flavanoid-like compounds have been implicated in the infection process (Becard and Piché, 1989; Becard *et al.,* 1992); these results are dealt with more thoroughly later on in this introductory chapter.

(ii) The root surface

When the fungal hypha eventually makes contact with a plant root a swollen appressorium may be produced (Garriock *et al.*, 1989) from which an infection peg usually arises. It seems clear that at this point 'recognition' of the host by the fungal partner occurs (see Smith and Gianinazzi-Pearson, 1988). It has been proposed that structures at the root surface may promote firmer attachment (appressorium formation) by the fungus prior to penetration of the root cells (see Harley and Smith, 1983; Anderson, 1988).

An infecting hypha may enter the root between or through epidermal cells or (more rarely) the root hair cells. This appears to occur anywhere along the root (Brundett *et al.*, 1985; Smith *et al.*, 1992).

(iii) Within the plant root

Once inside the root, hyphae grow through into the outer cortical cells where they branch and begin to spread longitudinally along the root. Frequently, two or more appressoria occur at discrete sites on a single root. These "infection units" (Cox and Sanders, 1974) spread through the epidermis and outer cortex with a fan-shaped morphology and may eventually overlap to form what appears to be a continuous infection. This phase of hyphal growth may be intercellular or intracellular with the formation of hyphal coils (Kinden and Brown, 1975). Hyphal spread in the outer cortex is usually restricted and most fungal growth occurs within the central and inner cortex of the root (Harley and Smith, 1983).

The mechanism of fungal penetration, mechanical and/or enzymatic, into epidermal or cortical plant cells is not known. Cox and Sanders (1974) were first to describe bulging of the cell wall around penetrating hypha. More recent ultrastuctural analysis has shown a clear breakdown of fibrillar components in the outer layers of the epidermal cell wall at the point of penetration whilst the inner layers appear to distend and envelop the invading hypha (Bonfante-Fasolo *et al.*, 1981; Bonfante-Fasolo and

Grippiolo, 1982). These results imply that penetration may be a result of mechanical pressure coupled with elasticity or extensibility (possibly induced) of the cell wall (Harley and Smith, 1983). Covalent cross-linking of the plant cell wall protein, extensin (Lamport, 1975), has been implicated with cell wall construction and rigidity (Chen and Varner, 1985). It has been postulated that proteolytic activity may be responsible for reducing the rigidity or disrupting the synthesis or assembing of this protein matrix (Harley and Smith, 1983; Anderson, 1988). This could result in an altered or incomplete cell wall structure amenable to distension and hyphal penetration. VA mycorrhizal infection pegs appear analogous to similiar structures that have been observed in many other fungal species; modified morphology (usually narrowing of the hypha) appears to be important in the infection process.

Pectinase activity has also been suggested as a mechanism to facilitate hyphal penetration through the epidermis (Garcia-Romero *et al.*, 1991). Production of cell wall-degrading enzymes (cellulases and pectinases) has been demonstrated *in vitro* with some ericoid and ectomycorrhizal fungi (Lindeberg and Lindeberg, 1977; Giltrap and Lewis, 1982; Cervone *et al*, 1988). There have been no reports demonstrating cellulase or pectinase activity *in planta* for VA mycorrhizal fungus.

It is within the cells of the inner cortex that the typical VA mycorrhizal structures, arbuscules and vesicles, are formed. Penetration of these cells starts as a constricted hyphal peg which causes the cortical cell wall to invaginate inwards. The invading hypha regains its normal size after penetrating the cell (Holley and Peterson, 1979) and immediately begins to branch dichotomously to form a distinctive 'tree-like' structure. The deposition of an amorphous layer or collar of host wall material around the point of penetration has been described (Dexheimer *et al.*, 1979; Harley and Smith, 1983; Bonfante-Fasolo, 1988a and 1988b). The cell wall becomes thinner and eventually discontinuous as the hyphae grow inwards, but the fungus does not penetrate nor appear to alter the cell plasmalemma (Bonfante-Fasolo *et al.*, 1981). These hyphal 'branches' eventually occupy much of the cell volume and form a very extensive

interfacial zone with the plant. This zone, comprised of fungal outer wall, an interfacial matrix (probably of host origin) and the cell plasmalemma, is thought to facilitate the bidirectional transfer of nutrients between fungus and plant, a feature of the VA mychorrhizal symbiosis (see extensive reviews by Gianinazzi-Pearson and Gianinazzi, 1983; Harley and Smith, 1983; Smith and Gianinazzi-Pearson, 1988). Not surprisingly, considerable interest has been centred on this zone and in particular the composition and origin of the interfacial matrix in the search to elucidate the mechanism of nutrient transfer.

The fungal wall at the arbuscular interface is much simplified. It is a thin amorphous structure whilst the extracellular fungal wall is thick and fibrillar (Bonfante-Fasolo and Grippiolo, 1982). The matrix appears to be a disorganised wall-like structure as it contains scattered fibrillar components and polygalacturonic acid (Dexheimer *et al.*, 1979; Bonfante-Fasolo *et al*, 1990).

The active life of the arbuscule is estimated to be only 4-15 days (Bevege and Bowen, 1975; Cox and Tinker, 1976; Alexander *et al.*, 1989). As they senesce, the hyphae become vacuoalated and eventually empty. Finally, the arbuscule collapses and aggregates. The ultrastructural organisation of the fungal walls appears to retain some of the same cytochemical characteristics as extracellular hyphae, although there is some suggestion of degradation (Bonfante-Fasolo *et al*, 1981). Absorption of the degenerating arbuscule or some of its contents by the plant cell, which remains active and reinfectable (see Harley and Smith, 1983) has been suggested as another mechanism of nutrient transfer (Scannerini, *et al.*, 1975). Cox and Tinker (1976) dismiss this suggestion as the senescing arbuscule is unlikely to provide enough phosphorous. The collapsed arbuscule may ultimately become encased in a polysaccharide fibrillar membrane, probably of plant origin (Dexheimer *et al.*, 1979). Intercellular hyphae show similar cytological changes, the hyphae become increasingly vacuolated with age and may become compartmentalised by the formation of septa (Harley and Smith, 1983).

Most VA mycorrhizal fungi form vesicles within or between the cortical cells which appear as large terminal swellings on inter- or intracellular hyphae. They contain lipid droplets (Holley and Peterson, 1979) and usually develop very late in mycorrhizal formation. They are probably storage structures.

The latter stages of mycorrhizal development are also associated with extensive growth of hyphae external to the root (Mosse and Hepper, 1975). This is presumed to be in response to the increased availability of nutrients which have been acquired from the host. These external hyphae have at least two major roles. They are intrinsic in the uptake and translocation of nutrients which may eventually be transferred to the host plant and they serve as a source of secondary infection along and between roots. For some VA mycorrhizal fungi, spores and vesicles can be produced on the external hyphae (Harley and Smith, 1983).

The infection of plant roots by VA mycorrhizal fungi is complex but appears well defined both temporally and spacially. This suggests that it is highly regulated and may be at least partly under plant genetic control .

1.5 Specificity and Recognition in VA Mycorrhizal Associations

Plants come into contact with vast numbers of micro-organisms during their lifecycles. The great majority of these encounters have no observable effect on the growth of the plant and yield or on reproductive ability because most potential invaders are only capable of infecting one or a few host species. Microbes inoculated onto non-host species are usually unable to breach the preformed physical barriers of the plant. If they do, the plant will often exhibit a resistance response which effectively halts the growth of the organism within the plant. Interactions characterised by the inability of a particular micro-organism to overcome the plant physical or biochemical barriers and colonise the host-plant tissue are usually termed incompatible associations.

Plant-microbial interactions which do result in extensive growth of the organism within the plant are termed compatible: they may be mutualistic, in which case both plant and micro-organism derive some benefit from the association, or pathogenic. The outcome of contact between a plant and a micro-organism appears to be at least partly dependent on a mutual perception between the interacting cells. It is presumed that specific extracellular molecules of each organism interact and that these events trigger cellular responses. The means by which these signals are transmitted and transduced within the cell are also poorly understood.

The concept of cell recognition arose because of the need to formulate a mechanistic basis for the discriminatory processes which appear to determine compatibility and possibly the nature of a particular symbiosis. Clarke and Knox (1978) have defined recognition as the "initial event in cell-cell communication that elicits a biochemical, physiological or morphological response". Sequeira (1984) suggested that it is "an early specific event that triggers a rapid, overt response in the host, either facilitating or impeding further growth of the pathogen". Defining in precise molecular terms the "initial" or "specific" event in any plant cell interaction has not proved possible to date and the mechanisms of plant cell communication are still poorly understood.

Molecular recognition and signalling resulting in defined cell reponses are probably fundamental to many plant biological processes; this includes cell division, cell growth and development, fertilisation and stress responses. Most researchers investigating these phenomena have approached the problem from a plant pathologists perspective, usually with the assumption that incompatibility (the resistance reaction) requires specific recognition events. Most reviews on the subject (see Sequiera, 1984; Collinge and Slusaranko, 1987; Lamb *et al.*, 1989 and Dixon and Lamb, 1990; Dixon *et al.*, 1994) reflect this bias. The reasons may be largely practical; pathogenic interactions are genetically well characterised and resistance responses are largely dominant gene-for-gene interactions. The more obvious benefits that are associated with plant

pathogen-related research (such as minimising agricultural losses) probably weigh largely as well.

The more complex the interaction, the greater the probability that a number of events determine the final outcome. It is likely that a succession of events; trophic or tactic responses; specific attachment molecules of host and/or microbial origin; and sensing or activational mechanisms, promote and define a compatible association. It should be noted that in the mutualistic *Rhizobium* -legume symbiosis, a series of recognition steps appear to condition compatibility (or susceptibility) to infection (see Bauer, 1981; Nap and Bisseling, 1990).

VA mycorrhizas are fundamentally different from other well characterised plant-microbial interactions (mutualistic and parasitic) in that they are persistent biotrophic interactions of very low or broad specificity. This implies that either a generalised or shared mechanism facilitating compatability has evolved in the majority of plant species or the plant simply does not 'recognise' the invader and a resistance (incompatability) response is not activated. The molecular basis of the processes controlling initial recognition and compatibility between plants and VA mycorrhizal fungi has engendered much discussion (see Harley and Smith, 1983; Bonfante-Fasolo and Spanu, 1992) but very little experimental data. The only clear evidence of recognition between VAM fungi and the potential host plant is the formation of the fungal appressorium. (Smith and Gianinazzi-Pearson, 1986). Transcriptional activation resulting in cutinase gene expression and subsequent penetration of the plant cuticle barrier has been reported in fungal pathogens including *Fusarium solani pisi* (Woloshuk and Kolattukudy, 1986). It seems likely that expression of VAM fungal genes which control appressorium development, possibly by transcriptional activation, must occur very quickly after contact between the two partners. Clearly it is a challenge to elucidate these molecular events.

Certain phenolic compounds which induce gene expression in plant-associated bacteria have been identified. Molecular analysis indicates that wound-induced monocyclic plant phenolics, particularly acetosyringone and some related compounds including chalcones, induce the expression of several virulence genes of *Agrobacterium tumefaciens* (Stachel *et al*, 1988; Bolton *et al.*, 1986). Expression of these genes is necessary for T-DNA transfer and subsequent tumour formation.

Similarly, one particular class of plant phenolics, the flavonoids, induce *Rhizobium* nodulation (*nod*) genes which are required for the formation of effective symbiotic associations with legumes (see Long, 1989a and 1989b). These compounds, some of which have been shown to be host-symbiont specific (Rossen *et al*, 1985 and 1987), are only found in the root exudate from zones where root hairs are emerging (Redmond *et al*, 1986). These zones are developmentally responsive to *Rhizobium* infection (Bhuvaneswari *et al*, 1981; Bhuvaneswari and Solheim, 1985). This type of evidence (see Innes *et al.*, 1885; Rolfe, 1988 and Rolfe and Gresshoff, 1988) shows that in at least some highly evolved symbiotic (parasitic or mutualistic) systems, signalling between the plant and microbe occurs before intimate or even direct contact is made.

There has been growing speculation that plant phenolic compounds may play a similar role and act as regulators of gene expression in VAM fungi-plant associations (Anderson, 1988; Becard and Piche, 1989; Peters and Verma, 1990; Phillips and Tsai, 1992). Some recent experimental evidence supports this contention.

The commercially available flavonones, hesperitin and naringenin, and the flavone apigenin were shown to enhance *in vitro* spore germination and/or hyphal growth of *Gigaspora margarita* by Gianiazzi-Pearson *et al.* (1989). The physiological relevance of these results is unclear, as these compounds had no significant effect on VA mycorrhiza formation in white clover (*Trifolium repens* L.) grown with two *Glomus* sp. (Siqueira *et al*, 1991). However, Siqueira *et al.* (1991) were able to demonstrate

that two isoflavanoids, formononetin and biochanin A, which had been isolated from white clover roots (Nair *et al.*, 1991) markedly increased VA mycorrhiza formation. Almost nothing is known about the modifications in plant gene expression which occur during the establishment of the symbiosis. Root cell-wall-bound peroxidase and chitinase activities appear to increase in leek seedlings during the early stages of mycorrhizal infection (Spanu and Bonfante-Fasolo, 1988; Spanu *et al.*, 1989), whilst the concentrations of phenolic compounds which have been correlated with antimicrobial activity (Mansfield, 1983) do not (Cordignola *et al.*, 1989). The isoflavanoids, glyceollin, coumestrol and daidzein rapidly accumulated in *Rhizoctonia solani* infected soybean roots, whilst *de novo* synthesis of these well characterised phytoalexins was only weakly induced by the mycorrhizal fungi *Glomus mosseae* and *Glomus fasciculatus* (Morandi *et al.*, 1984; Wyss *et al.*, 1990).

Gianinazzi-Pearson *et al.* (1990) compared the expression of the pathogenesis related protein gene, *PR-b* 1 (Van Loon *et al.*, 1985) in *G. mosseae* - and *Chalara elegans* -infected tobacco roots. This set of experiments were somewhat flawed because the authors used different membranes for the comparative total-RNA:PR-b1 cDNA hybridisations instead of loading all treatments on one filter. However, these results did indicate that the accumulation of *PR-b* 1 mRNA transcripts was higher in mycorrhizal roots than in the non-mychorrizal controls but very low in comparison to the pathogen infected roots. Franken and Gnadinger (1994) have detected similar responses in parsley. The protein was detectable using monoclonal antibodies in the *C. elegans* infected roots but the levels were either too low to detect or the mRNA's were not translated in the mycorrhizal roots. Using *in situ* hybridisation techniques, Harrison and Dixon (1994) have provided evidence that the expression of *Medicago trunculata* genes involved in the flavanoid/isoflavanoid pathway is elevated in (and only in) *Glomus versiforme* -colonised root cotical cells.

Nearly all research into recognition and compatibility in the VAM fungi-plant symbiosis has been somewhat derivative, taking ideas from the results obtained from

comparatively well studied plant-pathogen systems. It appears that plant phenolic compounds may activate or stimulate early hyphal growth in the rhizosphere and that (at least some) VA mycorrhizal fungi are capable of inducing a weak or transient general plant defence response during the early stages of infection. The generality of these observations remain undetermined and they may be of little biological significance; the flavanoid concentrations used in the spore germination/hyphal growth experiments were much higher than physiological levels; and the weak and apparently localised pathogen-like responses may simply be causally-related to harvesting stress or damage and the mechanical nature of fungal penetration into the root epidermis.

1.6 Effects of Mycorrhiza Formation on Phosphorous Uptake and Whole Plant Metabolism and Physiology

Ion uptake by plant roots has two major determinants; the movement or mobility of ions through the soil matrix and the absorption rate of the root (Nye and Tinker, 1977). Ions such as NO_3^-, SO_4^{2-} and Ca^{2+} can move rapidly by mass flow through the soil to the root surface. Uptake is limited by the plant root absorptive capacity. On the other hand ions such as $H_2PO_4^-$, NH_4^+, Zn^{2+} and Cu^{2+} are relatively immobile and move to the root surface by diffusion. In soils with low P, depletion zones can rapidly develop around the root zone (see Bieleski and Ferguson, 1983).

Mycorrhizal roots appear to be much more efficient at sequestering soil P than non-mycorrhizal roots (Cress *et al.*, 1979) because the external VA mycorrhizal hyphae increase the effective root surface area (Tinker, 1975a) absorb P from beyond the normal depletion zone and are able to translocate it to the plant root (see Bieleski and Ferguson, 1983). Six fold increases in the rate of P uptake have been reported (Sanders and Tinker, 1973).

Translocation studies using labelled N sources only accessible to the fungal hyphae show that VA mycorrhizal celery plants can also accumulate more ^{15}-N than non-mycorrhizal controls (Ames *et al.*, 1983; Ames *et al* , 1984). Smith *et al* , (1986)

provided corroborative evidence that external hyphae may also translocate soil N when they reported greatly increased N inflow into VA mycorrhizal leek plants. This aspect of nutrient acquisition in VA mycorrhizal roots remains poorly researched.

The development of functional mycorrhizas usually increases the uptake of P into host plant roots. The absence of mycorrhizas can result in P deficiency in non-mycorrhizal control plants. Direct comparisons between mycorrhizal and non-mycorrhizal plants are difficult, if not impossible to interpret because many of the growth responses observed in plants as a result of VA mycorrhiza formation (see Table 1) are directly or indirectly related to improved nutrition (Mosse, 1973) particularly P (Tinker, 1975b) but also Zn2+ and Cu2+ (Gilmore, 1971; Kleinschmidt and Gerdemann; Lambert *et al.,* 1979).

There is a paucity of information on the effects of P deficiency on specific plant metabolic pathways (Smith and Gianinazzi-Pearson, 1988). This notion can be expanded to include nutrient availability in general and needs to be kept firmly in mind when designing experiments to elucidate the mechanisms controlling plant responses to VAM fungal infection, particularly at the cellular level where small and/or temporary changes in P availability may have dramatic and confounding effects.

There are several excellent reviews (see Hayman, 1982; and Smith and Gianinazzi-Pearson, 1988) detailing the multitude of effects that VA mycorrhiza development has on whole-plant growth and development. Examples of these effects are presented below (Table 1) in order to emphasise the very broad range of modifications that can occur and to highlight the fundamental problem associated with comparative experimentation.

Table 1 Reported host responses to mycorrhizal infection

Effect on plant metabolism/physiology	Host plant	Reference
Increased photosynthesis	*Vicia faba*	Kucey and Paul (1982).
	Allium porrum	Snellgrove *et al.*, (1986).
	Bouteloua gracilis	Allen *et al.*, (1981).
Increased nitrate reductase and glutamine synthase activities	*Trifolium sp.*	Oliver *et al.*, (1983).
	"	Carling *et al.*, (1978).
	"	Smith *et al* , (1985)
Increased hydraulic conductivities	*Bouteloua gracilis*	Allen *et al.*, (1982).
	Glycine max	Safir *et al.*, (1971).
Decreased susceptibility to wilting	*Trifolium pratense*	Hardie and Layton (1981).
	Citrus jambhiri	Levy and Krikun (1980).
	Allium porrum	Nelson and Safir (1982).
Increased cytokinin levels	*Boutaloua gracilis*	Allen *et al.*, (1980).
Altered amino acid content	*Zea mays*	Young *et al.*, (1978)
	Citrus sp.	Nemec and Meredith (1981)
	Glycine max	Pacovsky (1989).
Increased tolerance to root pathogens	several species	Bartchi *et al.*, (1981)
		Schenck *et al.*, (1974 and 1975).
Lower root:shoot ratios	many species	Harley and Smith (1983).
Increased fresh weight:dry weight ratio	*Allium porum*	Snellgrove *et al.*, (1982).
		Tester *et al* , (1987).

1.7 Cellular Responses to Mycorrhizal Infection

Infection studies, using a variety of plant-VAM fungus combinations, have clearly demonstrated that VA mycorrhiza development is well defined and highly predictable (see Harley and Smith, 1983). Two types of plant root cell can be affected; the epidermal and outer cortical cells within which simple hyphal coils may develop and the inner cortical cells which usually contain the highly branched, haustorium-like arbuscules.

Ultrastructural and cytochemical analyses indicate that the fungal coils are surrounded by a thick layer of continuous and fibrillar material (interfacial matrix) which appears to be unmodified primary cell wall (Bonfante-Fasolo, 1988). The cytoplasm, central vacuole and amyloplasts appear normal (Bonfante-Fasolo, 1987). Cells containing arbuscules show distinct cytological modifications; the invading hypha penetrates the cell wall and invaginates the plasmalemma which substantially proliferates (Cox and Sanders, 1974; Toth and Miller, 1984) around the rapidly branching hyphae to accomodate the increased surface area and a thin layer of interfacial matrix is synthesised between the fungal cell wall and the plant plasmalemma (Scannerini and Bonfante-Fasoslo, 1979).

When compared to non-mycorrhizal inner cortical cells, the newly synthesised plasmalemma and cytoplasm adjacent to the interfacial matrix show some distinct modifications. Jabaji-Hare *et al.* (1990) used lectin-gold complexes to demonstrate that N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-mannose and sialic acid sugar residues were more abundant in arbuscular cells of *Glomus clarum* -infected leeks, particularly in the cytoplasm and the plasmalemma surrounding the plant-fungus interface. Increased neutral phosphatase activity, frequently associated with cell wall synthesis (Jeanmaire *et al.*, 1985) and ATPase activity (Marx *et al.*, 1982; Gianinazzi-Pearson *et al.*, 1991) has been localised (using Pb deposition) to this region of the plant plasmalemma. Physiological and biochemical studies strongly suggest that membrane embedded proton-pumping (H+) ATPases control many aspects of plant

physiology (see Serrano, 1989; Sussman and Harper, 1989) including solute transport coupled to the pH and electrochemical gradients generated by these enzymes (Serrano, 1989). There are some legitimate critisisms of the cytochemical procedures used to detect H^+-ATPase activity (see Katz *et al.,* 1988; Smith and Smith, 1990; Gianinazzi-Pearson *et al.,* 1991). However, it seems fair to conclude that these and other modifications which are not observable around the hyphal coils or in uninfected cells, indicate specialised alterations to the host membrane surrounding the fine arbuscule branches (Smith and Gianananzzi-Pearson, 1988) which are consistent with the argument that this is the site of bidirectional nutrient transport.

In a comparative study of nuclei from the roots of uninfected leek and leek infected by a *Glomus* sp., Berta *et al.* (1990) presented evidence that the nuclei from the mycorrhizal roots were significantly hypertrophied. The authors suggested that this was probably due to chromatin decondensation, an argument which they supported with transmission electron micrographs. They went on to speculate that these results may be related to increased transcriptional activity as a result of the *Glomus* infection.

As yet there is no quantitative data to support this hypothesis but two other lines of evidence do suggest modifications to plant-gene expression: the cytoplasm adjacent to the interfacial matrix appears to contain much higher concentrations of ribonucleic acid (RNA) than the cytoplasm of non-mycorrhizal cortical cells (Berta *et al.*, 1990) and the profiles of soluble proteins extracted from mycorrhizal roots of onion, tobacco (Dumas, *et al.*, 1989) and soybean (Pakovsky, 1989) show qualitative and/or quantitative changes in comparison to non-mycorrhizal roots. It is almost certainly true that some of the new proteins detected in these studies are artifactual and/or of fungal origin (Guttenberger and Hampp, 1992) or represent plant defence-related proteins (see Harrison and Dixon, 1994). Cytochemical localisation of proteins within developing mycorrhizas, using antibodies raised to these proteins may be the only way of unambiguously determining their origin. The purification of a putative acid chitinase

which is present, independent of the fungus involved in the symbiosis, is under way (see Dumas-Gaudot *et al.*, 1992).

1.8 Incompatibility in Plant-VAM Fungus Interactions

Whilst the overwhelming majority of plant species are susceptible to infection by VAM fungi, a number of plant genera do not appear to form typical or normal VA mycorrhizas. Unfortunately, much of the data collected on non-mycorrhizal or poorly-mycorrhizal plant genera has been obtained from field grown plants. As Tester *et al.* (1987) have pointed out, there could be several explanations for the apparent lack of infection in these samples; the plant roots may not have come into contact with viable (or compatible) inoculum; infection may be seasonal and the samples were collected at the wrong times; the local soil environment may not be conducive to, or may actively discourage infection (ie. high soil P, waterlogging, residual pesticide/herbicide activity); or the plants may be inherently non-mycorrhizal.

There is also the added problem of defining mycorrhizal status in order to score infection. Some VAM fungi-plant combinations do not form vesicles but do form functional mycorrhizas while others can lack normal arbuscules or appear as extensive but atypical infections (Harley and Smith, 1983; McGee, 1985) which may or may not be functionally active. Glasshouse experiments using controlled inoculum levels co-ordinated with detailed infection studies are absolutely necessary to accurately determine whether a particular species/genus/family of plants is capable of forming functional mycorrhizas (for a complete summary of these problems see Tester *et al.*, 1987).

Never-the-less it has been established that several plant families can be considered genuinely and generally non-mycorrhizal under controlled glasshouse conditions. Of these the Brassicaceae and the Chenopodaceae are the best characterised (see Hirrel *et al.*, 1978). Other important families include the Proteaceae (Khan 1978; Lamont 1982), the Zygophyllaceae (Khan 1974) and the Restionaceae (Lamont 1982). Several

non-mycorrhizal genera, most notably *Lupinus* (Morely and Mosse 1976; Trinick 1977), occur in families which are considered to be mycorrhizal.

How non-mychorrhizal plants resist or greatly restrict infection by VAM fungi is not known, but this incompatibility suggests that these species are fundamentally different from the majority of vascular plants. A substantial amount of work has concentrated on identifying fungitoxic compounds which accumulate within or are exuded from the roots of non-mycorrhizal plant species (see Koide and Schreiner, 1992).

Glucosinolate hydrolysis products, such as isothyocyanates (mustard oil) have received the most attention but the results are inconclusive as there does not appear to be a strong inverse relationship between root concentrations of these compounds and infection. Isothyocyanate-producing species (such as *Carica papaya*) which would be expected to display restricted infection, form normal mycorrhizas (Peterson *et al.*, 1985). *Brassica* sp. which display marked phenotypic variation in root glucosinolate concentration (7-524mmol/gf.wt) remain uninfectible (Glenn, *et al.*, 1985) while normal infections were produced in compatible hosts grown in close proximity to these plants, indicating that root exudates are probably not a determinant of VA mycorrhiza incompatibility in *Brassica* sp. either (Glenn, *et al.*, 1988). In contrast, root exudates from *Lupinus albus* do appear to hinder hyphal attachment and appressorium formation (Giovannetti *et al.*, 1993) and intergenic grafts between *Lupinus* and *Pisum* can induce resistance to VA mycorrhizal symbiosis in the pea roots, suggesting the presence of inhibitory systemic shoot factors in *Lupinus* sp. (Gianinazzi-Pearson and Gianinazzi, 1992).

Infection in non-host plants can sometimes be induced by the close proximity of an infected mycorrhizal host plant (see Hirrel *et al.*, 1978). In these cicumstances the infected host plant may provide compounds (nutritional or otherwise) critical in stimulating hyphal growth and development which allows the subsequent infection of a non-host plant (Tester *et al.*, 1887). These taxa may prove to be particularly important in elucidating the mechanisms which determine the outcome of VAM

fungus-plant interactions. There is very little known about the molecular or genetic basis of mycorrhizal development. The incompatibility observed in certain plant genera may reflect the absence or mutation of a particular plant gene product or the evolution of specific resistance mechanisms. The latter appears more likely given the apparent diversity of resistance stategies.

Recently, alfalfa (*Medicago sativa* L.), pea (*Pisum sativum* L.) and faba bean (*Vicia faba*..L.) genotypes defective in mycorrhiza formation have been reported (Bradbury *et al.,* 1991; Duc *et al.*, 1989). The true value and significance of these particular genotypes remains unclear because the classical genetic studies they undertook indicate that these mutations are tightly linked or involve recessive mutations to (as yet uncharacterised) *Rhizobium* symbiosis loci which clearly complicates further molecular or genetic characterisation of these particular plant genotypes: My interpretation of these data, and those presented by Gollotte *et al.* (1993) does not rule out the induction of a general root resistance-like response in these genotypes and in any event, the analysis of a biochemical pathway involved in the establishment of both the *Rhizobium* and VA mycorrhizal symbioses (both of which are often associated with dramatic nutritional effects), quickly becomes complicated. However, as Duc *et al.* (1989) point out, these plants could prove to be extremely useful for investigating genetic variabilty and specificity in VA mycorrhizal fungi.

What is urgently required is a set of plant genotypes, wild-type or induced, deficient at various stages of mycorrhizal development; ideally the altered phenotypes should range from very early infection stages (such as fungal attachment and appressorium development) through to the later stages (arbuscule and vesicle development). Preferably they should be derived from a non-leguminous plant species or cultivar which has been genetically well characterised so as to allow a comparative analysis of the two genotypes (see Smith, 1995).

1.9 Aims and Significance of this Project

There is good evidence that host plant cells are cytologically modified in direct response to infection by VA mycorrhizal fungi. It is reasonable to suggest that this is a result of altered gene expression (possibly by *de novo* transcriptional activation) within host plant cells.

The overall aim of this project was to develop a model system suitable for undertaking a molecular analysis of mycorrhiza development, and to determine if particular host plant genes are expressed during the early stages of infection. The types of questions to which answers are sought include:

(i) Is there an association between the early stages of mycorrhiza development and the accumulation of specific mRNA species?

(ii) How do these mRNA species accumulate temporally and spacially within the mycorrhizal plant root?

(iii) What are the functions of the mRNA translation products?

This project was divided into 3 distinct stages:

(i) The development of a model VAM fungus/plant system suitable for molecular analysis.

(ii) The production of a mutagenised plant population and subsequent identification of plant mutants deficient in mycorrhiza formation.

(iii) Cloning and characterisation of putative mycorrhiza-related mRNA species.

This research is significant for several reasons. It has the potential to identify some of the molecular events which occur in this association and would represent the first reports of this nature. The information obtained may ultimately be used to identify the biochemical events which control the symbiosis. It is possible that the mycorrhizal association represents a "basic compatibility" (Ellingboe, 1981) upon which the action

of specific resistance genes have been superimposed in plant/pathogen symbioses. Understanding the molecular basis of compatibility may help in elucidating how plants respond during incompatible interactions. Finally, the development of a well characterised model system and the isolation of plant genotypes deficient in mycorrhiza development and/or function could prove to be extremely valuable for future researchers wishing to carry on this work.

Chapter 2

Materials and Methods

2.1 Plant and Fungal Genetic Stocks

2.1.1 Plants

Seeds of *Hordeum vulgare* L. cv. 'Galleon' and cv. 'Schooner' were supplied by Dr. R.G. Lance, Dept. of Plant Science. Barley addition and ditelomeric lines were provided by Dr. Ken Shepherd, Dept. of Plant Science. The doubled haploid mapping populations were prepared by Drs Sue Logue (Chebec x Harrington and Galleon Haruna Nijo) and Rafique Islam (Clipper x Sahara).

2.1.2 Fungi

Glomus intraradices Schenk and Smith inoculum was obtained from pot cultures of *Trifolium subterraneum* L. grown in a soil:sand mix as described below. All pot cultures were supplied by Dr. S.E. Smith.

Gaeumannomyces graminis var. tritici (Ggt-800) which was isolated from *Triticum aestivum* at Avon, S.A. by A.D. Rovira and *G. graminis* var. graminis (Ggg-W2P) which was isolated from a *Paspalidium* sp. at Walcha, N.S.W. by P.T.W. Wong were obtained from Dr. Paul Harvey. These were supplied as pure cultures grown on PDA medium.

2.2 Plant Growth Conditions

2.2.1 Potting Medium

Plants were grown in a mixture of steamed sand and autoclaved soil (9:1 w/w). Plants were grown in either white plastic pots supplied by Disposable Plastic Products or containers made from black plastic bags. The pot and container sizes varied between experiments (see the relevant chapters) and neither contained drain holes.

2.2.2 Seed Sterilisation

Seeds were washed in NaOCl (O.5% v/v) for 2 minutes and then washed 2-3 times in autoclaved H_2O (the H_2O used in all of the investigations described herein was of nanopure quality). This was followed by 2x2 minute washes in 90% ethanol and a further 3 washes with autoclaved H_2O. The seeds were then placed in autoclaved glass Petri dishes containing 3 sheets of sterilised filterpaper and 15ml of sterilised H_2O. The Petri dishes were covered in aluminium foil and left at 22-25^oC for 36-48 hours.

2.3 Plant Growth

The growth conditions were either in a glasshouse without artificial light when the natural daylength was less than 11 hours or in a growth cabinet with 11 hours of artificial light (600-700mE). The day and night temperatures were 18-20^oC and 14^oC respectively.

All plants received mineral nutrients (-P unless otherwise stated) at the beginning of the growth period and at 2 weekly intervals. These nutrients were made from stock solutions (see Appendix 1) when required and applied at the rate of 10ml/kg of sand:soil. In some experiments (see Chapters 3 and 6) a range of phosphate (P) levels were used.

The following P levels apply where indicated:

P_0 :No added phosphate

P_1 :Phosphate added at the rate of 0.5mmoles/kg of soil:sand mix.

P_2 :Phosphate added at the rate of 1.5mmoles/kg of soil:sand mix.

P_{Add} :No added phosphate at the beginning of the growth period but phosphate was added at the rate of 0.5mmoles/kg of soil after 12 days growth.

2.4 Harvesting of Plant Material

Plants were harvested at the times indicated in each experiment.The roots were quickly but carefully washed in distilled water to minimise tissue damage and the accumulation of mRNA transcripts which might have been induced by the harvesting process. The roots were then blotted dry with tissue paper. Plant growth of roots and shoots was evaluated by fresh weight (immediately after careful blotting) or dry weight (after incubation at 80°C overnight). Root and shoot material required for nucleic acid extraction was snap frozen in liquid nitrogen within 2 minutes of harvesting.

2.5 Assessment of Mycorrhizal Colonisation

The extent of mycorrhizal infection in the roots was assessed as follows: Root samples were washed until free of the potting medium, cut into 10mm pieces and cleared by incubation in a 10% KOH solution for 4 hours at 55°C. The cleared root sub-samples were stained with Trypan Blue (Phillips and Hayman, 1970) and the extent of mycorrhizal colonisation (as a percentage of total root length) of each root sub-sample was determined using a grid intersect method (Giovannetti and Mosse, 1980). Mycorrhizal root sections were usually scored on a 1-3 scale which described the type of colonisation: (1) intercellular infection only, (2) arbuscules present and (3) arbuscules and vesicles present.

2.6 Other Materials.

A large number of enzymes, bacterial strains, media and solutions were used in the course of this work. The vast majority are those which are commonly used for investigations requiring recombinant DNA technology and have been described in Maniatis *et al.* (1983). I have listed those that I used and their composition (where applicable) in Appendix 1.

2.7 General Recombinant DNA Methods

2.7.1 *Escherichia Coli* Methods

2.7.1.1 Bacterial Cultures

Starter cultures and small scale plasmid extraction cultures were prepared by inoculating 10ml of culture medium (LB or TFB) containing the appropriate antibiotic with a single colony of *E. coli* from a fresh agar plate. They were then incubated 6-8 hours at 37°C with shaking.

Large scale cell cultures (50-250ml) were prepared by inoculating culture medium (LB or TFB) containing the appropriate antibiotic with 1-2ml of fresh starter culture and incubating as described above until the desired optical density of the culture was reached.

2.7.1.2 Preparation of *Escherichia coli* Competent Cells

LB (150ml) in a 250ml centrifuge flask was inoculated with the appropriate *E. coli* strain starter culture (1ml) and incubated at 37°C with shaking until the A_{600} was approximately 0.6. The cells were then cooled in iced water before being centrifuged (5000rpm/5 minutes/4°C). The supernatant was poured off and cold 50mM $CaCl_2$/10mM Tris (pH 8.0) solution (75ml) was added. The cells were gently resuspended and and left on ice for 15 minutes before being centrifuged (1000rpm/5 minute/ethanol precipitation (cells were then very gently resuspended in cold 50mM $CaCl_2$ (5ml) whilst swirling the flasks in iced water. The cells were either used for the transformation immediately or divided into 200µl aliquots and prepared for snap

freezing in liquid nitrogen and storage at -75°C after the addition of glycerol (30µl/aliquot).

2.7.1.3 *Escherichia coli* Transformations

Fresh competent cells or gently thawed frozen competent cells (200µl) were dispensed into Eppendorf tubes. Plasmid DNA (5µl) was added and the solution gently mixed. The cell/DNA mixture was then left on ice for 30 minutes before being heat shocked in a 42°C water bath for 2 minutes. The mixture was incubated on ice for 3 minutes and then at room temperature for 5 minutes. LB (800µl) was added and the cells incubated for a further 2 hours before spreading on LB 1.5% agar plates with the appropriate antibiotic to select for plasmid transformation and then incubated overnight at 37°C. A single colony was picked and cultured overnight in LB medium plus antibiotic (50µg/ml ampicillin when selecting for pTZ18U).

2.7.2 RNA Methods

2.7.2.1 Isolation of Plant Total RNA

The mortars, pestles, spatulas and glassware used for RNA isolations were oven-baked at 160°C for a minimum of 6 hours to inactivate exogenous RNase activity. Harvested root or shoot material (0.5-1.0gm) was snap-frozen in liquid nitrogen, placed in a mortar and ground to a fine powder. More liquid nitrogen was added to pool the root material at the bottom of the mortar before grinding for another 60 seconds. The frozen powder was transferred to a 30ml Corex centrifuge tube containing 2-5ml (100mM Tris HCl, pH 8.4; 10mM EDTA, 4% Sarkosyl) RE buffer. The slurry was vortexed (30 seconds) before centrifugation (11000 rpm/10 min/4°C) in a JA20 rotor. The supernatant was transferred to a 10ml Beckman ultracentrifuge tube containing 1g of CsCl added for each 1ml of supernatant. Three ml of a CsCl solution (0.975g/ml) was laid into the bottom of the tube using a sterilised Pasteur pipette. The tube was then centrifuged (30000 rpm/14-16 hours/6°C) in a Ti 70.1 rotor to pellet the total RNA.

After centrifugation, the sticky surface layer was removed using sterilised cotton buds and the supernatant removed with a baked Pasteur pipette. The sides of the tube were carefully and thoroughly wiped to remove as much of the residual supernatant as possible before the pellet was resuspended in 400µl of cold RE buffer (this was done on ice to minimise residual RNAse activity) and transferred to a 1.5ml Eppendorf tube containing phenol/chloroform/isoamylalcohol (400µl). After gentle vortexing (30 seconds) and centrifugation (12000rpm/5 minutes) the upper phase was transferred to a fresh tube and ethanol-precipitated with the addition of 3M sodium acetate (40µl) and 1ml of cold ethanol. The total RNA pellet was washed with 70% ethanol (1ml) before resuspension in an appropriate volume of RNase-free H_2O.

2.7.2.2 Synthesis of Double-stranded cDNA and Cloning into λgt10

Double-stranded cDNA with linked *Eco* R1/*Not* I adaptors was prepared using a cDNA synthesis kit purchased from Promega. The manufacturers protocols were followed precisely. The cDNA product was isopropanol precipitated and resuspended in 10µl of nanopure H_2O.

The linkered cDNA (2µl) was ligated for 12 hours at 15°C with *Eco*R 1 digested/dephosphorylated λgt10 arms (0.5µg) in a final volume of 5µl. The ligation mix was then ethanol precipitated and washed with 80% ethanol prior to packaging and transfection of competant cells.

2.7.2.3 Packaging and Transfection of Phage Library

Packaging of the cDNA/λgt10 ligation mix into infective bacteriophage λ particles was carried out according to the manufacturers (Promega) instructions. The packaging-reaction mixes were diluted to 500µl with SM buffer and 15µl of chloroform was added to prevent contaminating bacteria from growing.

The transfection of packaged DNA into *E. coli* strains C600 and C600*hfl* was carried out as follows:

A starter culture was prepared by inoculating LB (50ml) containing 10mM $MgSO_4$.and 0.2% maltose, with an appropriate *E. coli* strain and incubating the culture overnight at 37°C with shaking.

The recipient cells were prepared during the DNA packaging incubation period by inoculating LB medium (20ml) containing 10mM $MgSO_4$.and 0.2% maltose, with the overnight culture (500µl). The culture was incubated at 37°C with shaking until the optical density at 600nm was approximately 0.6 units. This incubation was carried out in 50ml flasks to ensure optimal aeration. The cells were then transfered to a 25ml centrifuge tube and pelletted (3000rpm/10minutes/4°C) in the SS34 rotor. The supernatant was discarded and the cells resuspended in cold 10 mM $MgSO_4$ (10ml). An aliquot of the cell suspension (100-300µl) was then inoculated with packaged DNA (100µl), gently mixed and incubated at 25°C for 15 minutes with no shaking. Molten warm Top Agarose (4-12ml depending on the plate size) containing 10mM $MgSO_4$ was added and the mixture gently vortexed before being overlayed onto a room temperature LB agar plate. The plate was incubated for 8-12 hours at 37°C.

2.7.2.4 RNA Gel Electrophoresis

All RNA separations were performed in a mini-gel apparatus under RNA denaturing conditions using a 1.2% agarose MOPS/EDTA gel matrix and loading buffer containing formaldehyde (2.2M) and formamide (50%). The RNA (5 or 10µg/lane) was dried under vacuum and resuspended in RNA load buffer (4.5µl) and formamide/formaldehyde (9.5µl). The sample was incubated at 70°C for 10 minutes to denature the RNA secondary structures and then chilled on ice for 2 minutes. Gel loading buffer (3µl) was added and well mixed before loading into the gel. Samples were separated at 30mA for 30 minutes and then 60mA for a further 1-2 hours.

2.7.2.5 RNA Gel Blotting, Prehybridisation and Hybridisation

RNA species (total or poly A+ preparations) were separated by RNA-denaturing electrophoresis and transfered to Hybond N^+ membrane by capillary blotting in 10xSSC. The membranes were fixed by baking at 80°C for 2 hours. Prehybridisation and hybridisation procedures were essentially the same as for DNA hybridisations except that RNA prehybrisation and RNA hybridisation solutions (see appendix 1) were used.

2.7.2.6 Synthesis of Radiolabelled First-strand cDNA

Total RNA (10-30 µg) or mRNA (0.1-0.5µg) was made up to a final volume of 10µl using RNase-free H_2O. One µl of oligo (dT) $_{12-18}$ (500mg/ml) was added and the mixture incubated at 70°C for 10 minutes before quick chilling on ice. First strand reaction mixture (see appendix 1) was prepared (8µl) and added to the RNA/oligo-nucleotide mixture. M-MLV H^- reverse transcriptase (200 units) was then added and the reaction mix incubated at 37°C for 45-60 minutes. After completion of the reaction, a 1µl sample was removed to a new Eppendorf tube and mixed with 9µl of TE buffer. Then 1µl of this solution was spotted onto one DE50 filter and 5µl onto a second filter. The incorporation of α-^{32}P-dCTP into the newly synthesised DNA was checked by the trichloroacetic acid precipitation of the DNA and comparison of the relative Cherenkov radiation signals (Maniatis *et al.* 1982). First strand cDNA probes with a specific activity in excess of 1×10^8 cpm/mg were purified using a Sephadex G-100 column.

2.7.3 DNA Methods

2.7.3.1 DNA Isolations from Plant and Fungal Tissues

(i) Root and fungal tissue

High molecular weight DNA (suitable for PCR analysis or restriction endonuclease digestion) was isolated from small amounts (10-100mg) of root tissue and fungal spores using a modification of the method descibed by Saghai-Maroof *et al.*, (1984).

The procedure, which uses cetyltrimethylammonium bromide (CTAB) buffer, is described at Appendix 2.

(ii) Leaf tissue

The isolation of high molecular weight plant DNA from leaf tissue was carried out as described at Appendix 3.

2.7.3.2 Plasmid Isolation from *Escherichia coli* Transformants

(i) Small scale isolation of plasmid DNA

Plasmids for immediate restriction-digest analysis were prepared by a modification of the alkaline lysis method (Birnboim and Doly, 1979). The protocol is described in Appendix 4. Plasmid DNA for sequencing was also prepared as described in Appendix 4 but the final pellet was further purified by PEG precipitation.

(ii) Large scale isolations of plasmid DNA

Plasmids for purification and storage were prepared from larger volumes (250 or 500ml) of cell cultures as described by the Maniatis *et al.* (1982).

2.7.3.3 Purification of Large-Scale Plasmid DNA Preparations

Large-scale preparations of plasmid DNA were purified on caesium chloride-ethidium bromide density gradients. The method was as described by Maniatis *et al.* (1982) except that only 600µl of ethidium bromide was added, no light parafin oil was used and the Beckman ultralock tubes were centrifuged at 50000rpm in the Ti-70.1 rotor for only 14-16 hours. The bands were visualised and extracted under long-wave ultra-violet light. Ethidium bromide removal, dialysis and DNA precipitation and resuspension were as described by Maniatis *et al.* (1982).

2.7.3.4 Isolation of Bacteriophage DNA

Bacteriophage lambda DNA was isolated using a modification of method described by Amersham in the cDNA cloning-kit manual and uses DEAE-52 cellulose to remove contaminating *E. coli* DNA from the lysate. The method is described in Appendix 5.

2.7.3.5 DNA Precipitations

(i) Ethanol precipitations

One tenth volume 3M NaAcetate (pH 4.5) was added to help all precipitations, except those being carried out in high salt buffers (eg. precipitation of minipreparation plasmid DNA). Two volumes of cold absolute ethanol were then added and the solution left to stand for at least 30 minutes at -20°C. The DNA was pelleted by centrifugation, washed with 70% ethanol to remove salts, vacuum dried, and resuspended in the desired volume of nanopure H_2O or TE buffer.

(ii) Isopropanol precipitations

One tenth volume of 3M KAcetate (pH 4.5) was added to all precipitation reactions. One volume of isopropanol was then added and the solution left to stand at room temperature for 5-10 minutes. The DNA was pelleted by centrifugation, washed twice with cold 70% ethanol, vacuum dried and resuspended in the desired volume of nanopure H_2O or TE buffer.

(iii) Polyethylene glycol precipitations

Preparations of DNA to be used for sequencing reactions included an extra precipitation using polyethylene glycol (PEG). Ethanol or isopropanol precipitated DNA was resuspended in 32µl of nanopureH_2O. 4M NaCl (8µl) was mixed with the DNA sample before the addition of 13% PEG (40µl). The solution was then incubated on ice for 30-60 minutes. The DNA was pelleted by centrifugation at 4°C, washed

twice with cold 70% ethanol, briefly vacuum dried and resuspended in 10-20µl of nanopure H_2O.

2.7.3.6 DNA Gel Electrophoresis

DNA fragment separations were carried out by submerged, horizontal gel electrophoresis in TBE agarose or TAE agarose gels in their respective conductive buffer solutions. The TAE agarose gels were used only for qualitative restriction-digest fragment analysis or for fragment purifications using the 'Gene-clean' system. Rapid sreening of restriction digests or DNA preparations was carried out using mini-gel apparatus. Overnight electrophoresis was carried out in large tanks. The DNA fragments separated by gel electrophoresis were stained with ethidium bromide (1 µg/ml) to enable visualisation or photography of the bands under short or long-wave length UV light .

2.7.3.7 DNA Gel Blotting, Prehybridisation and Hybridisation

(i) Plaque hybridisation studies

Recombinant λgt10 DNA was denatured and fixed to Hybond N^+ using a procedure similar to the bacterial colony transfer method described by Benton and Davis (1978): Phage were plated out as previously described using 13.5cm disposable Petri dishes. A dry, circular, 13cm diameter Hybond N^+ membrane filter was placed on the surface of the bacterial lawn containing the phage plaques and marked so as to permit unambiguous determination of the orientation of the membrane relative to the plate at a later time. The membrane was then carefully removed and briefly air dried, colony side up, before being treated, in assembly line fashion, by exposure to the following solutions: (1) denaturing (0.5M NaOH/1.5M NaCl) for 3-5 minutes, (2) neutralisation (0.5M Tris-HCl pH 7.5/1.5M NaCl) for 5-10 minutes and (3) 2x SSC for 1-2 minutes. These treatments were carried out in trays containing two sheets of Whatman 3 MM filter paper saturated in the appropriate solution. Membranes were treated

colony side up and never submerged as this usually caused streaking. The aim was to treat the transferred phage DNA by exposure to the solutions through the membrane.

After air drying on a single sheet of Whatman 3MM paper, the filter was placed in a 'Hybaid' hybridisation bottle and prehybridised in 20ml of DNA prehybridisation solution (see appendix 1) for 4-8 hours. The DNA prehybridisation solution was then replaced with 10ml of freshly prepared DNA hybridisation solution (see appendix 1) plus denatured radioactive probe (200µl/bottle) and salmon sperm DNA (100µg/ml final concentration). The membrane was left to hybridise with the probe for 18 hours at 65°C with constant rotation in the Hybaid oven. The filters were then removed from the bottles and agitated in 200ml of wash buffers 1 and then 2 (see appendix 1) at 68°C for 25 minutes to remove excess probe and hybridisation solution. The washings were repeated twice before the filter papers were exposed to X-ray film at -80°C for a minimum of 48 hours using intensifying screens.

(ii) Restriction digested-DNA hybridisation studies

DNA fragments were separated by 0.7-2% agarose TEB gel electrophoresis and then transferred to Hybond N+ membrane using the capillary blotting method (Southern, 1975). The membrane was placed in a Hybaid hybridisation bottle containing 20ml of DNA prehybridisation solution (see appendix 1) and prehybridised for 4-12 hours at 65°C in a Hybaid hybridisation oven with constant rotation.

The DNA prehybridisation solution was replaced with DNA hybridisation solution (10ml), denatured radioactive probe (200ml) and salmon sperm DNA (100ug/ml final concentration), the hybridisation was then allowed to proceed for 12-18 hours at 65°C in the oven with constant rotation. The membrane was removed and washed twice with constant agitation in wash buffer 1 (200ml/65°C/20 minutes) and once in wash buffer 2 (200ml/65°C/20 minutes) to remove the hybridisation solution and excess probe. Membranes which showed considerable background radioactivity after these

treatments were washed in wash buffer 3 (200ml/65°C/10 minutes) The membranes were then exposed to X-ray film at -80°C using intensifying screens.

2.7.3.8 Restriction Endonuclease Digests

Complete restriction digests were carried out in buffer conditions recommended by the enzyme supplier. Sufficient restriction enzyme (10-50u per digestion) was added to completely digest the DNA in 2 hours at 37°C.

2.7.3.9 Isolation of DNA Fragments from Electrophoresis Gels

The required DNA fragments were cut out of an agarose gel under long-wave UV light and the DNA purified using 'Gene-clean' as described by the manufacturer (BIO 101). The size and approximate DNA concentration of the excised fragments were then checked by electrophoretic separation and ethidium bromide visualisation against known standard concentrations.

2.7.3.10 Ligations

Ligation of restriction-fragment DNA into designated restriction sites of phosphatase-treated plasmid vector pTZ18U and cDNA into the Eco R1 restriction site of the bacteriophage vector λgt10 was carried out as described by Maniatis *et al.* (1982). Phosphatase treatments were also carried out as described by Maniatis *et al.* (1982).

2.7.11 DNA Radio-labelling for Hybridisation Studies

All probes for hybridisation studies were prepared by random primer radio-labelling of double stranded DNA with α-^{32}P-dCTP. The DNA template (80-100ng) plus random 6mer primer mix (3µl) was boiled for 5 minutes and then chilled on ice for 2 minutes. This was then added to a fresh Eppendorf tube containing 2x reaction buffer (12.5µl) and α-^{32}P-dCTP (1-3µl). The reaction mixture was made up to a volume of 24µl with nanopure H_2O before the addition of 1 unit of Klenow enzyme (1U/µl). Finally, the sample was incubated at 37°C for 30-60 minutes.

2.7.3.12 Purification of Radio-labelled DNA

A sterilised pasteur pipette was loaded with Sephadex G-100 in TE buffer and washed with TE buffer containing 0.1% SDS (1-2ml). The labelling reaction mixture then was made up to a final volume of 200µl with TE buffer, loaded onto the column and eluted with TE buffer in 200µl aliquots. The radioactivity of each aliquot was measured using a hand-held Geiger-Mueller counter and two peaks were always recorded for the elution curve. The first peak represented α-^{32}P-dCTP incorporated into the synthesised DNA, and the second unincorporated α-^{32}P-dCTP.

2.7.3.13 DNA Sequencing using *Taq* DNA Polymerase

Recombinant plasmids for sequencing were isolated and PEG precipitated as described above. The DNA concentration of each plasmid preparation was determined by gel electrophoresis and adjusted to approximately 500ng/µl. A 2µl aliquot was transferred to a fresh Eppendorf tube containing 9µl of nanopure H_2O. The diluted plasmid sample was used for the subsequent sequencing reactions. These were carried out using a modified dideoxy method (Sanger *et al.*, 1977) with fluorescent dye linked primers, buffer and *Taq* DNA polymerase supplied by Applied Biosystems The reaction products were combined, ethanol precipitated and briefly dried. Separation of the sequencing reaction products was carried out on a Applied Biosystem automatic sequencer by either Dr. N Shirley or Ms. J. Nield. The sequence data was analysed using SeqEd software (Applied Biosystems) running on a Macintosh Centris 650 personal computer.

2.7.4 Polymerase Chain Reaction (PCR) Methods

2.7.4.1 PCR Amplification of λgt10 Inserts

The bacteriophage vector λgt10 contains a unique *Eco* R1 site within the phage 434 immunity region (*imm*434). Cloning into this site allows selection for recombinant phage. The use of primers, specific for and complementary to the *imm*434 regions on

either side of this cloning site enabled reliable PCR amplification of DNA inserts up to 3 kilobase pairs.

The phage plaque was cored out using a sterile Pasteur pipette and transferred into an 1.5ml Eppendorf tube containing SM buffer (50-100µl). The tube was incubated at 4°C for a minimum of 2 hours to allow diffusion of the phage particles into the buffer. A 10µl aliquot of the phage sample was then transferred to a small PCR Eppendorf tube and boiled for 10 minutes to denature the phage protein coat and the DNA. The boiled sample was chilled on ice and spun down before the addition of the PCR buffer mix (15 or 40ml) which contained 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1.25 mM MgCl, 250 mM dNTPs, phage-F and phage-R primers (0.1 µg of each; see appendix 7 for all the primer sequences used in this work) and 0.5 units of *Taq* DNA polymerase. The reactions were carried out in an MJ-Research Thermal Cycler programmed as follows: denaturation of DNA at 95°C for 4 minutes followed by 32 or 36 cyles of 1 minute denaturation at 94°C, 2 minutes primer annealing at 55°C and 2 minutes DNA chain extension at 74°C. The chain extension for the final cycle was allowed to proceed for 7 minutes before the tubes were cooled to 4°C.

2.7.4.2 PCR of Plant Genomic DNA

Total plant genomic DNA (0.5 µg) was diluted 1:100 in SM buffer (see appendix 1). An aliquot (10µl) was transferred to a small PCR Eppendorf tube containing 40µl of PCR reaction buffer containing 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1.75 mM MgCl, 250 mM dNTPs, PCR primers (0.1 µg of each) and 0.5 units of *Taq* DNA polymerase. The reactions were carried out in an MJ-Research Thermal Cycler programmed as follows: denaturation of DNA at 95°C for 4 minutes followed by 36 cyles of 1 minute denaturation at 94°C, 2 minutes primer annealing at 45°C and 2 minutes DNA chain extension at 74°C. The chain extension for the final cycle was allowed to proceed for 10 minutes before the tubes were cooled to 4°C.

Plasmid or phage insert amplifications for qualitative analyses and production of DNA for probes were carried out using the same conditions as described above except that the thermal cycler was programmed as follows: Denaturation of DNA at 95°C for 3 minutes followed by 36 cyles of 1 minute denaturation at 94°C, 1 minute primer annealing at 55°C and 1 minute 30 seconds DNA chain extension at 74°C. The chain extension for the final cycle was allowed to proceed for 10 minutes before the tubes were cooled to 4°C.

Chapter 3

Growth Response and Infection Studies of the Hordeum vulgare - Glomus intraradices *Mycorrhizal Symbiosis.*

3.1 General Introduction

Vesicular-arbuscular (VA) mycorrhizal research has in the main centred on whole plant or gross cellular responses to plant colonisation by VA mycorrhiza fungi. A relatively small number of host plants have been used for much of this research; chosen because they are mycorrhizally responsive, easy to grow and have large root cells which make clearing, staining, sectioning and visualisation of anatomical structures comparatively straightforward. Spring barley (*Hordeum vulgare* L.) is an economically important cereal species, widely grown in South Australia and meets all the above criteria as well several others which are briefly discussed below.

This investigation of the mycorrhizal symbiosis will use recombinant DNA technology and ionising radiation mutagenesis and will require several additional features in the host plant; it must be amenable to nucleic acid extraction; it must be genetically well characterised, diploid and self-fertilising; and ideally, it should also be genetically transformable and re-generable from *ex planta* material. It is important that the extent of colonisation can be manipulated to ensure a large number of early-infection units are present in the tissue being used for the proposed analyses. This should maximise the chances of cloning and detecting low abundance mRNA transcripts involved in the establishment and maintenance of VA mycorrhizas.

The Waite Institute has a successful barley breeding program which has concentrated on cyto- and molecular genetic approaches in recent years. As a result chrosomomal mapping of cloned barley genes is relatively simple; barley cv. 'Betzes' addition and ditelosomic lines in a 'Chinese Spring' wheat background are available (Islam *et al.*, 1981) and the research group of Dr. P. Langridge has considerable experience in cereal nucleic acid manipulations and is well advanced in the preparation of a genetic map of barley using doubled haploid populations generated from several crosses. Barley transformation and *ex planta* regeneration are not yet routine techniques but two research groups at the Waite Institute are working towards these ends (Drs. A. Aryan and S. Logue respectively). This is an extremely important (although long term) consideration because the final, definitive test of a genes putative function will involve the transformation (complementation) of a suitable (usually mutant) phenotype to restore the putative function of that genes translation product to that particular plant phenotype. Finally, barley remains an important experimental organism for plant mutagenesis experimentation and considerable expertise is available in this through Dr. R. Lance.

Glomus intraradices Schenk and Smith, was chosen as the VA fungus for the experiments involving mycorrhizas described in this work because it readily colonises a broad range of host species (including barley) and forms morphologically and functionally 'normal' mycorrhizas (S. Smith pers. comm.; see Chapter 1).

Differential growth responses to VA mycorrhizal colonisation have been reported among cultivars of clover (Crush and Caradus, 1980), corn (Hall, 1978), cowpea (Rajapakse and Miller, 1987), pea (Estaun *et al.*, 1987), pearl millet (Krishna *et al.*, 1985), tomato (Bryla and Koide, 1990), soybean (Skipper and Smith, 1979) and wheat (Azcon and Ocampo, 1981; Manske, 1989). Genotypic variation also appears to influence the extent of root colonisation by VA mycorrhizal fungi (Azcon and Ocampo, 1981; Toth *et al.*, 1984; Krishna *et al.*, 1985; Young *et al.*, 1985; Estaun *et al.*, 1987; Heckman and Angle, 1987; Rajapakse and Miller, 1988; Manske, 1989;

Sreenivasa and Rajashekhara, 1989; Boyetchko and Tewari, 1990; Bryla and Koide, 1990; Mercy *et al.*, 1990; Toth *et al.*, 1990; Vierheilig and Ocampo, 1991). There are relatively few reports on VA mycorrhizal colonisation and growth responses in and between cultivars of *Hordeum spp*. However, Baon *et al.* (1992 and 1993) who worked concurrently with work discussed in this thesis, showed clear differences in the extent of mycorrhizal colonisation in a range of South Australian barley cultivars.

It has not been possible to always correlate the extent of colonisation with the variable growth responses observed because most of the studies described above have failed to take total root length into consideration. Accordingly, the variable responses described by these authors may reflect differences in extent of colonisation, mycorrhizal nutrient aquisition efficiency or phosphate utilisation efficiency. These serve to emphasise that the choice of host plant genotype is an important consideration and that any given plant genotype/fungus genotype combination needs to be evaluated, bearing the aims of the proposed investigation in mind, to maximise the chances of a sucessful outcome.

Field nutrition trials have indicated that 'Galleon', a South Australian barley cultivar, is more responsive (in terms of certain growth parameters) to phosphate amendments than many other South Australian barley cultivars, including 'Schooner' (Rob Wheeler, personal comm.). The growth and nutritional effects of VA mycorrhiza development are well established and have been extensively reviewed (Smith, 1980; Cooper, 1985; Hayman, 1982; Smith and Gianinazzi-Pearson, 1988). Increased growth in response to mycorrhiza development in soils with low available P concentrations indicates the formation of a functioning symbiosis; ie. the roots have become mycorrhizal and nutrient exchange has occurred.

The formation of a functioning or effective mycorrhiza also infers that the plant has responded 'normally' to colonisation by the VA mycorrhizal fungus in terms of the expression of genes which may facilitate and/or control VA mycorrhiza development or mediate nutrient transfer. A large growth response by either of these barley cultivars (relative to the other) could indicate greater differences in the expression of genes

necessary for effective mycorrhiza development and maintenance. This could reflect greater susceptibility to colonisation (and the total number of plants cells involved in the symbiosis) and/or greater changes in the up- or down-regulation of symbiosis-related gene expression within individual cells. Maximising host plant responses to VA mycorrhizal fungl colonisation within a given amount of tissue was an extremely important aim of this project because the experimental approach proposed for cloning prospective symbiosis-related genes has a greater chance of success if enriched tissues are used for the cDNA library preparation and subsequent differential screening.

The colonisation studies were also an important preliminary to the production and screening of a mutagenised barley population for mycorrhiza-deficient phenotypes (see Chapter 4). Jensen and Nittler (1971) reported striking varietal differences in leaf- and sheath-colour between and among barley seedlings grown with complete nutrient solution or a nutrient solution lacking in phosphorus. Leaf- and sheath-colour were documented during the experiment in torder to determine whether barley seedlings grown in low P soils without *G.intraradices* displayed P deficiency symptoms earlier and/or more severely than those grown with the VA mycorrhiza fungus. A simple system for nutritionally screening a mutant barley population for phenotypes which do not form effective mycorrhizas obviates the need to check each root system individually; the advantages in time and resources are clear.

The general aims of the two experiments reported in this chapter were to:

(i) confirm the mycorrhizal status of barley.

(ii) choose a cultivar suitable for these experiments.

(iii) establish a time course for colonisation of the roots which could be used to routinely produce mycorrhizal tissue of a predictable developmental stage for molecular genetic analyses.

(iv) determine whether phosphate deficiency could be visually detected in young seedlings and whether early mycorrhizal colonisation negated this effect.

3.2 Experiment 1: Variation between two *Hordeum vulgare* Cultivars in Response to *Glomus intraradices* Colonisation.

3.2.1 General Introduction

This first experiment compared the growth responses and extent of *Glomus intraradices* Schenck and Smith colonisation in 22 and 36 day old plants of the barley cultivars 'Galleon' and 'Schooner'. The specific aim was to ascertain if one or both of these cultivars were suitable host-plants for molecular-genetic analysis of the interaction.

The extent of root colonisation by the VA mycorrhizal fungus *G. intraradices* was measured at each of the harvests. The aim was to collect infection during this experiment to confirm that the plants became mycorrhizal under the growth conditions used and to provide information on the extent and physiological stage of VA mycorrhiza colonisation within the roots of each cultivar at certain chronological ages. Because the rates of VA mycorrhiza colonisation in developing barley roots are poorly documented, this information was necessary in order to design an colonisation-time course experiment to investigate more fully the development of *G. intraradices* induced mycorrhizas in the roots of the selected host plant.

3.2.2 Experimental Methods

There were two harvests (21 and 36 days after transplantation) and six treatments for each cultivar: the plants were grown with or without *G. intraradices* (M+ or NM) at three phosphate levels (P_0, P_1 and P_2). Each treatment was replicated three times for a total of 72 plants.

One hundred and fifty seeds (seventy five per cultivar) of approximately the same size were selected for germination. The seeds were surface sterilised, germinated and planted out after 36 hours (42 for each cultivar) into black plastic bags (one seed/bag)

containing 1.5kg of the soil:sand growth medium (see 2.2). The growth medium for the M+ treatments contained *G.intraradices* pot inoculum (10% w:w) whilst the phosphate treatments contained either no added phosphate (P_0); phosphate added at 0.5 mmoles/kg (P_1); or phosphate added at 1.5 mmoles/kg (P_2). Liquid Phosphate Solution was used for the phosphate amendments and Nutrient Solutions A-E were added at the rate of 10ml/kg of growth medium (see Appendix 1). All the nutrients were then watered into the soil. The plants were kept at field capacity (120ml/kg soil) by daily watering to weight.

The barley plants were grown in a glasshouse under natural light. The daylength was less than 11 hours and the day/night temperatures ranged from 18-24^{o}C and 10-15^{o}C respectively. These plants were visually assessed for phosphate deficiency 10, 12, 14, 16, 18, 20, 25 and 30 days after transplantation. Destructive harvests were carried out at 22 days and 36 days. At each harvest, the shoots were cut off at the soil level and weighed before drying at 75^{o}C. The roots were washed until free of the potting medium, cut off below the remains of the seed, surface dried and weighed. A small sample (20-40mg) was excised from each root system and weighed before being cleared by incubation in a KOH solution (see 2.5). The remainder of each root system was dried at 75^{o}C and weighed. The fresh-weight:dry-weight ratios of the residual root portions were used to calculate the dry weight of each whole root system. Three days later the cleared root sub-samples were used to determine the extent of mycorrhizal colonisation of each root sub-sample was determined using a grid intersect method (see 2.5).

3.2.3 Results and Discussion

All of the germinated seeds survived the transfer procedure and all plants inoculated with *G. intraradices* became mycorrhizal. The extent of colonisation as a percentage of total root length for each treatment are presented in Figures 3.1(a) and 3.1(b). Colonisation percentages were higher in 'Galleon' than 'Schooner' at both harvests and for all treatments. The extent of colonisation of the M+:P_1 and M+:P_2 plants

(both cultivars) was reduced compared to the M+:P_0 plants. Increased soil concentrations of P have frequently been shown to result in reduced colonisation (see Mosse, 1973; Abbott *et al.*, 1984; Amijee *et al.*, 1989; Koide and Li, 1990). The mechanism is unknown (see Koide, 1991) but it appears that plant-tissue P concentration may have a regulatory role in the development of colonisation (Sanders, 1975; Menge *et al.*, 1978 and Azcon *et al.*, 1978). There were no significant differences in the proportions of the various colonisation stages present in the roots of 'Schooner' or 'Galleon' (data for M+P_0 plants is shown at Figure 3.2). Stage (2) and (3) mycorrhizas (see 2.5) totalled approximately 65% and 85% of the mycorrhizal tissues of each cultivar (for any of the phosphate treatments) at harvests 1 and 2, respectively.

After 10 days growth the seedlings of both cultivars appeared to be uniform with respect to height and had normal (dark green) leaf-sheath colour. Discoloration of the leaf-sheath became noticeable in 25% of all P_0 and P_1 plants after 15 days growth. By the first harvest 71% plants had some degree of leaf-sheath discoloration and some 33% of the first and second leaf tips were chlorotic. It was not possible to Identify the low phosphorous or mycorrhizal treatments or the cultivar by monitoring the onset of P deficiency symptoms, it was apparent that early mycorrhizal colonisation had no effect on the leaf-sheaf discoloration caused by P deficiency in the barley cultivars 'Galleon' and 'Schooner'.

The NM:P_0 plants of both cultivars grew poorly. Total root and shoot fresh and dry weights and root and shoot fresh weight /dry weight ratios were lower relative to all other treatments and root/shoot ratios were higher than for all other treatments (Table 3.1). These data are indicative of severe P deficiencies (see Harley and Smith, 1983). The shoots and root systems of 'Schooner' were smaller (dry weight) than 'Galleon' for all treatments. VA mycorrhizas stimulated the shoot and root growth of all P_0 plants but had no significant effect on plants which received additional P. The M+:P_0 plants of 'Galleon' were more responsive to VA mycorrhiza formation with *G.*

intraradices than those of 'Schooner'. The M+:P_0 'Galleon' plants obtained 22 day old fresh and dry root/shoot weights which were much closer to those achieved by plants grown with additional P.

After the first harvest, the NM:P_0 plants of both cultivars grew very poorly in comparison with plants in other treatments. The NM:P_0 plants were much shorter and their leaf-sheath colour remained purple/red. Many first and second leaves became completely chlorotic and eventually necrotic. In stark contrast, most of the other plants (including all the 'Galleon' M+:P_0 plants) regained their normal sheath colouration by day 30. VA mycorrhiza formation was clearly correlated with alleviation of the P deficiency symptoms. There were three exceptions: two individual pots from the 'Schooner' NM:P_1 treatments and one from the 'Galleon' M+:P_0: treatment. By the time of the second harvest (36 days) nutrient deficiency symptoms were again visible in 83% of the plants (the plants are shown in Figure 3.3). All plants harvested at day 36 had large root sytems which filled the pot and had become matted at the bottom of the pots; it is quite likely that the accessable soil P or other nutrient reserves had been exhausted. The growth data collected at the second harvest paralleled those obtained from the first (see Table 3.2). However, these data should be considered less useful than those obtained from the first harvest because of inter-root competition and exhaustion of nutrients.

These results show that under any of the growth conditions used for this experiment, 'Galleon' plants had larger root and shoot sytems and higher levels of VA mycorrhiza colonisation than 'Schooner'. 'Galleon' was also more responsive than 'Schooner' (in terms of growth) to VA mycorrhiza formation in the P_0 growth medium. Whether this response represents more efficient aquisition of soil nutrients by the mycorrhizal 'Galleon' roots or better utilisation of the aquired nutrients by the 'Galleon' plant is unclear and would require further analyses (see Smith *et al.*, 1992; Baon *et al.*, 1992). What is clear, is that after only 22 days 'Galleon' formed effective mycorrhizas and more of the root system was infected than 'Schooner'.

The barley cultivar 'Galleon' was selected as the host plant for the remainder of this work because it best met the criteria discussed in the introduction to this experiment: 'Galleon' was responsive to mycorrhiza colonisation and 'Galleon' roots appeared to be a more enriched source of mycorrhizal tissues. Mycorrhizal, low P or adequate P status was not distinguishable by monitoring the onset of nutrient difficiency symptoms in either cultivar and so it was concluded that nutritional screening of the planned mutant barley population for the presence or absence of functional mycorrhizas would not be possible.

The levels of colonisation at the 22 day harvest showed that showed that good levels of colonisation (53%) could be achieved in 'Galleon' within 22 days but that most of the colonisation units contained arbuscules and/or vesicles. I decided to concentrate initially on genes involved in the early stages of colonisation (see Chapter 1) primarily because the symbiosis becomes more complex as the mycorrhizas develop. The mixture of new and physiologically older (including senescing) mycorrhizal tissue is likely to involve more complex and overlapping patterns of plant gene expression. Concentrating on the early stages also minimises the total amount of fungal tissue relative to the plant tissue and so reduces the chances of cloning fungal cDNA's. These problems were best overcome by working with mycorrhizal barley root tissues that contained as many early-infection units as possible, associated with minimal development of arbuscules and vesicules. It was clear from Experiment 1 that the optimal stage and time for harvesting root material was before 22 days. The next experiment was designed to investigate the time course of *Glomus intraradices* colonisation in 'Galleon' prior to 22 days growth.

FIGURE 3.1

Extent of*Glomus intraradices* colonisation (as a percentage of total root length) in 22 day old (3.1a) and 36 day old (3.1b) *Hordeum vulgare* cv. 'Galleon' and 'Schooner' plants grown at three levels of added phosphate; no added phosphate (P0); phosphate added at 0.5 mmoles/kg (P1); and phosphate added at 1.5 mmoles/kg (P2). Samples from three plants for each treatment were assessed for total colonisation and extent of colonisation containing developing arbuscules. The standard errors of the means are shown.

Figure 3.1a

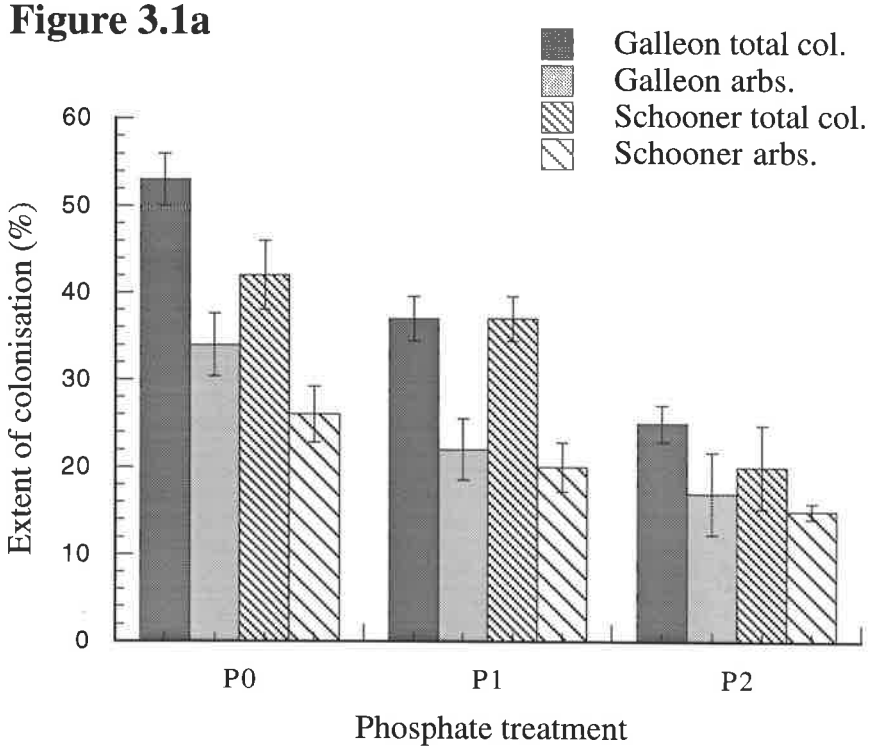


Figure 3.1b

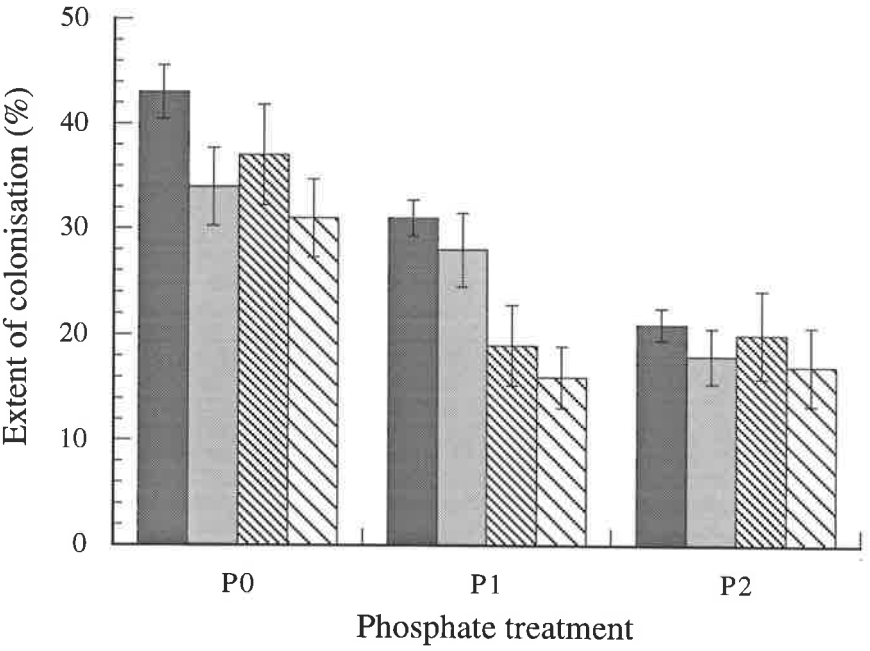


FIGURE 3.2

Proportion of mycorrhizal tissue containing arbuscules in 22 day old (Harvest 1) and 36 day old (Harvest 2) *Hordeum vulgare* cv. 'Galleon' and 'Schooner' roots colonised by *Glomus intraradices* and grown without additional phosphate (M+P0). Samples from three plants for each harvest were assessed for total colonisation and extent of colonisation containing developing arbuscules. The extent of mycorrhizas containing arbuscules is expressed as a proportion of the total colonisation. The standard errors of the means are shown.

Figure 3.2

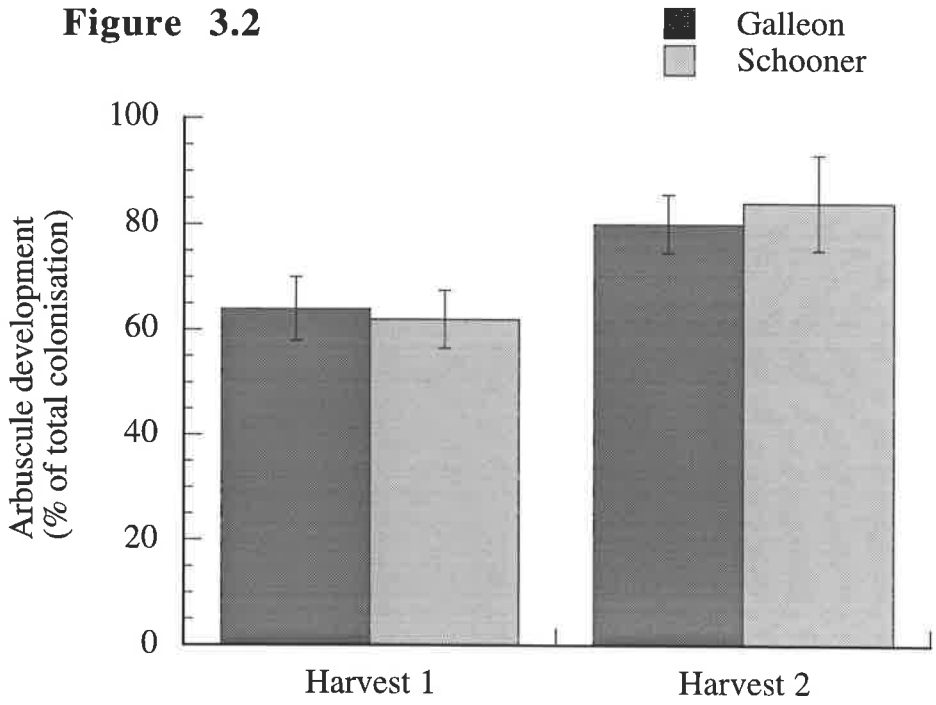

Galleon
Schooner
Arbuscule development
(% of total colonisation)
100
80
60
40
20
0
Harvest 1
Harvest 2

TABLE 3.1

Effects of *Glomus intraradices* colonisation and phosphate on shoot and root growth and shoot and root physiology of 22 day old *Hordeum vulgare* cv. 'Galleon' seedlings. The means and standard errors of the means (S.E.M.) of the three replicates of each treatment are shown. The treatments were nonmycorrhizal (NM), mycorrhizal (M+), no added phosphate (PO), phosphate added at the rate of 0.5 mmoles/kg (P1) and phosphate added at the rate of 1.5 mmoles/kg.

GALLEON

Growth characteristic:	Treatment:					
	NM:P0	NM:P1	NM:P2	M+:P0	M+:P1	M+:P2
Shoot fresh weight (g)	0.69	1.61	1.72	1.32	1.67	1.75
Shoot F/W S.E.M.	0.03	0.14	0.16	0.12	0.06	0.12
Shoot dry weight (g)	0.11	0.23	0.24	0.19	0.10	0.24
Shoot D/W S.E.M.	0.01	0.01	0.02	0.02	0.02	0.02
Shoot fresh/dry weight ratio	6.41	6.93	7.18	7.06	7.10	7.22
Root fresh weight (g)	0.52	0.84	0.91	0.77	0.93	0.94
Root F/W S.E.M.	0.05	0.09	0.07	0.02	0.04	0.03
Root dry weight (g)	0.08	0.11	0.11	0.10	0.11	0.11
Root D/W S.E.M.	0.01	0.01	0.01	0.01	0.01	0.01
Root fresh/dry-weight ratio	6.45	7.87	8.15	7.61	8.22	8.53
Root/shoot ratio (f.w.)	0.75	0.52	0.53	0.58	0.56	0.54

SCHOONER

Growth characteristic:	Treatment:					
	NM:P0	NM:P1	NM:P2	M+:P0	M+:P1	M+:P2
Shoot fresh weight (gms)	0.78	1.14	1.19	0.93	1.12	1.20
Shoot F/W S.E.M.	0.07	0.04	0.09	0.11	0.07	0.03
Shoot dry weight (gms)	0.08	0.16	0.15	0.10	0.14	0.14
Shoot D/W S.E.M.	0.01	0.01	0.01	0.02	0.01	0.01
Shoot fresh/dry weight ratio	10.29	7.08	8.22	9.78	8.02	8.61
Root fresh weght (gms)	0.60	0.64	0.68	0.54	0.63	0.67
Root F/W S.E.M.	0.01	0.04	0.06	0.06	0.11	0.06
Root dry weight (gms)	0.05	0.10	0.11	0.06	0.11	0.11
Root D/W S.E.M.	0.01	0.01	0.01	0.01	0.01	0.01
Root fresh/dry-weight ratio	12.57	6.31	6.16	9.27	5.66	6.19
Root/shoot ratio (f.w.)	0.78	0.56	0.56	0.58	0.56	0.56

TABLE 3.2

Effects of *Glomus intraradices* colonisation and phosphate on shoot and root growth and shoot and root physiology of 36 day old *Hordeum vulgare* cv. 'Galleon' seedlings. The means and standard errors of the means (S.E.M.) of the three replicates of each treatment are shown. The treatments were nonmycorrhizal (NM), mycorrhizal (M+), no added phosphate (PO), phosphate added at the rate of 0.5 mmoles/kg (P1) and phosphate added at the rate of 1.5 mmoles/kg.

GALLEON

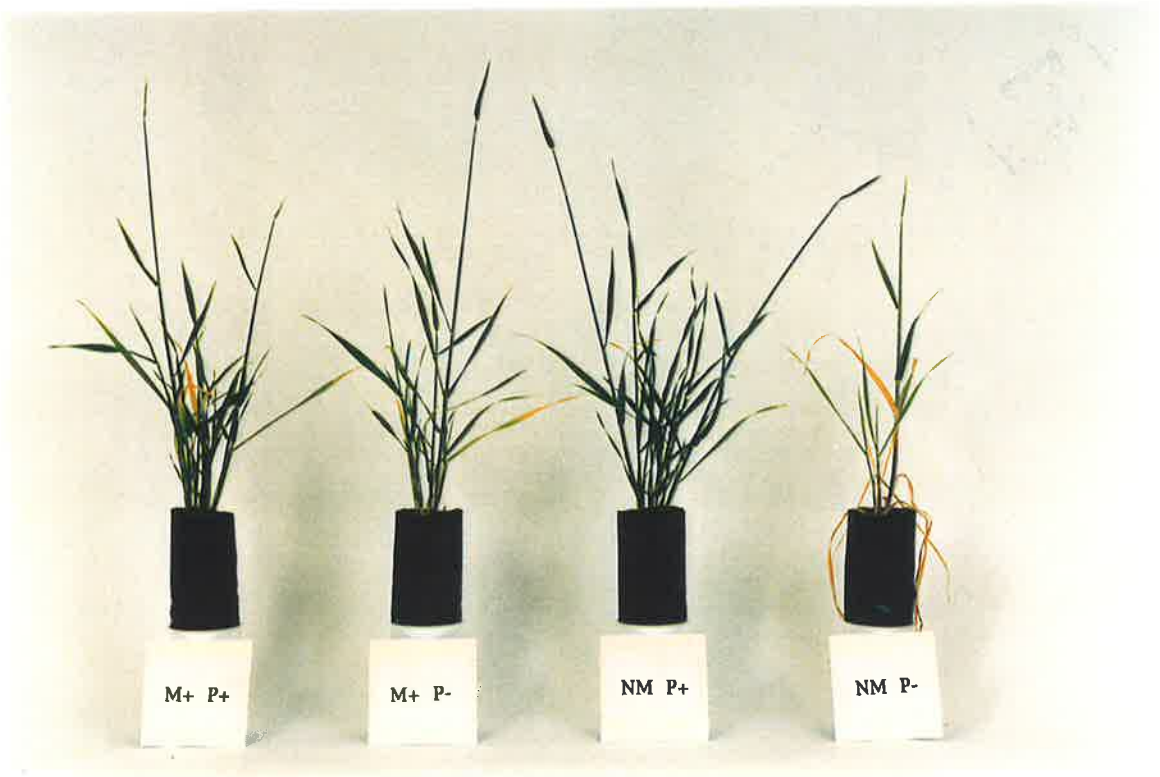
Growth characteristic:	Treatment:					
	NM:P0	NM:P1	NM:P2	M+:P0	M+:P1	M+:P2
Shoot fresh weight (g)	1.82	7.40	7.51	4.78	7.33	7.14
Shoot F/W S.E.M.	0.19	0.17	0.21	0.23	0.12	0.08
Shoot dry weight (g)	0.34	1.16	1.23	0.80	1.26	1.24
Shoot F/W S.E.M.	0.01	0.06	0.06	0.05	0.07	0.09
Shoot fresh/dry weight ratio	5.39	6.39	6.13	5.96	5.83	5.76
Root fresh weght (g)	0.62	1.03	1.10	0.81	0.94	1.14
Root F/W S.E.M.	0.04	0.06	0.05	0.02	0.03	0.09
Root dry weight (g)	0.10	0.15	0.17	0.12	0.14	0.17
Root D/W S.E.M.	0.00	0.01	0.02	0.01	0.01	0.01
Root fresh/dry weight ratio	6.03	6.90	6.63	6.62	6.85	6.78
Root/shoot ratio (F. wgt.)	0.34	0.14	0.15	0.17	0.13	0.16

SCHOONER

Growth characteristic:	Treatment:					
	NM:P0	NM:P1	NM:P2	M+:P0	M+:P1	M+:P2
Shoot fresh weight (g)	2.39	7.22	7.38	2.88	7.01	7.30
Shoot F/W S.E.M.	0.07	0.07	0.19	0.08	0.63	0.23
Shoot dry weight (g)	0.52	1.36	1.36	0.67	1.51	1.55
Shoot D/W S.E.M.	0.02	0.09	0.11	0.05	0.08	0.06
Shoot fresh/dry weight ratio	4.60	5.29	5.43	4.29	4.65	4.70
Root fresh weight (g)	0.55	0.87	0.84	0.47	0.74	0.79
Root F/W S.E.M.	0.03	0.05	0.03	0.06	0.04	0.01
Root dry weight (g)	0.10	0.15	0.14	0.08	0.13	0.13
Root D/W S.E.M.	0.00	0.01	0.01	0.01	0.01	0.00
Root fresh/dry weight ratio	5.74	5.89	6.17	6.27	5.74	6.07
Root/shoot ratio (F. wgt.)	0.23	0.12	0.11	0.16	0.11	0.11

FIGURE 3.3

The effect of VA mycorrhizal colonisation on shoot morphology and growth of *Hordeum vulgare* cv. 'Galleon' seedlings. The seedlings were grown for 35 days with (M+) or without (NM) *Glomus intraradices* and with (P+) or without (P -) of added phosphate (0.5 mmoles/kg).


M+ P+
M+ P-
NM P+
NM P-

3.3 Experiment 2 - Rate and Extent of VA Mycorrhiza Development in *Glomus intraradices* Colonised *Hordeum vulgare* cv 'Galleon' Roots

3.3.1 Introduction

The major objective of this project was to identify plant genes which mediate the development of the symbiosis so the experimental procedure adopted needed to be reproducible to ensure that I could obtain plant root material containing mycorrhizas of a reasonably predictable physiological age. The first experiment had been carried out in a glasshouse which varied in light intensity daily and seasonally. Light intensity can affect mycorrhiza development (Son and Smith, 1988) but does not appear to have an effect on spore germination or early colonisation events. On this basis a growth cabinet was used for this and all subsequent experiments involving the production of mycorrhizal 'Galleon' root tissue. The plants were grown in smaller (400 gm) plastic pots to minimise use of inoculum, the sand:soil growth medium and growth room space requirements. There is no evidence to suggest that a smaller volume of growth medium would dramatically alter the colonisation ontogeny during the early stages of VA mycorrhiza formation. The *G. intraradices* inoculum density was raised from 10% to 15% (w/w) in an attempt to increase the number of early colonisation units. Non-mycorrhizal control plants, with and without added P, were grown and compared with the M+ plants to ensure that 'Galleon' was still responsive to mycorrhiza colonisation (indicating the formation of a functional mycorrhiza) under the slightly altered growth conditions.

In this experiment, colonisation of the developing root systems of 'Galleon' seedlings by *G.intraradices* was followed more closely. The aims were:

(1) to develop a reproducible system for generating mycorrhizal 'Galleon' seedlings, predominantly at early stages of colonisation, and

(2) to identify the chronological age at which these roots should be harvested to meet the objectives discussed in the introduction to this experiment.

3.3.2 Experimental Methods

This experiment measured the extent of mycorrhizal colonisation as it developed over 23 days in newly germinated barley cv. 'Galleon' seedlings planted out into potting medium containing *G. intraradices*.

One hundred and forty 'Galleon' seeds were surface sterilised, germinated and transplanted (see 2.2) into individual pots containing 400gm of soil:sand (1:9 w/w) potting medium and *G. intraradices* inoculum (15% w/w) (M+:P_0). A further 40 seeds were transplanted into individual pots containing 400gm of the soil:sand potting medium only (NM:P_0) or 400gm of potting medium with P added at the rate of 0.5 mmoles/kg (NM:P_1). Nutrient solutions were added and watered into the soil as previously described. The plants were grown in a controlled growth cabinet as and kept at field capacity (48 ml/kg soil) by regular watering to weight.

Five M+:P_0, three NM:P_0 and three NM:P_1 seedlings were randomly selected for harvesting every second day from the day 1 to day 23 after transplantation. The shoot and root fresh weights were recorded before the extent of mycorrhizal colonisation within the roots was determined (2.5).

3.3.3 Results and Discussion

Slight leaf chlorosis and leaf sheath discoloration was noticable in 3 of the plants after 15 days growth and became evident in all (surviving) plants by day 21. The plants achieved slightly lower final fresh weights at day 25 (see Table 3.3) than those recorded at day 22 in Experiment 1. This could have been due to lower irradiance levels. The irradiance in the growth cabinet was not measured at the time at the time of the experiment but later measurements (A. Bruce pers. comm.) showed that it was much lower than in the glasshouse. Root/shoot ratios were higher in NM:P_0 control plants than in either the M+:P_0 or NM:P_1 plants, indicating physiological changes resulting from inadequate P availability. The growth response data indicates similar responses to the experimental conditions in this experiment as in the comparative study.

None of the NM control plants and all of the M+ plants developed normal mycorrhizas (see Figure 3.4). The first colonisations were not detected until 7 days after transplantation. Lag times before initiation of colonisation have been well documented (Saif and Khan, 1977; Carling *et al.*, 1979) and at least partially reflect the time required for infection-propagule germination, hyphal elongation and root penetration (Sutton and Barron, 1972; Sutton, 1973).

The extent of colonisation increased with the age of the plants to a maximum mean value of nearly 58% of the root length at day 21 before decreasing to 52 % at the end of the experiment. This decrease is not statistically significant but may reflect increased rate of growth by the developing root system. Figure 3.5 illustrates the ontogeny of VA mycorrhizal development over the course of this experiment. Mycorrhizal tissues with developing arbuscules were detected as early as day 9 but remained at less than 1% of the total root length scored (or 10% of the total mycorrhizal tissue) until day 13; the final value was 42% of the total root length (or 81% of the total mycorrhizal tissue).

Whilst scoring the roots for colonisation, it became apparent that very few (and in some samples none), of the thicker primary roots of 'Galleon' contained *G. intraradices* infection units. The lag time between transplantation and the detection of mycorrhizal infection units could therefore also reflect the growth pattern of the barley root system. The large primary roots clearly represent a large proportion of the root system during the first few days of growth and development. If they are only poorly susceptible to mycorrhizal formation, colonisation may not be possible until secondary roots are produced a few days after transplantation. This also suggests that VA mycorrhizas are not evenly distributed within barley root systems and that harvesting only secondary roots could provide an even more enriched supply of 'Galleon' cells involved in the interaction with *G. intraradices* .

'Galleon' and *G. intraradices* readily form functional mycorrhizal tissues under the growth conditions used. As Figure 3.5 clearly shows, strong early-stage colonisation (12-37% of the total root length) containing low proportions of arbuscules (less than 10%) can be obtained in 'Galleon' after 11-13 days growth under these experimental conditions. The low proportion of infection units observed in the primary roots required confirmation in view of the stated aim of using root tissue which contains the highest proportion of early stage colonisation for this study. This observation was followed up in Experiment 3.3.

TABLE 3.3

Increase in shoot and root fresh weights of *Hordeum vulgare* cv. 'Galleon' seedlings grown with (M+) and without (NM) the VA mycorrhizal fungus *Glomus intradices*. The plants were harvested and weighed every two days for 23 days, starting one day after transplantation into the growth medium. The means of each treatment (three replicates for the NM plants and five replicates for the M+ plants) and the standard errors of those means (S.E.M.) are shown.

Table 3.3

		Day of harvest											
		1	3	5	7	9	11	13	15	17	19	21	23
Shoot fresh weight (g)													
NM	Mean	0.03	0.09	0.14	0.16	0.23	0.33	0.39	0.42	0.48	0.55	0.66	0.73
	S.E.M.	0.01	0.01	0.01	0.01	0.03	0.01	0.06	0.01	0.05	0.04	0.08	0.06
M+	Mean	0.04	0.09	0.12	0.17	0.24	0.31	0.38	0.47	0.64	0.88	1.11	1.26
	S.E.M.	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.04	0.08	0.05	0.05	0.06
Root fresh weight (g)													
NM	Mean	0.03	0.09	0.13	0.19	0.26	0.39	0.44	0.49	0.62	0.62	0.69	0.72
	S.E.M.	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.01	0.06	0.01	0.02	0.02
M+	Mean	0.04	0.09	0.14	0.21	0.29	0.39	0.46	0.48	0.51	0.53	0.55	0.61
	S.E.M.	0.01	0.01	0.01	0.01	0.03	0.04	0.02	0.02	0.01	0.02	0.03	0.04

FIGURE 3.4

Ontogeny of VA mycorrhiza development in *Hordeum vulgare* cv. 'Galleon' roots. The roots were harvested after 7, 9, 11 and 21 days growth then cleared and stained as described at 2.5. The roots were photographed through a light microscope at 40X magnification.

Ar = arbuscule; Ap = appressorium; IH = intercellular hyphae; S = spore and V = vesicle.

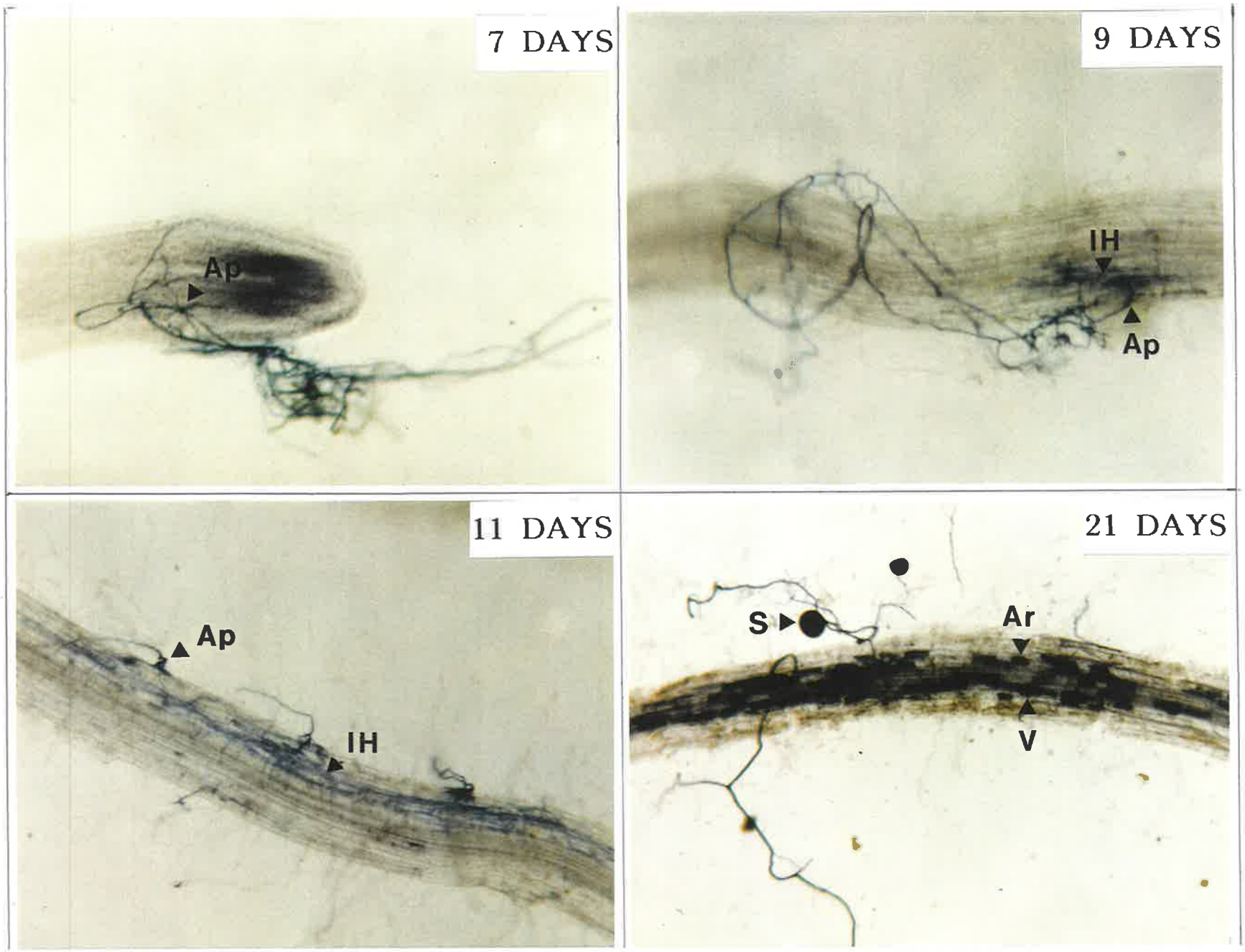
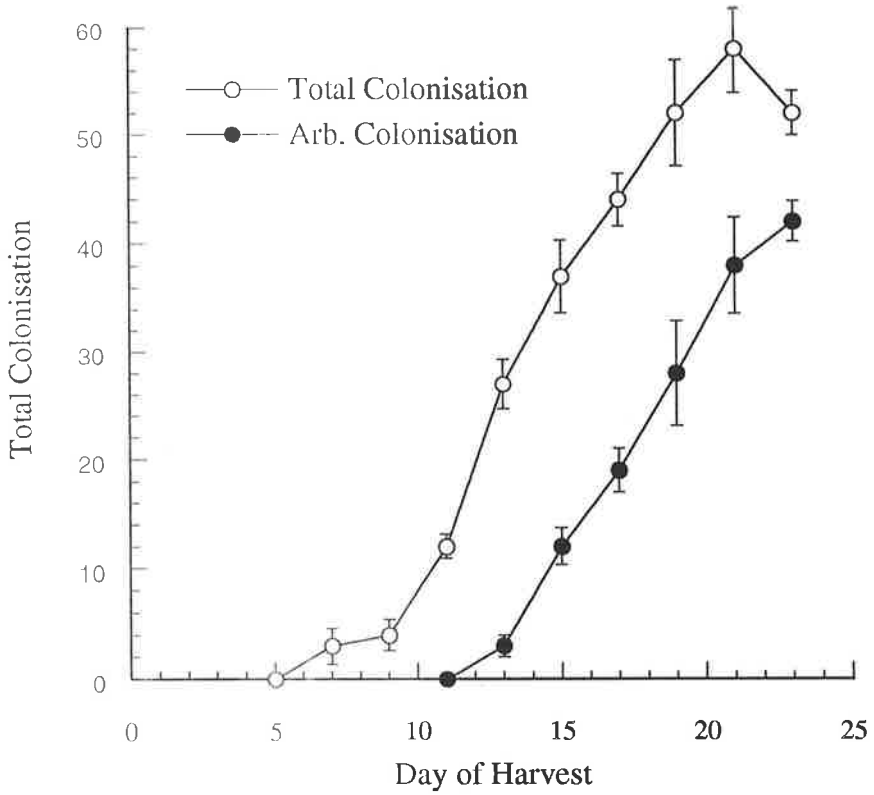

7 DAYS
Ap
9 DAYS
IH
Ap
11 DAYS
Ap
IH
21 DAYS
S
Ar
V

FIGURE 3.5

Graph showing the time course of colonisation in *Hordeum vulgare* cv. 'Galleon' seedlings inoculated with *Glomus intraradices*.. Five replicate plants were harvested every two days, starting one day after transplantation into the soil medium, then cleared, stained and assessed for total colonisation and extent of colonisation containing developing arbuscules as described (2.5). The means and standard errors of those means are shown.

Figure 3.5


Total Colonisation
Arb. Colonisation
Total Colonisation
Day of Harvest
0
10
20
30
40
50
60
0
5
10
15
20
25

3.4 Experiment 3 - The Distribution of *Glomus intraradices* Colonisation in the Roots of *Hordeum vulgare* cv. 'Galleon'.

3.4.1 Introduction

This experiment was undertaken to test:

(1) the observation that VA mycorhhizas developed primarily on the lateral roots of 'Galleon' barley, and

(2) the reproducibility of the results obtained in Experiment 2.

3.4.2 Experimental Methods

Forty 'Galleon' seeds of approximately the same size were surface sterilised, germinated and transplanted into individual pots containing 400 gm of soil:sand (1:9 w/w) potting medium and *G. intraradices* inoculum (15% w/w) as previously described (2.2). A further 7 seeds were transplanted into 400 gm of the sand:soil medium only. Nutrient solutions A-E were added at the rate of 10 ml/kg of growth medium and watered in. The pots were placed in the growth room and kept at field capacity by watering to weight (448 gm) every third day.

After 13 days, the shoots and intact root systems of 5 randomly selected plants from each treatment were carefully harvested and weighed. The root systems were cleared with 10% KOH and stained with Trypan Blue (see 2.5) prior to being laid out on a sheet of glass and immersed in a glycerol:H_20 solution (1:1 v/v) to imobilise the roots and stop them from drying out. The length of the individual primary roots was determined using the graph paper grid and the number and length of each individual lateral root on each primary root and the number of the VA mycorrhizal infection units on each of those lateral roots were recorded. The extent of colonisation, as a percentage of the total length was also assessed using the graph paper grid placed under the glass plate. The VA mycorrhizal infection units were clearly discernable by

eye. However, a dissecting miscoscope was used to check for the presence of very early stages of colonisation (attachment and appressorial swellings only). The infection unit data were expressed as a percentage of the root system using the total root length data.

3.4.3 Results and Discussion

The root and shoot fresh weight and root length data for the 'Galleon' plants (see Figures 3.6(a) and 3.6(b)) indicate that the NM and M+ plants were approximately the same size at the time of harvesting. No nutrient deficiency symptoms were obvious; all plants maintained a deep green sheath and shoot colour. The development, shoot and root size (fresh weight) of these plants were very similar to those of the plants harvested at day 13 in Experiment 2.

The colonisation data for the M+ plants is shown in Figure 3.7. The mean extent of colonisation of the whole root system for the 5 plants was 42%, which is higher than the value of 37% reported in 13 day old 'Galleon' in Experiment 2. This does not appear to be significant. The grid intersect method (Giovannetti and Mosse, 1980) tends to overestimate actual root colonisation (McGonigle *et al.*, 1990; Bradbury *et al.*, 1991) but may also underestimate it if the colonisation is unevenly distributed and a sub-sample is used to quantify VA mycorrhizal colonisation. It is clear from this experimental data that the extent of *G. intraradices* colonisation was unevenly distributed within the root system. It was highest in the lateral roots and in particular the first 5 laterals of each primary root.

The proportion of infection units with arbuscules was highest in the primary roots (where extent of colonisation was the lowest). It was also higher in the oldest five lateral roots in comparison with the whole lateral root system. The colonisation data from Experiment 2 suggests that the first infections occur at around 7 days. At least some of the VA mycorrhizal colonisation in the first five laterals (and the primary roots) of Experiment 3 could therefore be up to 8 days old. Arbuscules can develop

within this period of time (see Harley and Smith, 1983; Toth and Miller, 1984) and so, are likely to occur in greater proportions in the regions of older mycorrhizal tissue.

FIGURE 3.6

Shoot and root growth data (3.6a) and root length data (3.6b) for 14 day old *Hordeum vulgare* cv. 'Galleon' seedlings grown with (M+) and without (NM) the VA mycorrhizal fungus *Glomus intraradices*. Five replicates of each treatment were harvested after 14 days growth. The shoots and roots were weighed and the primary, lateral and total root lengths determined by immobilising the entire root systems in a large petrie dish which was placed over 1mm graph paper. The mean of each set of measurements and the standard errors of those means are shown.

Figure 3.6 (a)

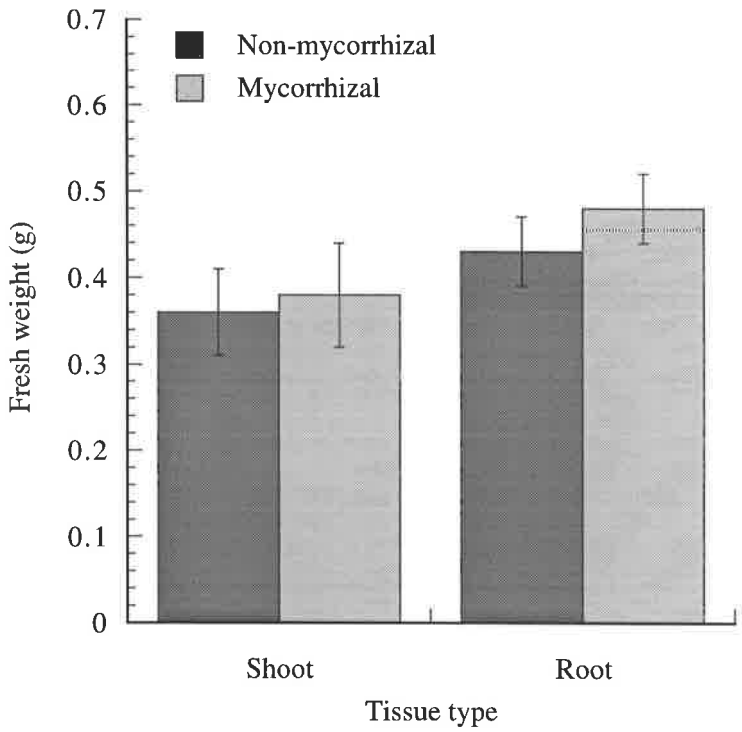


Figure 3.6 (b)

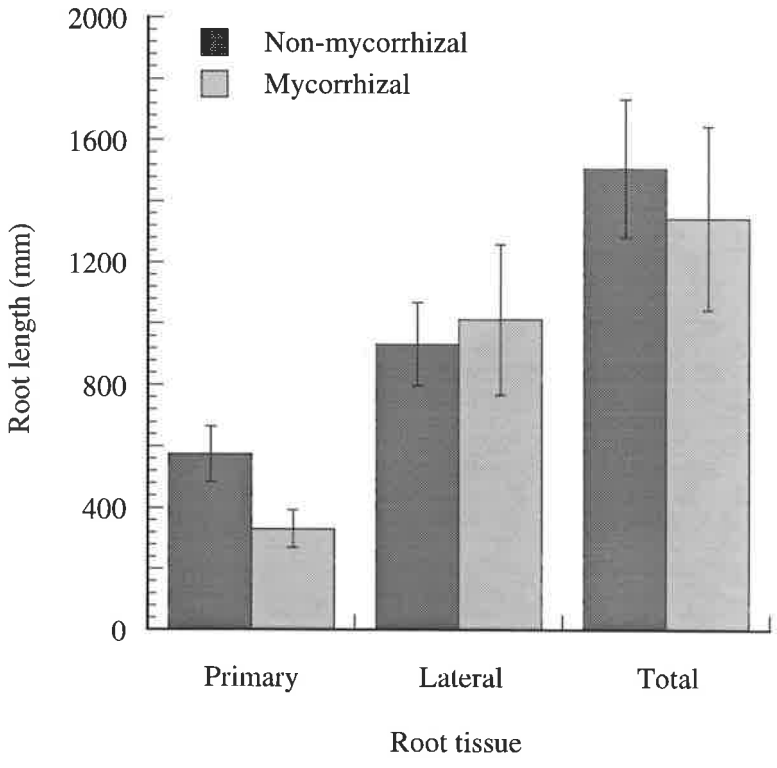
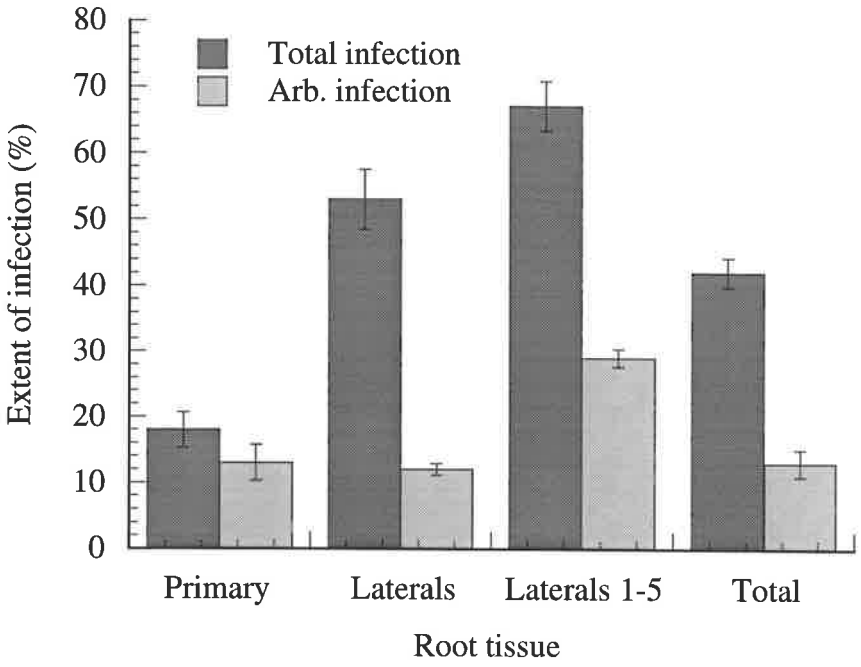


FIGURE 3.7

Distribution and extent of *Glomus intraradices* colonisation in 13 day old *Hordeum vulgare* cv. 'Galleon' roots. The intact root systems of five plants were harvested, cleared and stained as described (2.5). The intact root systems were then spread out in large petri dishes and placed over 1mm graph paper. The total colonisation and extent of colonisation containing developing arbuscules within the primary roots, the lateral roots, the first five lateral roots and the entire root system and distribution of colonisation was then determined. The means of these data and the standard errors of those means are shown.

Figure 3.7


80
70
60
50
40
30
20
10
0
Extent of infection (%)
Total infection
Arb. infection
Primary
Laterals
Laterals 1-5
Total
Root tissue

3.5 Summary and Conclusions

Experiment 1 established that the two *H. vulgare* L. cultivars, 'Galleon' and 'Schooner', were susceptible to colonisation by *G. intraradices* and formed effective VA mycorrhizas in the low P soil:sand growth medium: The levels of colonisation obtained in 'Galleon' were higher than in 'Schooner'.

Experiment 1 also showed that 'Galleon' was more responsive to VA mycorrhiza formation in terms of shoot and root growth. 'Galleon' was selected as the plant host for the remainder of this investigation because these results may also indicate greater responses in terms of the overall expression (up and down regulation) of symbiosis-related plant genes.

Experiment 2 showed that the optimal period for harvesting 'Galleon' root material for the proposed molecular anlyses was 11-15 days after transplanting freshly germinated seeds into the soil:sand growth medium inoculated with *G. intraradices* pot inoculum (15% w/w). During this period the proportion of VA mycorrhizal tissue containing arbuscules was low and the extent of colonisation was between 22-37%. At this stage of mycorrhiza development, the plants showed no significant differences in root and shoot growth.

Higher levels of colonisation could be obtained by harvesting later, but the proportion mycorrhizal tissue containing arbuscules quickly increased to over 70% by day 19. Leaf and sheath discoloration and chlorosis indicated P deficiency in many of the plants from day 19 onwards. The shoot and root growth data supported this conclusion. Unfortunately, early mycorrhiza development has no effect on the onset of these deficiency symptoms and so nutritional screening for mycorrhiza-deficient phenotypes is not possible.

Experiment 3 confirmed that the colonisation method used in Experiment 2 was reproducible. It also clearly showed that the distribution of VA mycorrhizal colonisation within the 'Galleon' root system 13 days after transplantation into the

inoculated soil was not uniform. The great majority of the colonisation was concentrated in the lateral roots. The first 5 lateral roots proved to be a source of highly-infected mycorrhizal root tissue but probably contained too many arbuscules for the preparation of a cDNA library to be used for the isolation of genes involved in the early stages of symbiosis establishment. The top half of M+ root systems (where colonisation is concentrated) were selected for the library prepartion as this appeared to be a reasonable compromise between using whole roots and further experimentation to optimise the level and type of colonisation in the oldest lateral roots.

Chapter 4

Isolation of Putative Mycorrhiza-deficient (myc -) Barley Phenotypes

4.1 General Introduction

Mutations are a source of variability in organisms. The availability of well characterised mutants provide valuable biological material for detailed molecular studies because complex metabolic pathways and developmental processes can be analysed using mutants which are defective at well defined and identifiable stages (see Cove, 1993).

Mutations occur spontaneously but can also be induced by a variety of physical and chemical agents (mutagens). Induction of mutation is a useful method for obtaining mutant genotypes for two reasons; the frequency of identifiable mutation events is high (up to several orders of magnitude greater than for a spontaneous mutation) and the basic genotype of the plant is usually only slightly altered. The generation of near isogenic lines in which many (if not most) attributes remain unaltered allows direct comparisons of mutant genotypes with a well characterised parental plant. Mutants which do display unwanted phenotypic characteristics can be relatively easily backcrossed to the nearly isogenic parent genotype and the progeny re-screened for plants with the desired mutation(s) but free of the unwanted trait(s).

A range of mutant plant genotypes, suitable for physiological, biochemical and molecular studies have been generated in several species (see Koorneef, 1991). Recent examples include cesium-insensitive, storage-protein accumulation and flower development mutants in *Arabidopsis thaliana* (Sheahan *et al.,* 1993; Nambara *et al.,*

1992; Bowman *et al.*, 1989), seed coat colour mutants in beans (Guimaraes *et al.* 1990), proanthocyanidin-free barley (Jende-Strid and Moller, 1981; see Jende-Strid, 1991), mitochondrial DNA mutants in tobacco (Bonnett *et al.*, 1993), and pigmentation mutations in *Antirrhimum* (Luo *et al.*, 1991).

Mutants have been particularly useful in the analysis of bacterial (*Escherichia coli* , *Agrobacterium tumefaciens* and *Rhizobium* spp.) and yeast (*Saccharomyces cereviseae*) gene-enzyme and developmental pathways (for recent examples see Evans *et al.*, 1993; Mantis and Winans, 1993; Roth and Stacey, 1989; Westenberg *et al.*, 1989; Folch *et al.*, 1989). The relatively well characterised legume-*Rhizobium* nitrogen fixing symbioses serve best to highlight both the potential and difficulty of plant mutagenesis experimentation. The primary focus of symbiotic nitrogen fixation research has been on the microbial partner and considerable progress has been made in identifying key molecular and biochemical events in *Rhizobium* spp. which lead to an effective association between the legume and the bacterial partner (see Dénaré *et al.*, 1990; Long, 1989a and 1989b; and Sprent, 1989). This is in no small way due to the relative ease of producing, identifying and isolating a range of suitable bacterial mutants.

Any plant species is inherently more difficult to mutagenise, manipulate and analyse than most bacteria. So it is not surprising that in contrast to *Rhizobium*, much less is known of the role that plant genes play in the regulation of symbiotic nitrogen fixation. Nodulins are specific proteins synthesised by plants during the morphogenesis and functioning of the root nodules responsible for nitrogen fixation in the Legume-*Rhizobium* symbiosis (Legocki and Verma, 1980; Van Kammen, 1984). It is clear that a complex series of developmental steps, involving co-ordinated regulation of plant and microbial genes, are required (see Nap and Bisseling, 1990) and to date, nearly fifty mutations at loci involved with nodule development and function have been identified using classical genetic studies. Unfortunately, no mutations have been detected for any of the known nodulins.

It is only relatively recently that molecular characterisation of genes involved with the legume-*Rhizobium* symbiosis has been made possible by advances in recombinant DNA technology (Rolfe and Gresshoff, 1988; Scheres *et al.*, 1990; Lobler and Hirsch, 1993; Miao and Verma, 1993). Already, a gene-for-gene complementarity-like system (Flor, 1971) has been reported in legume-*Rhizobium* spp. associations involving induced mutants of pea and lucerne (see Caetano-Anollés and Gresshoff, 1992). These plants may well provide excellent sytems for molecular analysis of plant genes involved in this symbiosis.

The use of induced mutations as an adjunct to investigations of fundamental plant-research questions will continue to grow. The "biological revolution" (Kerr, 1985) of the last 15 years has presented researchers with the tools to detect mutations at the molecular level and to analyse DNA sequences which may be responsible for the observed variations. The establishment of reliable and more general transformation and regeneration systems should eventually allow the elucidation of functions of cloned-genes via complementation of suitably characterised plant mutants. Generating and identifying plants which form a range of atypical mycorrhizal associations from genotypes that are normally mycorrhizal (and preferably non-leguminous) is an important research priority. Our understanding of the molecular and genetic processes underlying recognition and compatibility between plant species and VAM fungi is probably dependant upon a successful mutagenesis program .

4.2 Barley Mutagenesis

Barley remains one of the chief experimental plants for mutagenesis research. It is diploid, self-fertilising, has a small number (2n = 14) of large chromosomes and is genetically well characterised. These features have allowed the identification and subsequent characterisation of an array of mutants at the whole plant or cellular level (Nilan, 1987; Nilan *et al.*, 1981). Induced mutations in barley have been used to elucidate the pathway of flavanoid biosynthesis (see Jende-Strid, 1991), modify powdery mildew (*Erysiphe graminis*) resistance (Torp and Jørgensen, 1986; Robbelen

and Heun, 1990), and improve agronomic traits such as early maturation, reduced lodging and increased yield (see Sigurbjörnsson, 1975). The choice of barley as the host plant for these studies was greatly influenced by these characteristics (also see Chapter 3) and because of the considerable volume of work describing approaches to barley mutagenesis. The choice of mutagen, also a very important consideration, is discussed in detail in the next section.

4.3 Mutagenic Agents.

(i) Chemical mutagens

The number of chemical mutagens is large but only a few are useful. The effective ones are classified into four groups: base analogues, antibiotics, azides and alkylating agents.

Base analogues, such as 5-bromo-uracil (BU), 2-amino-purine, 8-ethoxy caffeine and maleic hydrazide (MH), are chemically related to the DNA bases, adenine, guanine, cytosine and thymine. They appear to be incorporated into DNA without hindering replication (see Heslot, 1977), but because of their modified stuctures they are able to induce pairing errors (base changes) and chromosome breaks. Both BU and MH have been identified as effective plant mutagens (Jacobs, 1964; Darlington and Mcleish, 1951). A number of antibiotics such as actinomycin D, mitomycin C and streptoniogin have also been found to possess mutagenic properties (see Heslot, 1977). The usefulness of base analogues and antibiotics as mutagens is limited because the frequency of mutation is so low as to make screening impractical.

Azides (N_3^-), particularly sodium azide (SA), can generate mutations at high frequencies with negligible chromosome aberrations (Kleinhofs *et al.*; 1974). The mode of action of sodium azide is still not well understood (Nilan, pers. comm.) as azide itself is not mutagenic and is metabolically converted by O-acetyl-serine(thiol)-lyase to the compound b-azidoalanine, as neither racemer of b-azidoalanine is mutagenic, further biochemical activation must occur (Nilan, pers. comm.).

Alkylating agents such as ethyl methanesulphonate (EMS), diethyl sulphate and methyl nitroso urea form the largest and most important class of mutagenic chemicals. Like SA, they are very effective plant mutagens, characterised by high frequencies of induced mutation with minimal chromosomal aberrations and low frequencies of lethal genotypes (Gaul *et al.*, 1972; Gottschalk and Wolff, 1983; Nilan *et al.*, 1981). They are also easy to prepare and use but must be handled with caution. When these

compounds (and SA) are used in conjunction with certain modifying factors such as presoaking in H_2O or with certain metal ions (ie. Zn^{2+} or Cu^{2+}) or dimethyl sulfoxide (see Heslot, 1977), they are extremely efficient at inducing mutations. These mutagens react with DNA by alkylating both the phosphate groups associated with the deoxyribose and the pyrimidine and purine bases. Some recent data suggests that EMS may be capable of inducing structural alterations in plant genes (deletions) in addition to the point mutations usually ascribed to alkylating agents (Okagaki *et al.*, 1991).

(ii) Physical mutagens

The most successful and widely used physical mutagens are gamma rays, X rays, fast and thermal neutrons. These types of radiation can release discrete units of energy, ionizations or ion pairs, as they pass through organic matter. These ionizing events can cause chemical changes which are capable of inducing single and, more importantly, double strand chromosome breaks. These may lead to structural re-arrangements (see Salganik and Dianov, 1992) of chromosomes (ie. translocations, inversions, duplications and deletions) and the subsequent loss or gross alteration of gene expression and protein function.

X-rays and gamma-rays are forms of electromagnetic radiation that have been widely used as mutagens. X-ray machines accelerate electrons which are then stopped abruptly by a target (usually tungsten or molybdenum) to produce a wide range of radiation wavelengths of variable energies (50-300 keV). In contrast, the wavelengths of gamma-rays are far more homogenous than X rays and, being of shorter wavelength, they possess more energy/photon (up to 4 MeV). Monoenergetic gamma radiation is also relatively easy to obtain from radioisotope sources such as ^{60}Co and ^{137}Cs. While it is possible to filter out the longer wavelengths generated by-X ray machines and leave only the penetrating high-energy radiation, this type of X-ray source can be expensive and difficult to access (for a complete discussion see Briggs and Constantin, 1977). For practical reasons, gamma rays are often preferred to X-rays.

Neutrons have been shown to be highly effective plant mutagens. Fast neutrons (0.5-2 MeV) are usually obtained from ^{235}U reactors undergoing nuclear fission. By using moderators, such as carbon and hydrogen, the energy of fast neutrons can be reduced to about 0.025 KeV to produce thermal neutrons. Initially, the lack of adequate and uniform dosimetric techniques (Smith, 1961) hampered the use of neutrons in mutagenesis studies. Increased public accountability of nuclear facilities has stimulated nuclear research in recent years and as a consequence, many of the early dosimetry problems (again see Briggs and Constantin, 1977) have been solved. A major difficulty with using this type of radiation for mutagenesis is gaining access to a neutron source.

4.4 Choice of Mutagenic Agent

This mutagenesis study was part of a molecular genetic investigation of mycorrhizal interactions. The aim was to generate stable barley lines that did not form mycorrhizas or formed abnormal mycorrhizas. One of the long term aims is to use *myc-* plants in physiological and molecular investigations in order to ascribe functions to morphological structures. (eg. arbuscule-less mutants could be used to assess the importance of the arbuscule in nutrient transfer between the symbionts) and cloned symbiosis-related genes. These aims were kept very much in mind when deciding what type of mutagen was to be used. Practical issues were also a major consideration.

The vast majority of chemically induced mutations are thought to contain single DNA base pair changes with commensurate amino acid substitutions (see Gottschalk and Wolf, 1983). The translated protein is either inactive or has reduced and/or altered biological activity. These genotypes provide excellent material for plant breeding and biochemical studies. Unfortunately, they may not be particularly useful for molecular analyses because very few point mutations will affect transcription of the mutated gene or the transcript length of the mature mRNA. Gross effects are almost essential for the detection and isolation of specific genes using current recombinant DNA technology.

Physical mutagens also have practical limitations: It can be difficult to access suitable sources of radiation and a high frequency of lethal mutations can be expected (R. Lance, pers. comm.), necessitating a large initial M1 population. However, they often induce the types of mutations detectable using recombinant DNA or cytological techniques.

In the experiment described here gamma radiation was used as the mutagen because a ^{60}Co source was available, courtesy of the Royal Adelaide Hospital, and the barley breeders at the Waite Agricultural Reserach Institute provided the technical assistance and field space to plant out a large population of gamma irradiated barley seed.

4.5 Generation of M2 Populations from ^{60}Co Gamma-ray Irradiated *Hordeum vulgare* cv. 'Galleon' Seed.

Two M1 populations (6000 seeds each) of gamma irradiated *Hordeum vulgare* cv. "Galleon" seeds were prepared using a ^{60}Co radiotherapy unit at the Royal Adelaide Hospital which emmitted approximately 650 Rads of gamma-rays/minute. The seeds were irradiated in 10 x 10 x 5cm clear perspex, open top containers which allowed an even, shallow (1cm) spread of the seeds. Population 1 (P1) received 2 x 15 minute (20 kRads total) exposure to the gamma-rays and population 2 (P2) received 2 x 19 minute (25 kRads total) exposure to the radiation. The initial and final doses were given 24 hours apart to maximise the efficacy of the treatment and the seeds were thoroughly mixed and carefully respread over the trays between the treatments to average out the dose for each seed (Borojevic *et al.*, 1977). The irradiations were carried out at room temperature ($22^{o}C$).

Approximately 3500 seeds from each population were planted out in separate field plots in May 1990, using a direct-drilling seeder. These seed plots were separated from the Waite Institute barley breeding lines by a 2 metre gap of bare earth which was in turn surrounded by a 2 metre wide apron of parental *H. vulgare* cv. "Galleon" plants. The M1 plants developed normally through the growing season and received

no fertiliser or pesticide treatments. In February 1991, the plants were harvested individually and the M2 seeds collected from each plant were placed in brown paper bags and numbered.

Both populations had high mortality rates; approximately 25% of P1 and 80% of P2 failed to germinate or mature. A further 2.5% of P2 failed to set viable seeds. These data indicated that the gamma irradiation treatments had induced mutations in both populations but also that the large amount of genetic damage to P2 may have reduced the usefulness of that population. The high mortality rate had only left 279 P2 families for screening, which may have been too few (statistically) to detect useful mutations.

The results obtained from the growth and colonisation experiments (see Chapter 3) showed that preliminary screening for non-mycorrhizal 'Galleon' plants could not be done using phosphate deficiency symptoms. This greatly reduced the rate at which the plants could be screened because the only way of determining the mycorrhizal status of the plants in the mutagenised populations was to check the roots of each plant individually.

4.6 Screening of the Gamma-irradiated *Hordeum vulgare* M2 Populations for *myc* Mutant Phenotypes

Twelve seeds were selected from each family for screening; this number of seeds gives a 95% probability of detecting homozygous recessive mutations. The twelve seeds from each M2 family were sterilised, germinated and planted out in a small plastic pots containing 250 grams of potting soil and 10% *Glomus intraradices* pot inoculum as described (2.3). The seedlings were grown for 21 days in the greenhouse before each plant was non-destructively sampled. Root samples were taken using a 1 cm corer, and used to determine the extent of mycorrhiza development within roots (2.5). Initially, arbuscular development was assessed subjectively. Putative arbuscule mutants were then reassessed quantitatively; the root pieces were re-scored for the presence of *G. intraradices* and the presence of arbuscules within the segments. Arbuscular development was expressed as a proportion of total colonisation. The extent of colonisation and arbuscular development within the M2 roots was then compared with that of roots collected from four parental 'Galleon' plants grown at the same time and under the same conditions. Plants which varied from the parental 'Galleon' controls by more than 25% for total root colonisation or had not developed arbuscules were repotted into a rich soil mix and grown on to collect the M3 seeds.

In all, forty M2 families from P1 and one hundred and twenty M2 families from P2 (for a total of 1880 plants) were screened in this manner. Four of the P2 families (F11, F14, F52 and F144) continued to segregate for lethal chlorophyll (albino) mutations. A total of 34 putative *myc*- mutants from sixteen families were identified in the initial screening (see Table 4.1). All of the putative mutants were from the P2 population. Six families (F4, F24, F25, F61, F62 and F104) contained three or more plants which appeared to form abnormal mycorrhizas. In the majority of these putative *myc* plants, the extent of colonisation was lower than the parental 'Galleon'. Three plants from

F24 and one from F109 had very little arbuscule development and two plants from F131 were extremely heavily infected in comparison to the control plants.

FAMILY	PLANT	TOTAL INFECTION (%)	VARIANCE FROM CONTROL PLANTS (%)
F4	4-4	8	- 46
	4-8	7	- 53
	4-10	2	- 86
	4-12	9	- 40
F11	11-11	9	- 43
F14	14-8	9	- 43
F24 *	24-4	25	+25
	24-5	20	0
	24-11	24	+20
F25	25-1	12	- 40
	25-5	11	- 45
	25-10	9	- 55
	25-12	12	- 40
F52	52-5	19	- 25
F61	61-3	12	- 33
	61-7	11	- 38
	61-9	14	- 22
F62	62-3	8	- 55
	62-4	11	- 38
	62-9	6	- 66
	62-10	9	- 50
	62-11	11	- 38
F67	67-7	13	- 27
F91	91-2	14	- 26
	91-5	14	- 26
F97	97-9	15	- 21
F101	101-6	15	- 32
F104	104-3	11	- 50
	104-9	13	- 41
	104-10	12	- 45
F109 *	109-6	21	- 5
F131	131-7	55	+ 175
	131-12	48	+ 140
F144	144-10	7	- 65

Table 4.1

List of putative *myc* barley mutants identified from primary screening of 1880 M1 plants. Twelve seedlings from each of 160 M1 families of *Hordeum vulgare* cv. 'Galleon' (obtained by 20 or 25kRad gamma-irradiation) were inoculated with the VA mycorrhizal fungus *Glomus intraradices* . The roots were non-destructively sampled after 21 days growth and assessed for mycorrhizal colonisation (% of the root length sampled). The plants which varied in extent of colonisation from parental controls by more than 25% or appeared not to have formed arbuscules (shown with *) are listed.

The M3 seeds from each of the putative *myc*- mutant lines (six per line) were rescreened as described above except that the whole root systems were harvested and weighed. The fresh weights were used as an indicator of variances in total root length for each M3 line. The mean fresh root weight of one M3 family (F4), was 21% lower than the 'Galleon' control plants. The root systems of three plants from this M3 line appeared to be much smaller than the others and were also morphologically different from the control plant root systems; they were stunted and slightly thickened. The rest of the putative *myc*- families did not significantly differ in mean fresh root weight from the control plants.

The extent of mycorrhizal colonisation was not significantly different, quantitatively or qualitatively, in the great majority of putative mutants when compared with the parental 'Galleon' in the second screening. However, one line (F25-10) showed a small but significant decrease in the extent of mycorrhizal colonisation within its root system (see Figure 4.1). The two putative *myc* mutants, F4-10 and F25-10 were repotted into a rich soil medium and grown on in the glasshouse. The seeds (M4) were collected and stored.

Line F4-10 segregated into two distinct sub-populations, one (F4-10A) contained 3 plants which were very poorly infected. These plants had much lower root fresh weights (35% compared to the controls) and displayed the unusual root morphology described above. The mean colonisation percentage and the root fresh weights of the other sub-population (F4-10A) was not significantly different from the parental controls. A graph showing the extent of colonisation for F4-10A, F4-10B and F25-10 compared to the parental 'Galleon' control plants is shown in Figure 4.1.

The M3 line, F25-10, appeared to be phenotypically the same as parental 'Galleon'. It grew normally and set seed. The F4-10A type plants began to display unusual shoot morphology and leaf chlorosis indicative of nutrient deficiency after 14-17 days growth (see Figure 4.2). All such plants became necrotic and died without setting seed. A further two F4-10A type plants were identified from ten F4-10 seeds grown as

described above. These plants were passed on to the Dept. of Plant Science glasshouse manageress in the hope that she could keep them alive. They showed the same unusual shoot morphology and nutrient deficiency symptoms and died within 30 days of germination.

Both of the putative *myc* mutants formed mycorrhizas but at significantly lower levels of colonisation under the growth conditions described above. Both phenotypes contained all the structures associated with normally developed and functional *G. intraradices - H. vulgare* mycorrhizas. It appeared that the putative mutations did not involve mechanisms mediating compatibility between the symbionts (ie. resistance reactions) or the formation of the gross structures. No ultrastructural analyses were carried out and alterations to the fungal - plant cell wall interfaces cannot be ruled out. However, it seems unlikely that mutations to the ultrastucture of mycorrhizas could affect overall development to such a large extent. Presumably the plant genes involved in these stages of mycorrhiza formation are determinants of functional (eg. nutrient transport) mechanisms rather than compatability and gross fungal morphology.

The gene mapping projects in the laboratory of Dr. P. Langridge, which were being carried out concurrently with this research, had indicated that some seed stocks of the barley cultivar 'Galleon' were not pure. Polymorphisms were detected between and within plants expected to be homozygous. It is possible that early stocks of 'Galleon' seed may have been contaminated with another cultivar or that pollen drift from other cultivars had resulted in cross breeding rather than selfing.

The comparison between the barley cultivars 'Schooner' and 'Galleon' (see Chapter 3: Experiment 1) showed significant differences between the two genotyptes with respect to their response to and extent of mycorrhizal colonisation. As discussed previously, this is in agreement with reports which describe similar differences between cultivars of many other species. It is possible that the differences in the extent of mycorrhizal colonisation observed between parental 'Galleon' and the M2 lines 4-10A and

particularly 25-10, are a result of genotypic differences caused by 'genetic contamination' and not by induced mutation.

FIGURE 4.1

Extent of colonisation by the VA mycorrhizal fungus, *Glomus intraradices,* of *Hordeum vulgare* cv. 'Galleon' and three putative mutants derived from gamma-irradiated 'Galleon' (F4-10A, F4-10B and F25-10). The values represent the means of six samples taken from the roots of six plants of 'Galleon' and the putative mutant F25-10 and three samples taken from the roots of three plants of the putative mutants F4-10A and F4-10B. Error bars represent the standard errors of these means.

Figure 4.1

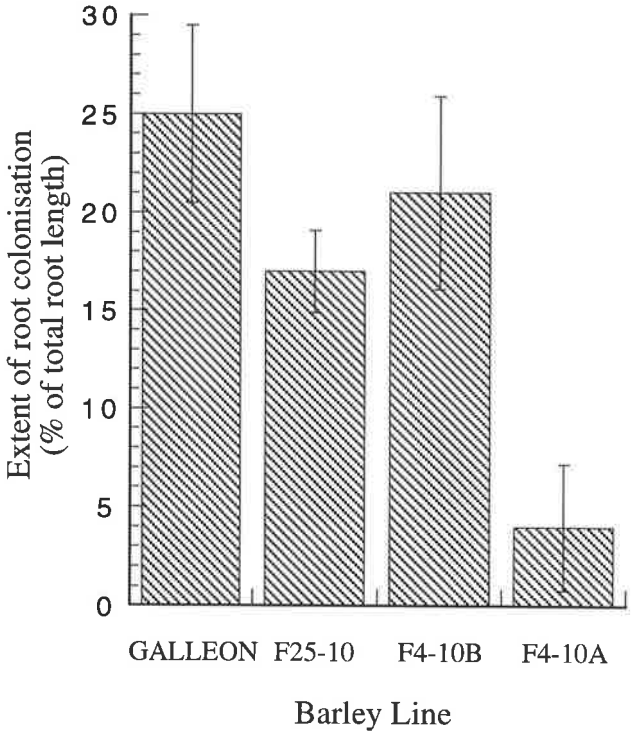

30
25
20
15
10
5
0
Extent of root colonisation
(% of total root length)
GALLEON
F25-10
F4-10B
F4-10A
Barley Line

FIGURE 4.2

Growth responses of the parent *Hordeum vulgare* cultivar 'Galleon' and two putative mutants derived from gamma-irradiated 'Galleon' (F4-10A and F25-10) to additional P (0.5 mmoles/kg) or inoculation with the VA mycorrhizal fungus *Glomus intraradices* . The plants were photographed after 21 days growth.

GALLEON
-M +P

GALLEON
+M -P

4-10
+M -P

4-10
+M -P

25-10
+M -P

25-10
+M -P

MYCORRHIZA DEFICIENT (*MYC*-)
M4 'GALLEON' LINES

4.7 Polymerase Chain Reaction (PCR) Analysis of the Putative Barley *myc*- Mutants, 4-10A and 25-10.

Conventional varietal indentification is based on morphological description (Stegemann, 1984). This is time consuming and not always accurate due to environmental effects. Biochemical markers (ie. isozymes) are available which provide quicker and more accurate assessments (see Cooke, 1988). DNA-based techniques such as restriction fragment length polymorphism (RFLP) and PCR have also been successfully used to detect polymorphisms in cereals (Weining and Langridge, 1991). The PCR-based techniques have two major advantages; they allow the detection of a larger number of polymorphisms which helps to distiguish between closely related varieties; and they are cheaper, quicker and easier than RFLP analyses.

The polymerase chain reaction (PCR) involves the *in vitro* amplification of DNA fragments using DNA polymerase (Saiki *et al.*, 1985; Mullis and Faloona, 1987). Short oligonucleotides (primers) complementary to the DNA flanking the target sequence, hybridise to opposite strands of the DNA molecule with their 3-OH ends pointing towards each other. The primers are enzymatically extended to synthesise DNA complementary to each strand of the target sequence. Repeated cycles of heat denaturation of the DNA, primer annealing and primer extension, result in the amplification of the DNA sequence defined by the 5-^{P} ends of the primers. The extension products also serve as templates and essentially double the number of target DNA fragments produced in each cycle. This results in rapid and exponential amplification (up to several millionfold) of specific target sequences.

Several PCR-based approaches are available for consideration. The simplest methods use the presence or absence of a DNA fragment band or band sequence length polymorphisms as determinants. For example, it is possible to design (or purchase) short oligonucleotide primers complementary to highly conserved DNA regions which allow the amplification of variable sequences which lie between them (Skolnick and

Wallace, 1988). Degenerate primers, coupled with lower annealing temperatures (see Arnheim and Erlich, 1992) or microsatellite primers (Litt and Luty, 1989; Beckman and Soller, 1990) can also be used to produce DNA product banding patterns which allow the detection of polymorphisms at the plant species and cultivar level (Morgante and Olivieri, 1993; Rafalski and Tingey, 1993) .

Recently, Weining and Langridge (1991) described the use of primers, designated R1, E2 and E4, to distinguish between a large number of barley cutivars and local (South Australian) breeding lines. These three primers and four microsatellite primers (see Appendix 6 for the sequences), which also produce polymorphic banding patterns for barley cultivars usually grown at the Waite Institute (A. Karakousis pers. comm.), were used to generate DNA fragment banding patterns using template DNA extracted from:

(i) the putative *myc*- mutant lines 4-10A and 25-10,
(ii) 10 plants grown from randomly selected seed of the parental 'Galleon' population,
(iii) a plant positively identified as 'Galleon',
(iv) and the wheat variety 'Chinese Spring'.

The PCR amplifications were carried out as described (2.7.4.2) and the results are shown in Figures 4.3 and 4.4. Figure 4.5 is an example of the polymorphic banding profiles of amplified products produced by the primers E4 and AT when used with genomic DNA extracted from several local barley cultivars. The banding profile was different for each primer set and no polymorphisms were detectable between any of the barley plants. 'Chinese Spring' was clearly distinguishable from barley for all primer combinations. Thus the lines are all at least putatively 'Galleon'.

As no evidence of cross pollination was detected using the PCR-based identification method (cross pollinated plants, in effect heterozygotic F1 plants, should be clearly discernable) it was concluded that the phenotypic differences observed between F4-10

and F25-10 were not caused by 'genetic contamination' of the original parental 'Galleon' seed. It seems most likely that the observed phenotypic differences can be ascribed to the gamma irradiation treatment.

FIGURE 4.3

Agarose gel electrophoresis of polymerase chain reaction amplification products of DNA from four barley lines and one wheat line using the primers AT, ST, E4, R1, LA and CP.

Lane 1 in each set of reactions is the parent *Hordeum vulgare* cultivar 'Galleon'; lane 2 is a plant positively identified as 'Galleon'; lanes 3 and 4 are the putative *myc* mutants F4-10A, and F25-10 respectively, which were derived by gamma irradiation treatment of the parent cultivar; and lane 5 is the *Triticum aestivum* cultivar 'Chinese Spring'.

The DNA markers (shown as lane M in the first set of reactions) are *Hind* 111 digested λDNA. The 580bp marker bands are indicated at the right hand side of the figure.

Figure 4.3

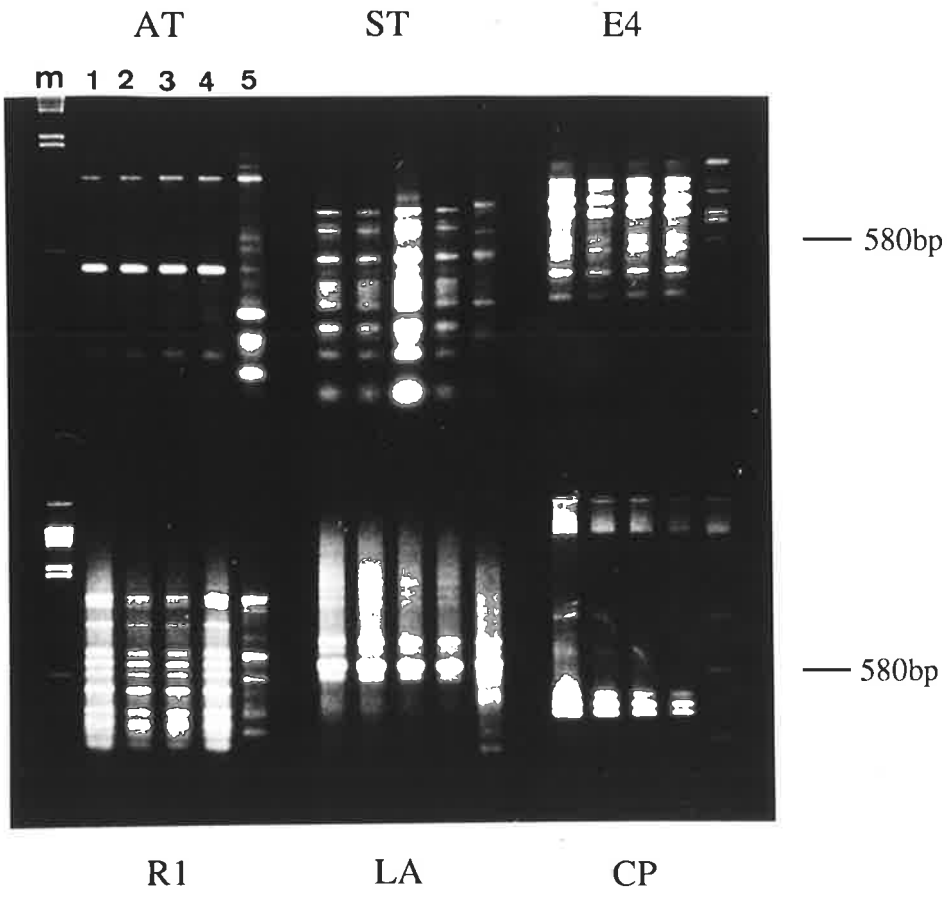


FIGURE 4.4

Agarose gel electrophoresis of polymerase chain reaction amplification products of four barley lines and one wheat line using the primers B1, B1+E4, E1 and E2.

Lane 1 in each set of reactions is the parent *Hordeum vulgare* cultivar 'Galleon'; lane 2 is a plant positively identified as 'Galleon'; lanes 3 and 4 are the putative *myc* mutants F4-10A, and F25-10 respectively, which were derived by gamma irradiation treatment of the parent cultivar; and lane 5 is the *Triticum aestivum* cultivar 'Chinese Spring'.

The DNA markers (shown as lane M in the first set of reactions) are *Sau* 3A digested pTZ18U. The 603bp marker bands are indicated at the right hand side of the figure.

Figure 4.4

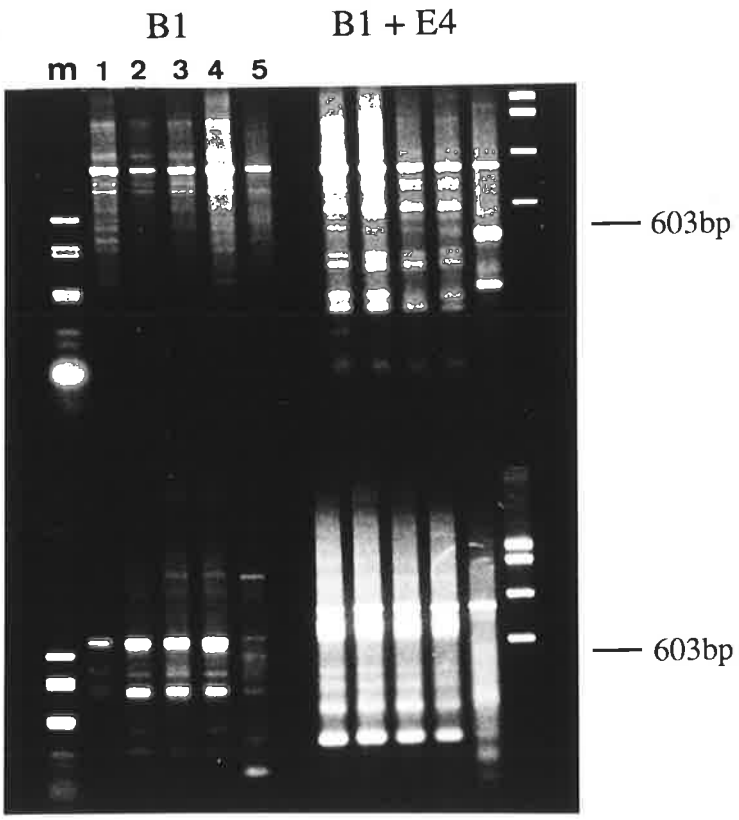


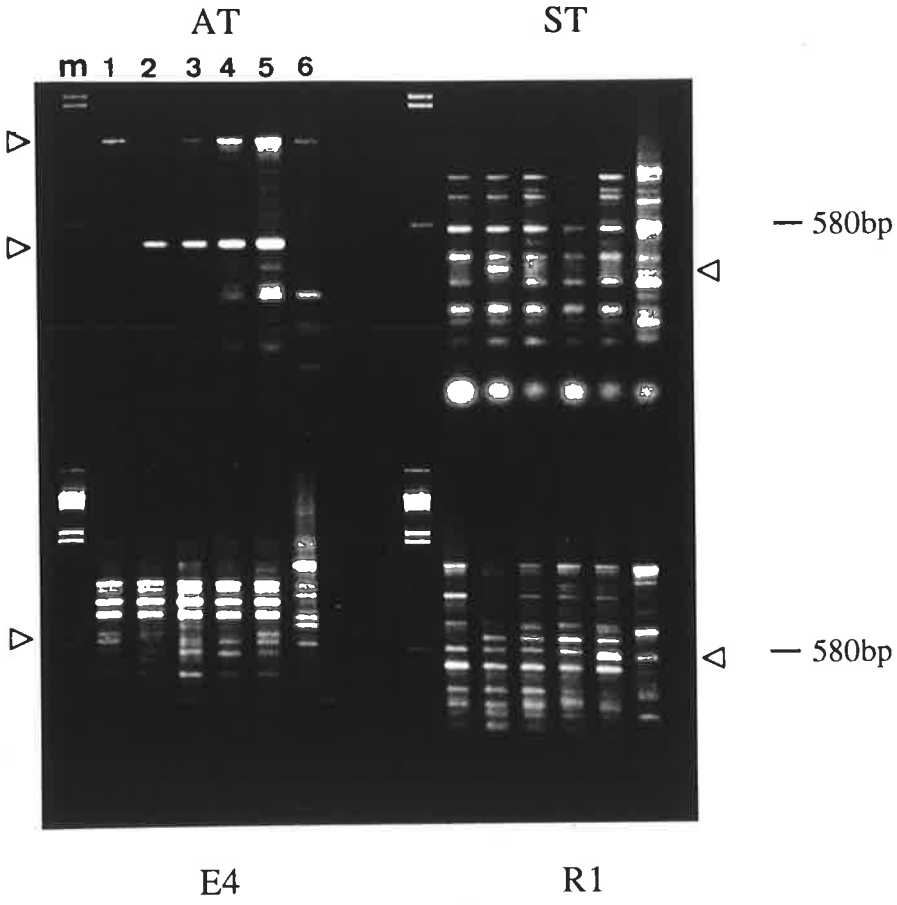
FIGURE 4.5

Agarose gel electrophoresis of polymerase chain reaction amplification products of five barley lines and one wheat line using the primers AT, ST, E4, R1.

Lane 1-5 in each set of reactions are the *Hordeum vulgare* cultivars 'Schooner', 'Clipper', 'Galleon', 'Haruna Nijo' and 'Betzes' repectively, and lane 6 is the *Triticum aestivum* cultivar 'Chinese Spring'. Polymorphic bands are indicated by the arrow heads.

The DNA markers (shown as lane M in the first set of reactions) are *Hind* 111 digested λDNA. The 580bp marker bands are indicated at the right hand side of the figure.

Figure 4.5



4.8 Discussion and Conclusions

The high mortality rate observed for the M1 seed of P2, suggests that considerable genetic damage occurred to the seeds which received the 25 kRad gamma irradiation treatment; 80% of the mutations were lethal. The M1 seeds of P1 were less affected (in terms of lethal mutations) than those of P2. No direct comparisons can be made to the parental population survival rate because 'Galleon' mortality rate was not measured at the time. However, data from subsequent experiments indicated a survival rate of over 95%.

The PCR analyses of the original 'Galleon' population and the 2 putative *myc*- mutants did not indicate any 'contamination' from another genotype, so it seems probable that the phenotypic variations observed between 'Galleon', 4-10A and 25-10 with respect to the extent of mycorrhizal colonisation, arose from induced mutation(s).

The lower colonisation percentage in F25-10 may reflect slower development within the root (caused by slight differences in nutrient availability and/or transfer) or a lower number of initial colonisation points (indicating greater resistance to root penetration). Line F25-10 was only slightly less susceptible to VA mycorrhizas than parental 'Galleon' and because there were no obvious differences in the pattern of development, experimental error cannot be ruled out as the source of the observed variation. A larger sample of this M3 population needs to be critically evaluated with regard to the extent and development of colonisation.

The F4-10A line segregates for a lethal mutation in the M3 population (11:5). The population tested is too small to accurately determine the segregation ratio; it may be 2:1 or 3:1. The low levels of normal colonisation in F4-10A established that VAM colonisation is possible in this plant line and that in all likelihood, the mutation responsible for this phenotype does not involve genes controlling structural changes to plant cells during the establishment of the symbiosis. The chlorosis and subsequent necrosis of leaves in F4-10A, after what appears to be normal early growth, may be

indicative of the slow 'starvation' of this genotype following the exhaustion of seed nutrient reserves. This phenotype could be the result of radiation induced changes in a gene encoding a component of the epidermal root-cell wall. Neumann *et al.*, (1994) have reported root cell hardening and root inhibition in response to salinity stress. They postulated that biochemically regulated changes to the visco-elastic properties of cell walls (see Passioura and Fry, 1992) may be responsible for the limited uptake and supply of essential nutrients associated with salinity stresses (Zidan *et al.*, 1992). An induced mutation affecting cell wall visco-elasticity could render these cells not only impermeable to soil solutes but almost totally impenetrable by *Glomus intraradices*.

Arabidopsis thaliana has become the major experimental plant for molecular and genetic analysis of plant development and function. It is amenable to large scale screening and has a small genome, facilitating chromosome walking (see Koornneef, 1991). Unfortunately, it is one of the few non-mycorrhizal plants. *Myc-* mutations had previously been identified in pea (Duc *et al.*, 1989) but they appear to be linked with nodulation (*nod-*) mutations which may have complicated the results, not to mention the difficulties in setting up a model system involving two micro-organisms. Barley was used for this work because it appeared to be a good compromise for molecular, genetic and mutant screening.

Yields for single gene mutations in *A. thaliana* can be in the order of 1 mutant per 40,000 (Cabrera y Poch *et al.*, 1993) and even lower for barley (see Briggs and Constantin, 1977). The number of families screened was not large (150) but the yield of *myc* mutants (1.3 mutants per 100 families) suggests that a large number of genes may affect the rate and/or extent of root colonisation in barley by VA mycorrhizal fungi. Isolating a large number of mutants defective in mycorrhizal development may not be particularly difficult. The two putative *myc-* mutant lines, F25-10 and F4-10A, are less susceptible to VA mycorrhizal colonisation than parental control plants but neither appear to be developmental or functional mutants. Identifying the genes which directly control the interaction will prove much more difficult.

Chapter 5

Molecular Cloning and Isolation of Barley -mycorrhiza Related Plant cDNA's

5.1 Introduction

Elucidating the molecular mechanisms underlying the VA mycorrhizal symbiosis will require both biochemical and molecular genetic data. Making direct biochemical comparisons between mychorrizal and nonmycorrhizal roots is difficult because individual VA mycorrhizas are almost impossible to detect and excise without staining, and so whole root systems (which contain uncolonised sections of root tissue and VA mycorrhizas of varying physiological stages) must often be used in order to obtain enough material. This may confound the results obtained, as do the physiological and biochemical changes induced by the improved P uptake usually associated with mycorrhizal roots (see Chapter 1).

These limitations also apply to current molecular genetic methods but to a lesser extent. Techniques for investigating temporal and spatial gene expression within plant tissues are extremely sensitive relative to most biochemical assays; this means that smaller amounts of root tissue can be used for most nucleic acid analyses.

Broadly speaking, the molecular genetic stategies aimed at isolating genes can be classified into two groups: Targeted and Non-targeted.

5.1.1 The Targeted Approach to Gene Cloning

The targeted approach requires the knowledge (or at least the assumption) that specific genes are involved. Suitable genomic or cDNA libraries can then be directly and rapidly screened with protein or heterologous DNA probes obtained from other sources to isolate the corresponding gene(s). The VA mycorrhizal symbiosis is still poorly understood at the molecular level, nonetheless the biochemical and cytochemical evidence (again see Chapter 1) suggests there are potential candidate genes for direct screening; these could include genes which encode proteins involved in cell wall and cytoskeleton synthesis (Dexheimer *et al.,* 1979; Scannerini and Bonfante-Fasolo, 1979; Bonfante-Fasolo and Grippiolo, 1982; Bonfante-Fasolo *et al.,* 1990) and sugar metabolism (see Smith and Gianinazzi-Pearson 1988), and genes which encode ATPases (Marx *et al.,* 1982; Gianinazzi-Pearson *et al.,* 1991). The major drawback of this approach is that it is unlikely to reveal new or novel features of the system under analysis: It can only confirm or refute previous thinking.

5.1.2 The Non-targeted Approach to Gene Cloning

The non-targeted approach does not require any knowledge of gene function or involvement. It relies on the increasingly sophisticated techniques available for modifying, manipulating and identifying nucleic acids *in vitro* and *in vivo*. There are several potential strategies:

(i) 'Shotgun' Cloning and Transformation.

This approach involves the cloning of complete and functional genes from partially digested genomic DNA and the subsequent transformation of specific (typically mutant) phenotypes with those clones. Monitoring the transformants for altered (or restored wild-type) phenotypes can allow elucidation of the biochemical and/or

physiological functions of the transforming genes. 'Shotgun' cloning stategies have proven successful in isolating genetic sequences and confirming gene functions in several bacterial and fungal species (for examples see Nester *et al.*, 1984; Djordjovic *et al.*, 1987; Staskawicz *et al.*, 1984; Leong and Holden, 1988).

Cloning plant genes by complementation in suitably characterised plant phenotypes is logistically difficult if not impossible. Plant genomes are orders of magnituide larger than those of bacteria and fungi, necessitating very large libraries of clones (see Brown, 1991) and the efficiency of transformation in even the most amenable plant species is extremely low. Complementation of plant DNA sequences in suitable *E. coli* and *S. cerevisiae* phenotypes has been used to identify several plant genes (see Gibson and Somerville, 1993). Whilst this approach appears suitable for cloning genes involved in basic cell metabolism, it is unlikely to prove useful for identifying genes involved in plant developmental processes (ie. cell differentiation) or plant-microbe interactions.

(ii) Gene tagging

Many genes are dominant and the insertion of DNA sequences into them should lead to a loss of gene function. Gene tagging involves the creation of a population of mutant plants by the random insertions of a transposable elements (see Balcells *et al.*, 1991) or *Agrobacterium* -mediated T-DNA (see Koncz *et al.*, 1989; Feldmann, 1991). The target-gene sequences of interest which flank the transposable element or T-DNA sequence can then be isolated by using the inserting sequences as a hybridisation probe. Insertion-sequences have been used to identify and isolate, amongst others, nitrogen fixation and nodulation genes from *Rhizobium* species (see Djordjevic *et al.*, 1987), genes of the anthrocyanin pathway from maize (Federoff *et al.*, 1984), and genes which affect trichome (*GLABROUS1*) and chloroplast (*ch-42*) development in *Arabidopsis thaliana* (Marks and Feldman, 1989; Koncz *et al.*, 1990). The insertional mutation approach has several problems which will only be

very briefly discussed here (for more complete discussions see Ellis *et al.*, 1988; Walden *et al.*, 1991; Walbot, 1992).

Only two plant species, *Zea mays* L. (maize) and *Antirrhinum majus* L., have genetically well defined and characterised transposable elements. To overcome this obvious limitation, transgenic plants containing an active transposable element from maize have now been obtained in several species including *A. thaliana* (Van Sluys *et al.*, 1987), carrot (Van Sluys *et al.*, 1987), tobacco (Baker *et al.*, 1986), tomato (Yoder, 1990), and rice (Murai *et al.*, 1991). Clearly progress is being made but many important crop species (including barley) are not yet amenable to this type of genetic manipulation.

Maize is also subject to very high natural mutation frequencies. As Ellis *et al.* (1988) point out, the number of background mutations detected for each insertional mutation could range from 33 to 4000 in a population considered large enough to be "reasonably sure" of obtaining at least one transposable element-derived mutation event. Maize also contains multiple copies of sequences which hybridise to the transposable elements suitable for this type of experimental approach. Separating the background mutations from the insertion events and characterising the many false positives that are likely to be detected when transposable element sequences are used as probes is impractical for any but large teams of researchers.

Agrobacterium- mediated transformation (T-DNA insertion) shows considerable promise as a method for gene-tagging in dicot plant species which are most amenable to transformation and whole plant regeneration techniques (*A..thaliana*, tobacco and tomato). Transformation and plant regeneration is not yet efficient enough in monocot species (with the possible exception of rice; see Christou *et al.*, 1992) for this type of approach to be given serious consideration for this project.

(iii) Differential screening.

Complementary DNA's (cDNA's) are doublestranded DNA copies of the messenger RNA (mRNA) molecules found in cells which serve as templates for protein biosynthesis. Apart from non-coding regions at the 5' and 3' ends, eukaryotic mRNA is free of the non-coding regions (introns) which usually disrupt the coding regions (exons) of eukaryotic genes (Darnell, 1982).

Thus cDNA is a source of intron-free genetic information. This is a necessity when the aim of gene cloning is to produce eukaryotic-protein expression in a bacterial-cell system. Bacterial genes do not possess the characteristic intron-exon structure of eukaryotic genes and appear not to have the mechanisms for post-transcriptional processing of RNA's containing internal non-coding regions. Even if protein production is not an express aim, cDNA's are still an extremely useful source of genetic material; cDNA clones are usually much smaller than their corresponding genomic clones making cloning, manipulation and subsequent analyses of the DNA sequences easier and more efficient; they represent only the expressed genes and this is only a very small percentage of the total genomic DNA; and they make very useful probes for hybridisation experiments, particularly in gene-expression studies.

Differential screening essentially involves comparing the relative abundances of individual mRNA species in different RNA populations. A cDNA library is made from one population of RNA, the individual clones are plated out, transferred to a membrane in duplicate and hybridised with single-stranded labelled cDNA obtained by reverse transcription of RNA from the original plant material and the material being used for the comparitive analysis. By comparing the relative signal intensities of clones within and between the two populations it is possible to draw conclusions regarding the levels of expression of an individual clone in each of the two populations. This approach has allowed the cloning of many tissue specific,

developmentally regulated and symbiosis-associated plant genes from a range of plant species (see Table 5.1 below).

Table 5.1 Examples of plant genes isolated by differential screening

Gene(s)	Plant Species and Tissue	Reference
Auxin-inducible genes.	Strawberry receptacles.	Reddy *et al.*, 1990.
Calmodulin-like gene	Tobacco leaves	Monke and Sonnewald, 1995.
Cotton fibre developmental genes	Cotton fibres	John and Crow, 1992.
Light-inducible gene.	Rice shoots.	de Pater *et al.*, 1990.
Grain-development genes.	Aleurone layer of barley seeds.	Olsen *et al.*, 1990.
Gibberelic acid-induced endochitinase	Aleurone layer of barley seeds.	Swegle *et al.*, 1989.
Myo-inositol synthase	Citrus leaves	Abu-Abied and Holland, 1994.
Pathogen-induced genes.	*Erysiphe graminis* - infected barley leaves	Davidson *et al.*, 1987.
	Nematode infected potato roots	Gurr *et al.*, 1991.
Pollen-abundant putative actin-depolymerising gene	Maize pollen.	Rozycka *et al.*, 1995.
Nodulin genes.	Pea root nodules.	Govers *et al.*, 1987.
Fruit-development genes.	Ripe tomato fruit.	Pear *et al.*, 1989.

The differential screening approach was selected for the analysis of mycorrhiza-related genes for several reasons; this method has been succesful in isolating symbiosis:pathogenesis-related plants genes from both pathogenic and mutualistic systems; it is not derivative of other research and so there is the potential to isolate

and identify novel genes; and the methods required are technically well developed and readily adaptable to the *Glomus intraradices* -barley model system.

5.2 Construction, Characterisation and Differential Screening of a cDNA Library Prepared from Mycorrhizal *Hordeum vulgare* cv. 'Galleon' Roots.

The aims of this series of nucleic acid manipulations were:

(1) to constuct a cDNA library from 12-14 day old mycorrhizal (M^+) barley root tissue;

(2) to differentially screen this library with α-^{32}P-dCTP-labelled (radio-labelled) cDNA reverse transcribed from M^+ and non-mycorrhizal (NM) 14 day old barley roots;

(3) to identify plant cDNA clones which detect mRNA's that increase or decrease in abundance during the establishment of the symbiosis and;

(3) to confirm the results of the differential screening using DNA:RNA (Northern) hybridisation techniques.

5.2.1 Synthesis of cDNA

Forty *H. vulgare* cv. 'Galleon' seeds were sterilised and germinated as described (see 2.2). Twenty four germinated seeds of approximately equal size and development were selected; half were transplanted into pots containing 400 g of sand:soil (1:9 w/w) growth medium and the remainder were transplanted into pots containing 400 g of growth medium inoculated with *Glomus intraradices* pot culture (15% w/w). Nutrient solutions A-E were added (10 ml/kg of growth medium) to each pot and the plants were watered to weight (12% w/w) every 2 days (see Appendix 1).

After 14 days growth the whole root systems of 3 plants from each treatment were harvested and assessed for *G. intraradices* infection (2.3): None of the NM plants were infected; the extent of the infections in M^+ plants were 16%, 17% and 21%. The roots of all remaining plants and the leaves of the M^+ plants were harvested, carefully washed free of the growth medium and snap frozen in liquid N_2.

The results described in Chapter 3 show that the majority of VAM development is in the first few lateral roots of 14 day old 'Galleon' plants. Accordingly, the bottom half of each root system was excised and discarded. The root samples of each treatment were then pooled, weighed and divided into 3 approximately equal portions (100-150mg). Total RNA was extracted from each of the 6 individual subsamples as previously described (2.7.2.1). The yield of RNA from each subsample was determined by spectrophotometry (Maniatis *et al.*, 1982) and 1µg of each sample was separated on an ribonuclease (RNAse) free 1.5% TAE agarose gel, stained and photographed (2.7.2.4). Non-denaturing electrophoresis was used because of the improved staining of non-denatured RNA (which is usually associated with secondary structures) in comparison to denatured RNA. The electrophoresis gel (see Figure 5.1) indicated only slight degradation of the ribosomal RNA in all samples; this can prossibly be attributed to low levels of exogenous RNAse activity in the electrophoresis tank and gel and/or residual endogenous RNAse activity in the samples which is not inhibited by the buffering conditions (RNA samples run in less stringently prepared RNAse free systems were almost invariably more degraded than those run in very strictly prepared RNAse free sytems).

The yields from the total RNA extractions (see Figure 5.2) showed that the concentration of RNA (µg/mg f.w.) was higher in M^+ root tissue than in NM root tissue. This observation was supported by a considerable amount of data collected over the course of this project. The fresh weights, extents of infection, and RNA yields were determined for all tissues used in paired extractions (NM and M^+) of

approximately the same fresh weight: Total RNA yields were always greater from M^+ roots than NM roots when the extent of total infection was higher than 10%. This could indicate increased gene expression in the M^+ root tissues due to *in vivo* transcriptional activation and protein translation which corroborates the data reported by Pacovsky (1989) and Wyss *et al.*, (1990) showing that protein levels are higher in M^+ roots than in NM roots. However, some recent unpublished results suggest that P starvation may increase RNase activity in plant roots (communicated by S. Smith) which could lower total RNA concentrations in NM roots by increased rates of RNA degradation. The observed increase in RNA yield could also reflect higher concentrations of RNA in the fungal hyphae relative to the barley roots. As the proportion of fungal tissue is unlikely to exceed 10% of the total fresh weight in even the most heavily colonised mycorrhizal roots (see Harley and Smith, 1983), the RNA concentrations within the colonising hyphae would need to be at least 2-4 times higher than that of the root tissues to account for yield increases ranging from 10-40%. A combination of all three explantions cannot be ruled out.

5.2.2 Construction and Characterisation of a M^+ cDNA Library

RNA sample 6 was selected as the material for the synthesis of cDNA (see Figure 5.1). A cDNA cloning kit was obtained from Pharmacia; this kit utilises Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase, which unlike Avian Myeloblastosis virus reverse transcriptase, is not inhibited by ribosomal RNA (Kotewicz *et al.*, 1985). Consequently, total RNA preparations rather than purified poly A^+ RNA can be used as the template for 1st strand synthesis. This might reduce the total number of clones obtained but may increase the number of full length cDNA's as the poly A+ RNA purification step involves buffering and temperature conditions which may subject the RNA to further degradation by any contaminating RNAses.

The cDNA synthesis was carried out using 50µg of RNA template as described by the manufacturer except that α-^{32}-P-dCTP (3µC) was added to the first strand reaction mix. Samples were taken from the reaction mix after completion of the first and second strand reactions (5µl and 10µl, respectively) and separated on an RNAse-free 1.5% TAE agarose gel which was then dried down in a vacuum oven and autoradiographed for 4 hours at -80°C. The developed autoradiograph (see Figure 5.3) indicated that cDNA:RNA hybrid molecules and doublestranded cDNA's ranging from 200 bp to 4000 bp had been synthesised.

The cDNA's were ligated with *Eco* R1/*Not* 1 linkers, cloned into the *Eco* R1 site of the insertion vector λgt 10 (Huynh *et al.*, 1984) and packaged into infective phage using extracts supplied by Promega. Two insert/vector ratios were used for the ligations (the molar ratios were unknown because the yield of cDNA was not known) and λgt 10 arms (1µg) were ligated with 0.5µg of *Eco* R1 digested pSP64 plasmid (supplied by Promega) as a ligation control. All ligations were carried out as previously described (2.7.3.10). Undigested non-recombinant λgt10 DNA (100ng) was used as a control to test the efficiency of the packaging reaction. The 2 cDNA ligations and the pSP64 control ligation were used to transfect *E. coli* host strain C600*Hfl* which carries a high frequency lysogeny mutation (*Hfl* A150). Recombinant λgt10 phage (which contains a disrupted *imm* 434 gene) produce plaques very efficiently in this stain whilst non-recombinants (with an intact *imm* 434 gene) do not (see Winnacker, 1987). The non-recombinant λgt10 was used to transfect *E. coli* host strain C600. The titres of the packaging reactions were determined (see Table 5.2) as previously described (see 2.7.2.2) which indicated the ligation and packaging reaction 1 contained approximately 6.6 x 10^4 cDNA clones. The entire library was then plated out, amplified (Maniatis *et al.*, 1982) and stored as 1ml aliquots.

Dilution	10^3	10^4	10^5
Packaging control	confluent	568	9
Ligation control	628	67	5
Ligation 1	66	9	0
Ligation 2	55	8	1

Table 5.2 Titration of cDNA library and control reactions.

Half of each packaging reaction mixture (100µl final volume) was serially diluted and 100µl from each of the dilutions was used to transfect 100µl of competant *E. coli* which were then plated onto 90mm petri dishes containing LB medium. The plaques were allowed to develop for 12 hours at 37°C and then counted to determine the titre.

The estimated titre of phage in ligation 1: $= \frac{66\text{pfu} \times 10^3}{0.1\text{ml}} = 6.6 \times 10^5$ pfu/ml

$= 6.6 \times 10^4$ clones in 100µl total volume

The quality of the cDNA library was checked by analysing the size of the cDNA inserts using the Polymerase Chain Reaction (PCR) and hybridising a proportion of the library with a ribosomal RNA clone to calculate the number of cloned ribosomal sequences.

Two 12 base oligomer primers which anneal to the regions flanking the *Eco*R1 site into which the cDNA's were cloned (see Figure 5.4) were synthesised by D. Hein. The insert sizes of fifty phage plaques were determined. The PCR's were carried out as described (2.7.4.1) and the results (some of which are presented at Figure 5.5) indicated that 95% of the clones contained inserts and that they ranged in size from 120bp to 3.5kbp. The majority of inserts were 450bp to 700bp in size.

Approximately 10,000 clones were plated out (2,500 pfu/plate), transferred to Hybond N+ hybridisation membranes and hybridised with a wheat rinbosomal DNA clone (2.2.18). Less than 1% of the clones (56 clones out of approximately 10,000) hybridised with the wheat ribosomal DNA (data not shown).

The PCR amplifications and hybridisation analysis indicated that the majority of the cDNA's contained inserts of over 400bp in length and that less than 5% contained no insert or ribosomal DNA. However, the number of clones obtained from the first library was lower than expected, as good quality total RNA was readily available, a second cDNA library was prepared and characterised as described above using polyA$^+$ RNA purified from 100µg of the M$^+$ total RNA. The purification was carried out using a Promega PolyATtract mRNA isolation kit. The second library contained many more clones than the first (5 - 6 x 10^5) but the average insert size was lower (200bp to 350bp; results not shown) and 45% of the phage contained undetectable or no inserts.

FIGURE 5.1

Non-denaturing gel electrophoresis of total RNA (1µl from each sample/lane) extracted, using a standardised proceedure, from 14 day old non-mycorrhizal (lanes 1-3) and *Glomus intraradices* -colonised (lanes 4-6) roots of *Hordeum vulgare* cv. 'Galleon'. The extent of colonisation in the mycorrhizal roots was estimated to be 18% of the total root length.

The sizes of some of the DNA marker bands (*Hae* III digested λdv DNA) are shown at the right hand side of the figure.

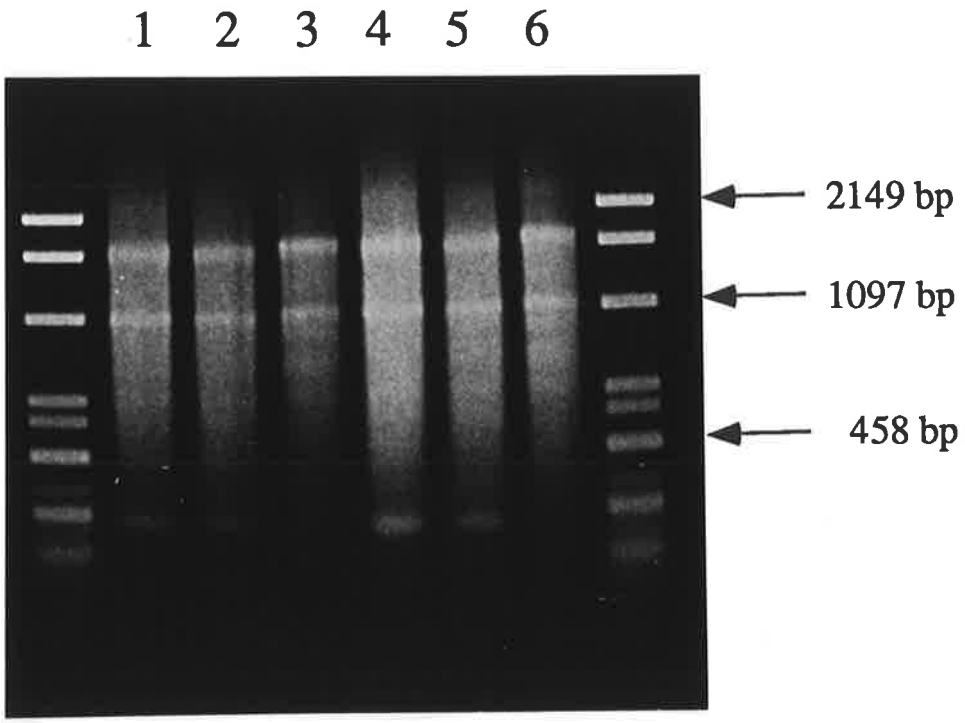

1 2 3 4 5 6
2149 bp
1097 bp
458 bp

FIGURE 5.2

Yield of total RNA extracted from 14 day old non-mycorrhizal (lanes 1-3) and *Glomus intraradices* -colonised (lanes 4-6) roots of *Hordeum vulgare* cv. 'Galleon'. The extent of colonisation in the mycorrhizal roots was estimated to be 18% of the total root length. This graph represents the means of three samples for each treatment of approximately equal weight and the standard errors of those means. Each sample was extracted using a standardised proceedure.

Figure 5.2

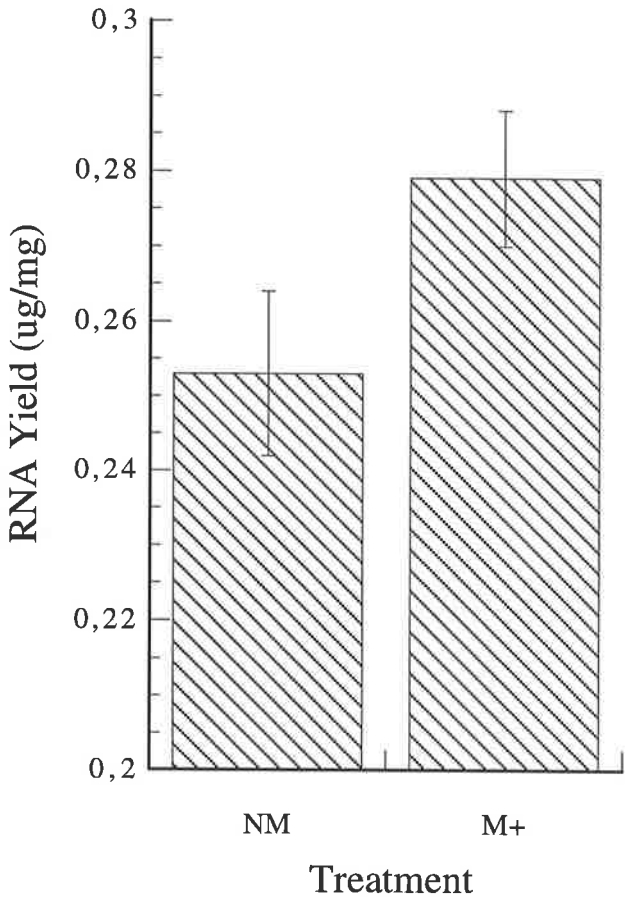


FIGURE 5.3

Synthesis of first (lane 1) and second strand (lane 2) cDNA reverse transcribed from total RNA extracted from 14 day old *Glomus intraradices* -colonised *Hordeum vulgare* cv. 'Galleon' roots.

Aliquots were taken from the reaction mixes at the completion of each cDNA strand synthesis and electrophoresed on a 1.2% TAE agarose gel. The gels were wrapped in 'Gladwrap' plastic after the electrophoresis and exposed to X-ray film at room temperature for 6 hours. The gels were then stained with ethidium bromide to determine the position of the DNA markers (*Hind* III digested λ DNA).

The approximate positions of some of the DNA markers are shown at the right of the figure.

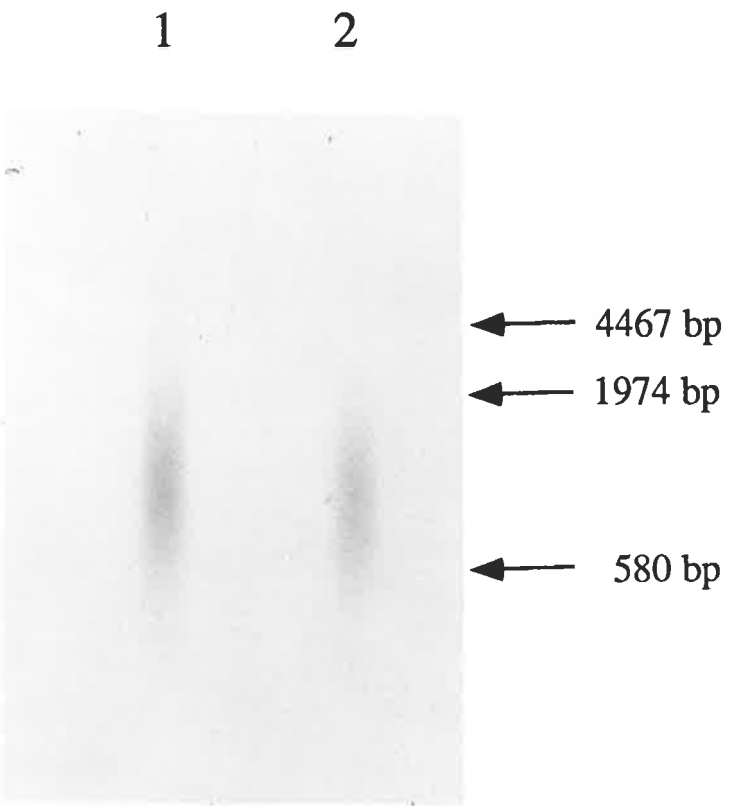

1
2
4467 bp
1974 bp
580 bp

FIGURE 5.4

DNA sequence from the *Imm* 434 region of the phage vector λgt10 and the two primers used to amplify cDNA inserts by the polymerase chain reaction. The *Eco* R1 cloning site is in bold print and the primer sequences (which anneal to the complementary strands) are in bold print and underlined. The arrows indicate the direction of the*Taq* polymerase-mediated DNA synthesis.

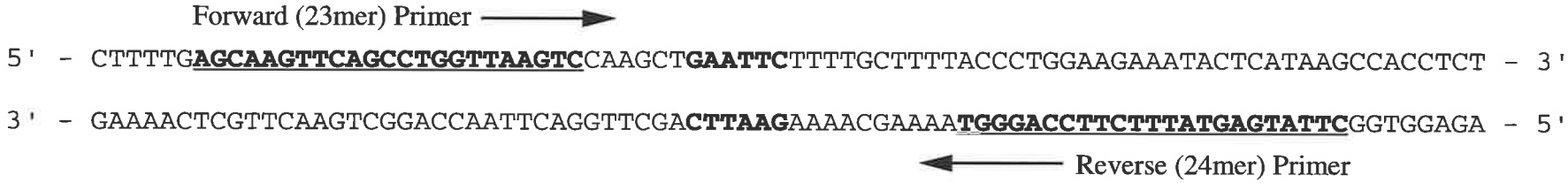
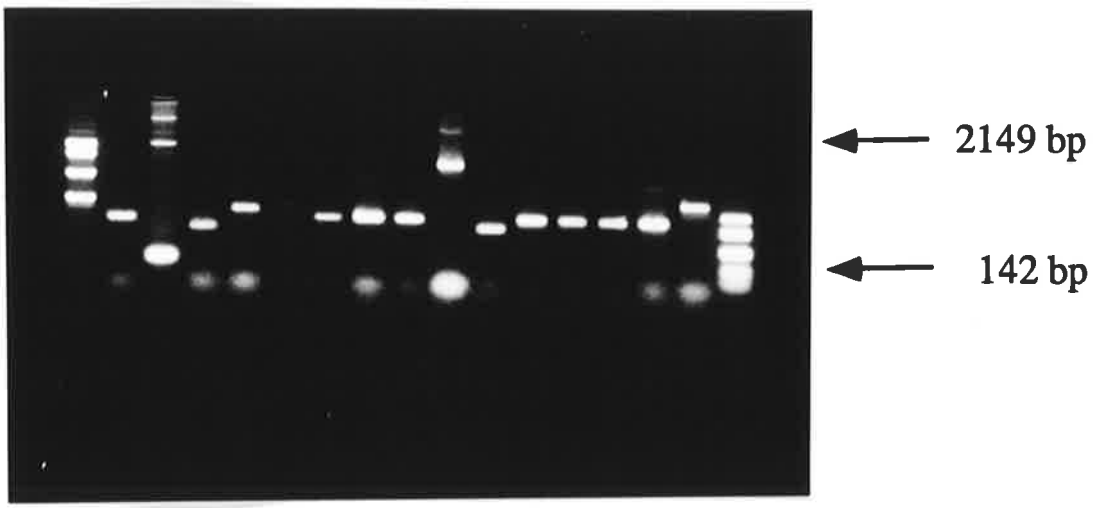

Forward (23mer) Primer
5' - CTTTTGAGCAAGTTCAGCCTGGTTAAGTCCAAGCTGAATTCTTTTGCTTTTACCCTGGAAGAAATACTCATAAGCCACCTCT - 3'
3' - GAAAACTCGTTCAAGTCGGACCAATTCAGGTTCGACTTAAGAAAACGAAAATGGGACCTTCTTTATGAGTATTCGGTGGAGA - 5'
Reverse (24mer) Primer

Figure 5.4

FIGURE 5.5

Gel electrophoresis of polymerase chain reaction amplified inserts from 15 phage randomly selected from a cDNA library prepared from reverse transcribed total RNA extracted from 14 day old *Glomus intraradices* -colonised *Hordeum vulgare* cv. 'Galleon' roots.

The DNA markers were *Rsa* 1/*Dra* 1 digested pTZ18U (left hand side) and *Sau* 3A digested pTZ18U (right hand side). The sizes of two of the marker fragments are shown at the right hand side of the figure.


2149 bp
142 bp

5.2.3 Isolation of Putative Barley-mycorrhiza Related cDNA Clones

In view of the much better quality of the first cDNA library, it was used for the initial differential hybridisation. Approximately 2.5 x 10^4 clones were plated out (2,500 pfu/plate) and transferred (in duplicate) to Hybond N^+ hybridisation membranes (2.7.3.7). Radio-labelled first strand cDNA was prepared from total RNA (25µg), extracted from the first 10 lateral roots of 14 day old NM and M^+ barley roots, using Superscript H^- reverse transcriptase (Bioprobe). One set of membranes was then hybridised with NM-root derived cDNA and the other with M^+-root derived cDNA. After 16 hours of hybridisation, the membranes were stringently washed (see 2.7.3.7) and then autoradiographed for 7 days at -80°C with intensifying screens. The autoradiographs were developed and the hybridisation patterns of duplicate membranes compared to identify clones with differential signal intensities (see Figure 5.6). The plaques of 512 putative barley-mycorrhiza-related (BMR) clones were identified in this manner and cored out with a Pasteur pipette, expelled into 100µl of SM buffer and allowed to diffuse into the buffer for 12 hours at 4°C. Each clone was then numbered.

The membranes were then stripped of the remaining probe (Maniatis *et al.,* 1982) and each set of membranes was re-hybridised with radio-labelled first strand cDNA prepared from total RNA (25µg) extracted from either 14 day old NM or 14 day old M^+ barley leaves. This hybridisation was carried out because systemic responses to pathogenic symbioses have been reported in plants (see Ryals *et al.*, 1994). No differential hybridisation signals were observed in the cDNA population analysed and (not surprisingly) nearly 90% of the clones did not hybridise with the reverse transcribed shoot RNA transcripts. It was concluded that most of the cDNA clones in the library were homologous to root-specific mRNA species and that none of them were detectable as being systemically induced in barley leaves in response to the mycorrhizal infection process.

Top agarose bacterial lawns were overlaid on LB medium in 135mm Petri dishes (as per 2.7.2.3 but without adding any infective phage) and incubated for one hour at 37)C before 1µl of the SM buffer containing each putative BMR clone was spotted onto the plates in a regular pattern using a 1cm grid. The phage were then allowed to mutiply and lyse for 12 hours. These plaques were transferred to Hybond N^+ in duplicate and differential hybridisations were performed as described above except that 40µg of freshly extracted plant total RNA was used for each of the reverse transcription reactions.

The hybridisation patterns of the duplicate membranes (for an example see Figure 5.7) indicated that the majority of the putative BMR clones were false positives but variations in signal intensity were observable in 121 of the putative BMR clones so these were collected and renumbered; the remaining clones were discarded. None of the clones displayed hybridisation patterns consistent with specific expression of genes during mycorrhiza development but both increased and decreased accumulation of specific RNA transcripts were observed. Both sets of membranes were then stripped of the probe (Maniatis *et al.,* 1982) and re-hybridised with radio-labelled first strand cDNA reverse transcribed from shoot RNA as described in the previous paragraph. The results were very similar to those described above; over 95% of the cDNA clones represented RNA species which are specific to plant roots and none appeared to be differentially expressed.

The putative BMR clones remaining after the second round of differential screening were then used to generate DNA probes by PCR amplification of the phage inserts as previously described. Unfortunately, it was found that nearly 50% of the phage samples generated mutiple bands, indicating a mixture of phage particles (see Figure 5.8). Many of these mixed phage samples were replated on to 90mm petri dishes at low plaque formation densities (100-200 pfu/plate) and six plaques from each plate were isolated and checked by PCR amplification of the inserts. All samples

containing single inserts obtained in this manner were numbered and stored for later use as DNA probes.

FIGURE 5.6

Identification of putative barley-mycorrhiza related cDNA clones by differential screening of the mycorrhizal root cDNA library.

Phage DNA (approximately 25,000 plaques in all) was transferred, in duplicate, onto two sets of Hybond N+ nylon membranes. One set of membranes were then hybridised with ^{32}-P-labelled single stranded cDNA prepared from non-mycorrhizal (NM) root total RNA (25µg) whilst the other set of membranes were hybridised with ^{32}-P-labelled single stranded cDNA prepared from *Glomus intraradices* -colonised (M) root total RNA (25µg). The membranes were washed and then autoradiographed at -80°C for 8 days with the aid of intensifying screens.

By comparing the hybridisation signal intensites of matched pairs of membranes, it was possible to identify 512 cDNA clones that appeared to increase or decrease in abundance during the earliest developmental stages of VA mycorrhiza formation. These cDNA clones, which correspond to genes which could be either up-regulated (u) or down-regulated (d), are indicated by the arrows and were isolated and used for a secondary differential screening.

FIGURE 5.6

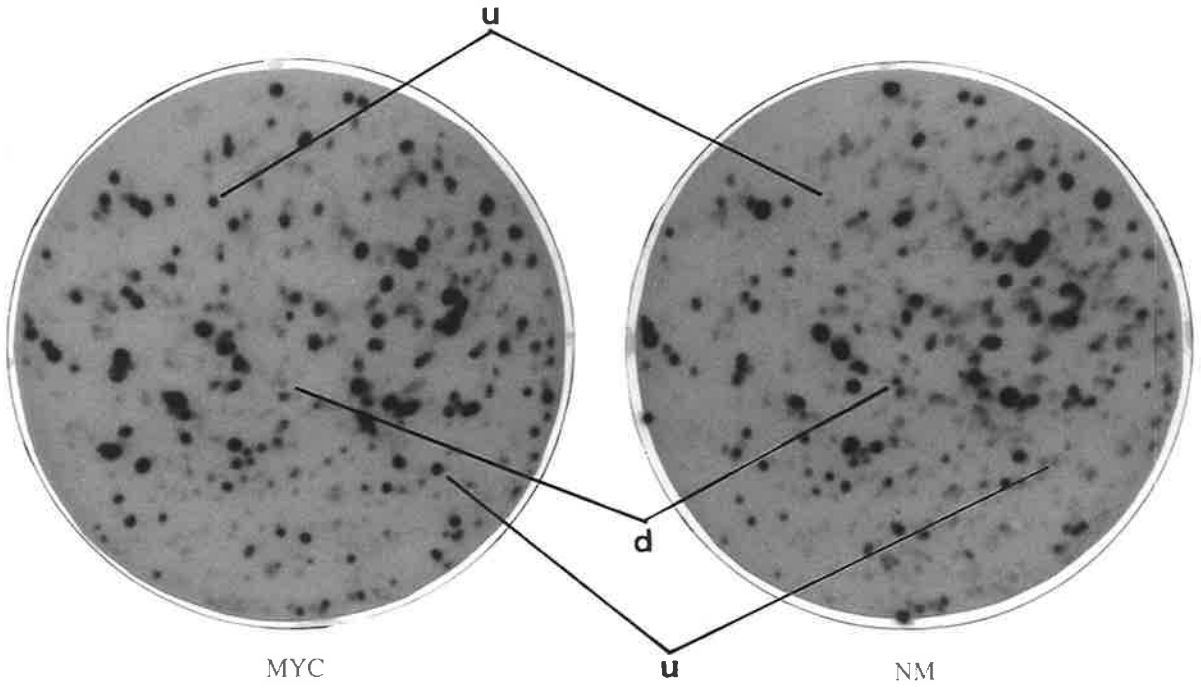


FIGURE 5.7

Secondary differential screening of the putative barley mycorrhiza related (BMR) clones identified during the primary screening.

The putative BMR phage-clones isolated during the primary screening process were used to tranfect a bacterial lawn and form large plaques (0.7-1.0cm). The phage DNA was transferred to Hybond N+ nylon membranes, in duplicate, and each set of membranes was hybridised with either ^{32}-P-labelled single stranded cDNA prepared from non-mycorrhizal (NM) root total RNA (40µg) or ^{32}-P-labelled single stranded cDNA prepared from *Glomus intraradices* -colonised (M+) root total RNA (25µg). The membranes were washed and autoradiographed at -80^{o}C for 6-8 days with intensifying screens.

Putative BMR clones which are either upregulated (u) or down-regulated (d) are indicated by the arrows.

FIGURE 5.7

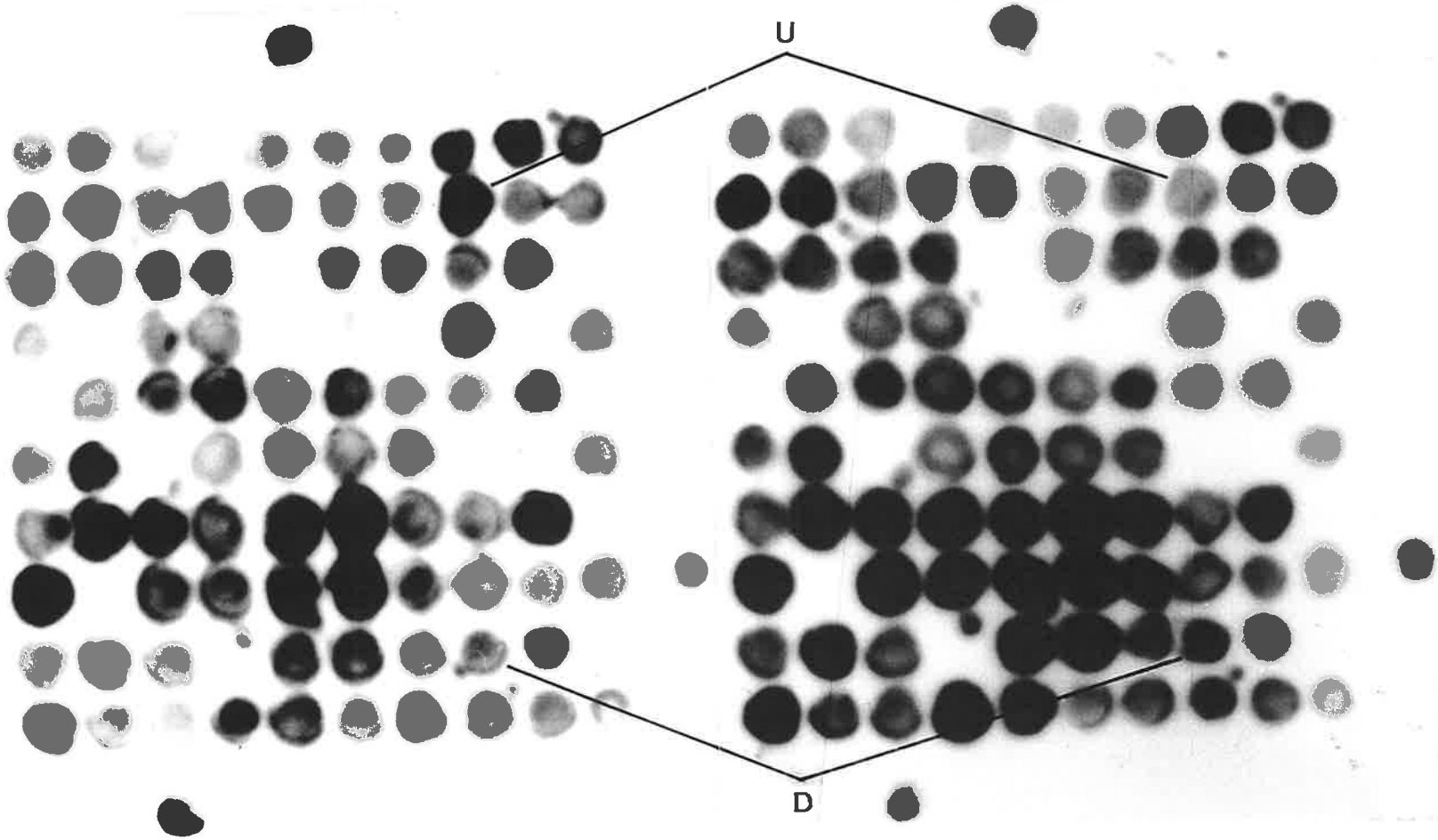
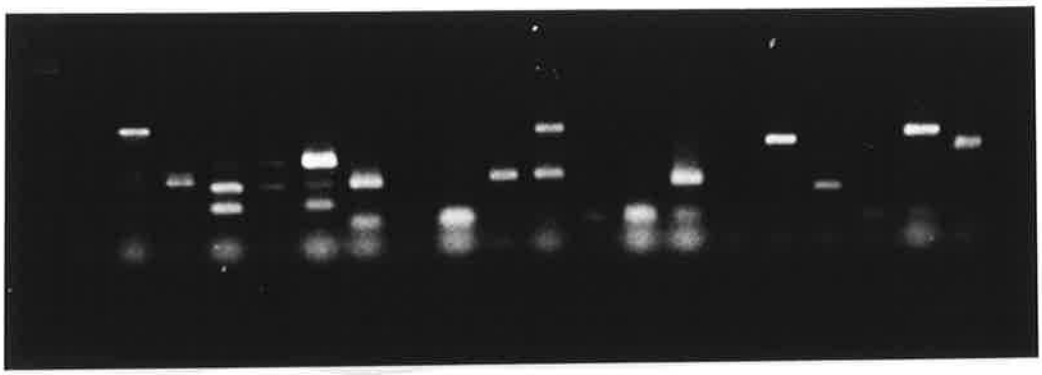


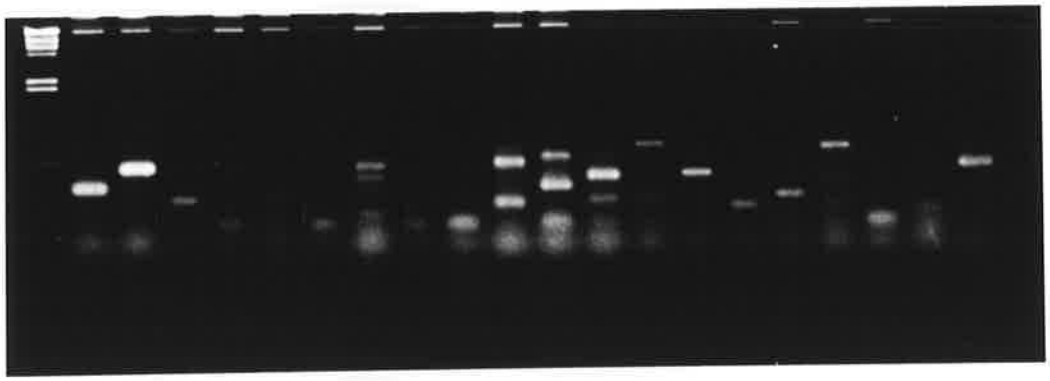
FIGURE 5.8

Gel electrophoresis of polymerase chain reaction amplified inserts from 40 phage randomly selected from the pool of putative barley-mycorrhiza related clones.

The clones containing multiple bands were replated at low densities of plaque-formation (50-100 pfu/plate) and rescreened to ensure only single bands were present in each phage preparation. All unique bands from each rescreening were kept for analysis.

The DNA marker was *Hind* 111 digested lambda DNA and can be seen at the left hand side of the gels. The sizes of two of the marker fragments are shown at the right hand side of the figure.


1973 bp
580 bp


1973 bp
580 bp

5.2.4 DNA:RNA (Northern) Hybridisation Analysis of Putative BMR cDNA Clones

The single band cDNA clones were used to compare the accumulation and abundance of their respective homologous RNA transcripts in total RNA samples extracted from the top half of whole root-systems of 7 and 14 day old NM and M^+ barley roots and 14 day old M^+ barley shoots by DNA:RNA (Northern) hybridisation. The extent of total infection in the mycorrhizal barley plants was calculated to be 0% and 21% in the 7 and 14 day old plants respectively (see 2.5). These same plants were also used for the 14 day old M^+ shoot RNA extractions. Lateral roots were not specifically used for the preparation of these RNA membranes because non-denaturing electrophoretic separations of these samples indicated that slightly greater degradation occurred in lateral root RNA samples than whole root samples, probably reflecting the longer time required for the root excision/extraction process prior to the addition of CsCl to preparations before the overnight centrifugation.

The five RNA samples (10µg/sample; 4 gels at a time) plus marker DNA were electrophoresed under denaturing conditions (2.7.2.4) and transfered to Hybond N^+ hybridisation membrane (2.7.2.5). These membranes were then hybridised to PCR-generated putative BMR probes (2.7.4.1) which had been isolated and purified (2.7.3.9) from a 1.5% agarose TAE buffered electrophoresis gel (2.7.3.6). The approximate sizes of the hybridising RNA transcripts were determined by comparing the migration of the hybridising band with a DNA size marker. A total of fifty six hybridisations were carried out and each membrane was used for a maximum of three hybridisations. The ribosomal RNA bands on the membranes were stained with methylene blue (3%) in acetic acid (50%) after they had been used for the final time. This allowed an estimation of the amounts of RNA that had been transferred to the membrane and acted as a further aid to transcript length determination. New

membranes were always prepared from fresh extracts of total RNA. The mycorrhizal plants always appeared uninfected after 7 days growth whilst the extent of infection ranged from 15-22% after 14 days growth when 15% *G. intraradices* inoculum was used. Freshly extracted RNA was used for each set of hybridisations to guard against artefacts caused by contamination to an individual plant and to ensure reproducibility of the results. The RNA extracted from the 7 day old material (uninfected in both NM and M^+ plants) served two purposes; it was a further control against contamination-induced changes in one group of plants; and it allowed the possibility of detecting transcripts of genes which are up- or down-regulated prior to or at the very early (undetectable) stages of mycorrhiza development. The leaf total RNA served as a negative control to identify cross hybridisation (particularly with ribosomal RNA) and background hybridisation. It may also have identified systemic responses not detected during the differential screening process.

Forty one of the cDNA probes used for the Northern analyses detected only very slight differences in the accumulation of the homologous RNA transcripts between the NM or M^+ roots. These differences were most probably due to slightly uneven loading or transfer of the gels (the large number of false positives obtained all through the screening process highlights some of the difficulties involved in using untargeted cloning strategies). No obvious differences in transcript accumulation were detected by any of the cDNA probes between NM and M^+ 7 day old root RNA samples. Seven of the clones hybridised very weakly with the total root RNA; the transcript levels of those particularly RNA species appeared to be at the limit of detection.

One cDNA probe (BMR37) detected a root-specific 1100b RNA transcript which accumulated to much higher levels in 14 day old roots of NM or M^+ plant than in 7 day old roots of NM or M^+ plants (Figure 5.9). Two cDNA probes (BMR64 and BMR96) detected root-specific RNA transcripts (1300b and 200b in length,

respectively). While equivalent in abundance in 7 day old roots of either NM and M+ plants these sequences appeared to much less abundant in 14 day old M^+ roots than in NM roots of the same age (Figure 5.9).

Finally, two probes (BMR6 and BMR78) detected RNA transcripts (1100b and 3500b respectively) which were more abundant in the M^+ 14 day old roots than in NM roots of a corresponding age (Figures 5.10 and 5.11). The genes which encode these RNA species appear to be constitutively expressed at a low level (7-10 day exposure times were required for the autoradiographs) but are up-regulated during the formation of mycorrhizas. It appears that they may be actively involved in the response to or formation of VA mycorrhizas. However, it should be noted that the increased expression of the genes detected by BMR6 and BMR78 correlates with the onset (and as shown for BMR6, the extent) of dramatic fungal development. That these two cDNA clones are of fungal or even bacterial origin (see review on mycorrhizal helper bacteria by Garbaye; 1993) cannot be ruled out. It is unlikely but possible that the constitutive levels of expression observed in the young barley roots were caused by cross-hybridisation of both these cDNA clones with a barley-root RNA of approximately the same size as a fungal or bacterial RNA. To ensure that the clones were monitoring the increased accumulation of specific plant mRNA species and not simply fungal development, further experiments were undertaken to confirm that the cDNA clones, BMR6 and BMR78, were of plant origin.

5.2.5 DNA:DNA (Southern) Hybridisation Analyses of the two cDNA Clones BMR6 and BMR78.

High molecular weight genomic DNA was extracted from leaves of *H. vulgare* cv. 'Galleon' leaves, *H. vulgare* cv. 'Betzes' leaves (2.7.3.1), 21 day old heavily mycorrhizal (40-50%) 'Galleon' roots, which were grown as previously described, and sterilised *G. intraradices* spores (as per 2.2.2) which were kindly supplied by A.

Tassie. The plant leaves and mycorrhizal roots yielded 50-70µg of DNA, the quality of DNA extracted, as determined by minigel electrophoresis, was excellent. The *G. intraradices* spores yielded 7-8µg of what appeared to be good quality, high molecular weight genomic DNA.

A sample of DNA from the three plant sources (8µg) and all the *G. intraradices* DNA was digested with the restriction enzyme *Eco* R1. Each DNA digestion reaction was divided into two equal samples and the fragments were separated by overnight gel (1.5% TAE agarose) electrophoresis (2.7.3.6). The DNA fragments were transferred to Hybond N+ membranes and hybridised (2.7.3.7) with radiolabelled PCR-generated insert (2.7.4.1) from either BMR6 or BMR78 before washing and exposure to X-ray film at 80°C for three days. The results of the hybridisations are shown in Figure 5.11.

Both probes hybridised to the *Eco* RI digested DNA extracted from the two barley cultivars and the mycorrhizal 'Galleon' roots. BM6 detected two bands (of approximately 4.0 kbp and 5.0 kbp in size) in the 'Galleon' samples and a single band of approximately 4.0 kbp in 'Betzes'. BMR78 detected a single band of approximately 3-3.5 kbp in the two 'Galleon' samples and a slightly larger in 'Betzes'; this may have been an artefact caused by the gel. The hybridisation signals were weaker for 'Betzes' DNA than both the 'Galleon' DNA samples for both the probes, indicating that the loading of the wells may not have been even. No signal was detetable in the fungal spore DNA lane (which almost certainly has a much smaller genome), even after 10 days exposure (not shown). Subsequent reprobing of the membranes with radiolabelled fungal DNA showed that the fungal DNA had been efficiently transferred to the membranes (not shown). It is likely that the cDNA clones were derived from plant RNA's and are not of fungal or bacterial origin.

FIGURE 5.9

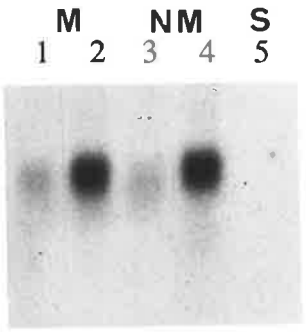
Autoradiographs of DNA:RNA hybridisations using ^{32}-P-labelled inserts from the putative barley-mycorrhiza related clones BMR37, BMR64 and BMR93 to probe total RNA extracted from the tissues of barley of differing age and mycorrhizal status.

Total RNA (10µg/lane), extracted from the roots of 7 day old (lane 1) and 14 day old (lane 2) *Glomus intraradices* -colonised *Hordeum vulgare* cv. 'Galleon' , 7 day old (lane 3) and 14 day old (lane 4) non-mycorrhizal 'Galleon' and the shoots of 14 day old (lane 5) mycorrhizal plants, was gel electrophoresed under denaturing conditions and transferred to Hybond N+ membranes. The probes were generated using the polymerase chain reaction and labelled by random priming. The membranes probed with BMR37, BMR64 and BMR96 were autoradiographed at -80°C with intensifying screens for 8, 5 and 7 days respectively.

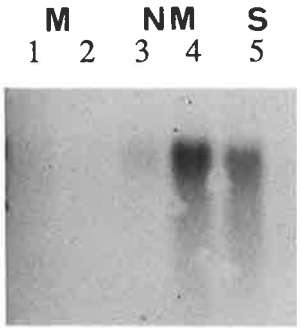
The approximate sizes of the hybridising RNA bands, shown at the right of the figure, were determined by comparing the distances of migration of the hybridising bands with those of the 28S and 18S ribosomal RNA bands.

The lane order for the membrane probed with BMR93 has been rearranged to match the other two autoradiographs.

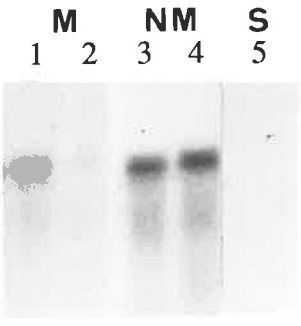
FIGURE 5.9



BMR37



BMR64



BMR93

FIGURE 5.10

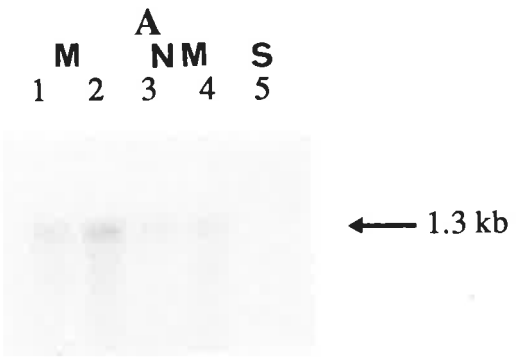
Autoradiographs of DNA:RNA hybridisations using ^{32}-P-labelled insert from the putative barley mycorrhiza related clone BMR6 to probe total RNA extracted from the tissues of barley of differing age and mycorrhizal status.

A: Total RNA (10µg/lane), extracted from the roots of 7 day old (lane 1) and 14 day old (lane 2) *Glomus intraradices* -colonised *Hordeum vulgare* cv. 'Galleon', 7 day old (lane 3) and 14 day old (lane 4) non-mycorrhizal 'Galleon' and the shoots of 14 day old (lane 5) mycorrhizal 'Galleon', was size fractionated by gel electrophoresis (under denaturing conditions) and transferred to Hybond N+ membranes. The phage cDNA insert was generated using the polymerase chain reaction (PCR) and labelled by random priming using the Klenow fragment of DNA polymerase 1. The membranes were autoradiographed for 7 days at -80°C with intensifying screens.

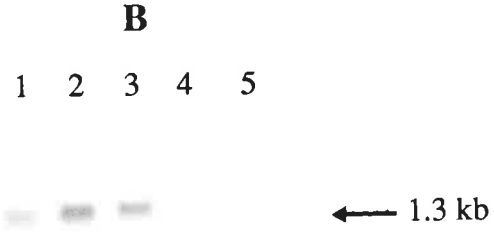
B: Total RNA (15µg/lane) was extracted from the roots or shoots of 14 day old *Hordeum vulgare* cv. 'Galleon' plants with varying extents of *Glomus intraradices* colonisation: roots which were 10% colonised (lane 1); roots which were 25% colonised (lane 2); roots which were 30% colonised (lane 3); roots which were not colonised (lane 4); and shoots from plants with roots which were 25% colonised (lane 5). The RNA (10µg/lane) was size fractionated by gel electrophoresis (under denaturing conditions) and transferred to Hybond N+ membranes. Phage cDNA insert was PCR amplified and labelled as above. The membranes were exposed for 8 days at -80°C with intensifying screens

The approximate sizes of the hybridising RNA bands, shown at the right of the figure, were determined by comparing the distances of migration of the hybridising bands with those of the 28S and 18S ribosomal RNA bands.

FIGURE 5.10



BMR6



BMR6

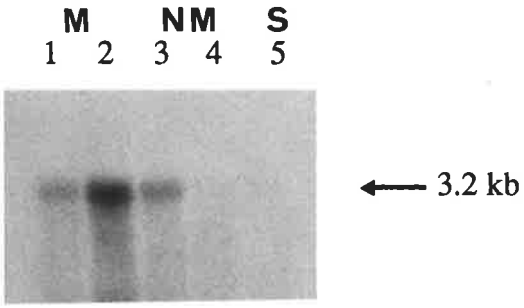
FIGURE 5.11

Autoradiograph of DNA:RNA hybridisation using ^{32}-P-labelled insert from the putative barley mycorrhiza related clone BMR78 to probe total RNA extracted from the tissues of barley of differing age and mycorrhizal status.

Total RNA (10µg/lane), extracted from the roots of 7 day old (lane 1) and 14 day old (lane 2) *Glomus intraradices* -colonised *Hordeum vulgare* cv. 'Galleon', 7 day old (lane 3) and 14 day old (lane 4) non-mycorrhizal 'Galleon' and the shoots of 14 day old (lane 5) mycorrhizal 'Galleon', was gel electrophoresed under denaturing conditions and transferred to Hybond N+ membranes. The phage cDNA insert was generated using the polymerase chain reaction and labelled by random priming using the Klenow fragment of DNA polymerase 1. The membrane was autoradiographed for 5 days at -80°C with intensifying screens.

The approximate sizes of the hybridising RNA bands, shown at the right of the figure, were determined by comparing the distances of migration of the hybridising band with those of the 28S and 18S ribosomal RNA bands.

FIGURE 5.11



BMR78

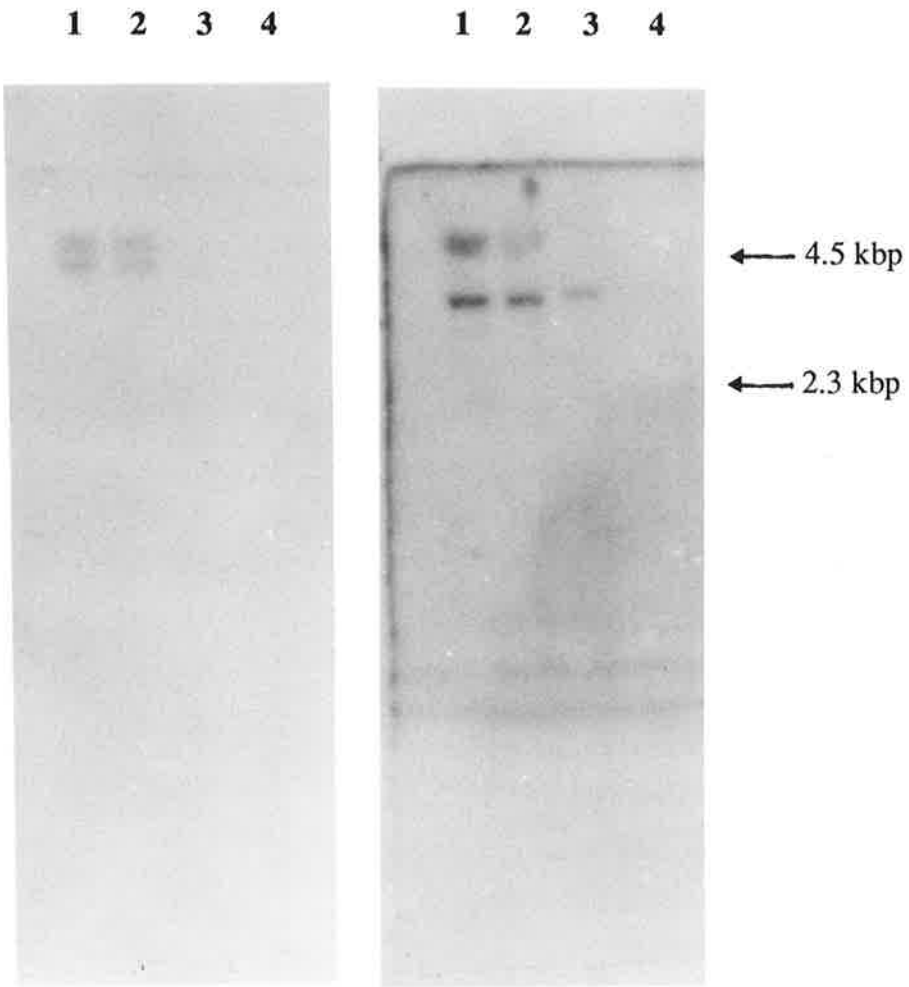
FIGURE 5.12

Autoradiograph of DNA:DNA hybridisations using ^{32}-P-labelled inserts from the putative barley mycorrhiza related clones BMR6 and BMR78 to probe genomic DNA from two barley cultivars and the mycorrhizal fungus, *Glomus intraradices.*

High molecular weight DNA (4-5µg), extracted from the shoots (lane 1) and *Glomus intraradices* -colonised roots (lane 2) of *Hordeum vulgare* cv 'Galleon', the shoots (lane 3) of *H. vulgare* cv. 'Betzes' and spores (lane 4) of the mycorrhizal fungus *Glomus intraradices* , was digested with *Eco* R1 and the fragments size fractionated by gel electrophoresis. The DNA was transferred to Hybond N+ membranes and hybridised with either BMR6 or BMR78 which had been labelled by random priming using the Klenow fragment of DNA polymerase 1. The membranes were then autoradiographed for three days with intensifying screens.

Relevant DNA size markers are shown at the right hand side of the figure.

FIGURE 5.12



5.3 Discussion

The approach taken to identify BMR genes was to differentially screen a cDNA library prepared from *G. intraradices* colonised barley roots. This sucessfully identified a number of interesting clones that fell into several categories based upon patterns of gene-expression associated with colonisation of the roots by the mycorrhizal fungus.

One clone, BMR37, detected a gene that showed increased expression in older roots. Seven day old 'Galleon' root systems are much smaller than fourteen day old 'Galleon' roots and have already developed many lateral root primordia (see Chapter 3). As such, the seven day old root systems may have a much greater proportion of active root tip cells to the metabolically less active transport cells distal to the growing tips than fourteen day old root systems. The increase in expression of the gene which encodes the BMR37 RNA-homologue in fourteen day old root systems, may reflect an increase in the proportion of cells (for example the distal transport cells) expressing this gene as the root system ages and expands. Spatial analysis of the transcript distribution of the BMR37 RNA-homologue within the root by either Northern or *in situ* hybridisation methods or PCR-based detection should answer this question.

The observed decreases in abundance of RNA transcripts in M^+ root material detected by BMR64 and BMR96 are not easily explained. Similar expression patterns have been detected by *Pisolithus tinctoris* cDNA clones during ectomycorrhiza formation with *Eucalyptus globulus* roots (Tagu and Martin, 1993). The down regulation of the genes from which the RNA's are transcribed appears to be related to the differentiation of the fungus from the free-living state to the symbiotic state (Murphy and Tagu, unpublished). At the cellular level, root cells also undergo considerable cytological change during mycorrhizal development (see my

comments in Chapter 1) particularly in cells containing arbuscules. These changes appear to involve metabolic activation of the plant cells; for example, the synthesis of membranes and (presumably) trans-membrane transport machinery.

It is tempting to speculate that the RNA transcripts detected by these two cDNA probes encode regulatory gene products: The mycorrhizal fungus could directly or indirectly repress these regulatory genes, which in turn derepress other genes, the translation products of which are necessary for functional mycorrhiza establishment. Such an elaborate regulatory system is not inconceivable in what appears to be (in evolutionary terms) an extremely old symbiotic association. The possibility that they detect mRNA's which encode proteins involved in plant defence systems that are repressed during mycorrhiza development or induced in the non-mycorrhizal roots because of contaminating micro-organisms, cannot be ruled out. The Northern analyses indicated that both mRNAs accumulated at low to moderate levels in non-mycorrhizal roots. This may reflect gene expression induced by the harvesting process. Pathogenic infection and wounding can result in the rapid accumulation of specific RNA species, particularly those encoding enzymes of phenolpropanoid biosynthesis (Lamb *et al.*, 1989), and protein-synthesising capacity (Davies and Schuster, 1981). Sequencing these cDNA clones could very quickly confirm this hypothesis as defense-related genes are relatively well characterised and many DNA sequences are now available (see Dixon and Lamb, 1990; Bowles, 1990). This should enable a DNA data base search to establish the identities of cDNA's with homologies to any of the well characterised defense-related genes.

BMR6 and BMR78 detected the RNA transcripts of genes which are constituvely expressed at a low level (5-8 day exposure times were required for the autoradiographs) and upregulated during the formation of mycorrhizas. The genes homologous to these cDNA's appear to be actively involved in the formation of, or response to, VA mycorrhiza development. These two clones were analysed in much

greater detail in order to ascribe a putative function to the translation products. Chapter 6 describes the preliminary molecular characterisation of the two cDNA clones, BMR6 and BMR78.

Chapter 6

Molecular Characterisation of the Barley Mycorrhiza-related cDNA's: BMR6 and BMR78

6.1 Introduction

The cDNA clones, BMR6 and BMR78 were shown to detect mRNA transcripts which increase in abundance during the early stages of mycorrhiza development in *Glomus intraradices* infected *Hordeum vulgare* cv. 'Galleon' roots (see Chapter 5). The aims of the series of experiments described in this chapter were to characterise these clones in more detail and, to ascribe a putative biological function to the products of the genes represented by these cDNA clones. Both clones were subcloned into the plasmid, pTZ18U, and used as probes in further DNA:RNA (Northern) hybridisation experiments to determine whether the increased accumulation of the corresponding mRNA species was specific to the development of mycorrhizal roots or was a general response induced by pathogens and/or water stress. It was also important to determine if the accumulation of mRNA transcripts was influenced by the phosphate nutrition of the non-mycorrhizal barley plants, because of the important influence of mycorrhizal colonisation on phosphate uptake from the soil (see Chapter 1). The clones were also used to map the positions of the corresponding genes to the barley genome using DNA:DNA (Southern) hybridisation analysis of barley addition lines and doubled haploid mapping populations. Finally, the sub-clones were sequenced and the sequences compared to available DNA and protein sequence data banks because high degrees of homology between the sequences of BMR6 and BMR78 and previously characterised genes could provide some strong evidence of their biological functions.

6.2 Subcloning of BMR6 and BMR78 into the Plasmid Vector pTZ18U

Insert (approximately 250ng) obtained from large scale phage preparations of BMR6 and BMR78 was ligated into the *Eco* R1 restriction site of the mutifunctional plasmid vector, pTZ18U which is based on the vector, pUC18 (see Yanisch-Perron, 1985). The ligation products were used to transform competant *Eschereshia coli* DH5a (see 2.7.1.3) which were then spread onto LB agar plates containing ampicillin (75mg/ml), X-gal and ITPG (see Maniatis *et al.,* 1982).

Twelve white colonies from the BMR6 transformation and all 5 white colonies from the BMR78 transformation were picked from the plates and used to prepare plasmid minipreparations (2.7.3.2). The insert size of the isolated plasmids was checked by *Eco* R1 digestion and visualisation of the restriction fragments after separation by gel electrophoresis (2.7.3.6). All of the BMR6 plasmids contained inserts of approximately 650bp which matched the size of the original phage insert. All of the BMR78 transformants contained plasmid DNA but only one contained an insert that was recoverable by *Eco* R1 digestion. The insert size of this clone matched that of the original phage insert (approximately 580bp). Subsequent re-culturing of this clone, both on LB plates and in LB liquid medium containing ampicillin (75µg/ml) indicated that the pBMR78 insert was unstable in *E. coli* DH5a. Consequently, further ligations and transformations were carried out (as above) using *E. coli* strains HB101 and ER1647: Strain ER1647 yielded 5 stable pBMR78 clones.

The restriction maps prepared for each clone (see Zehetner, 1987) have been incorporated into the sequencing-strategy diagram (see Figure 6.6).

6.3 Expression of the BMR6 and BMR78 Genes

6.3.1 Aims

The aim of the experiment described in this section was to grow plants under a variety of conditions in order to test whether factors other than mycorrhizal colonisation resulted in the increased accumulation of the mRNA species detected by BMR6 and BMR78.

Two biovars, Ggt-800 and Ggg-W2P, of the root fungal pathogen *Gaeumannomyces graminis* (often known as 'Take-all') were used to colonise barley seedlings for one part of this experiment. Both strains of *G. graminis* colonise the epidermal and cortical cells of the root but Ggt-800 can also invade the stele. Consequently Ggt-800 is virulent to many barley cultivars because it blocks the vascular tissues resulting in a marked reduction in the growth of the above ground parts (particularly the seed) of the plant. Ggg-W2P is considered avirulent with barley but colonises the outer root cells as much or even more (as judged by root blackening) than Ggt-800 (Harvey, 1994).

Another group of barley seedlings were subjected to water-stressing as an example of environmental stress. Increased accumulation of the transcripts detected by BMR6 and/or BMR78 under any or all of the treatments used in this experiment, would suggest that the genes corresponding to the two cDNA's are up-regulated by conditions more general than just mycorrhizal development. As such they could be related to a large group of genes which are known to be induced as a general response to pathogen colonisation and/or environmental stresses such as drought and wounding by feeding insects or herbivores. This group includes the genes that encode the PR, chaperonin and polyphenolpropanoid pathway proteins (see Dixon and Lamb, 1990; Linthorst, 1992; and Ellis, 1990).

Barley seedlings were also grown under different phosphate regimes to see if phosphate availability (which is probably largely responsible for the mycorrhizal growth responses described in Chapter 3) induced increased accumulation of these

RNA transcripts. The transcripts may be produced from phosphorous metabolism-associated rather than mycorrhiza-development genes.

Non-mycorrhizal and mycorrhizal control plants, without any added phosphate, were grown as previously described in Chapter 2. A list of the treatments used in this experiment and the number of replicates for each treatment are shown in Table 6.1.

Treatments	Replicates
Non-mycorrhizal with no added phosphate (NM:P_0)	5
Non-mycorrhizal with added phosphate (0.5 mmoles/kg; NM:P_1)	5
Mycorrhizal with no added phosphate (M+:P_0)	5
NM with phosphate added (0.5 mmoles/kg) after 12 days (NM:P_{Add})	5
NM:P_0 and water-stressed	25
NM:P_0 and grown with virulent *G.graminis* (Ggt-800)	5
NM:P_0 and grown with avirulent *G.graminis* (Ggg-W2P)	5

Table 6.1
List of treatments for the experiment designed to assess the effect of infection by virulent and avirulent 'Take-All', phosphate nutrition and water stress on the expression of the genes corresponding to the cDNA clones BMR6 and BMR78.

6.3.2 Plant Growth

The *G. graminis* inoculations of plants were carried out by burying a $1cm^3$ agarose block excised from a petri dish covered with actively growing fungus of either biotype, approximately 5cm below the soil medium surface in each treatment pot (see Riggleman *et al.*, 1985) five days before transplanting of the barley seedlings. The plants were harvested 14 days after transplantation. The extent of infection in the *G. graminis* infected plants was not quantified, but extensive black discolouration (over 50% of the root length), which is associated with 'Take-all' infection, was clearly discernible on all of the root systems that were harvested. The shoots of all the plants appeared to be normal when visually compared with NM:P_0 and M+:P_0 control plants (see Chapter 3). Total RNA was then extracted from the top half of those root systems (2.7.2.1) with extensive colonisation by either the virulent and avirulent *G. graminis*

biotypes.

Twenty plants were used in the water-stress component of this experiment. All plants were set up as previously described for NM:P_0 treatments (see Chapter 3). Water-stress was applied by discontinuing daily watering to five randomly selected plants after 7/8/9 or 10 days growth. These plants were visually compared with the control plants (see above) which were watered to weight (12% w/v) every day throughout the experimental period. All of the unwatered plants became visibly water-stressed within 6 days of the discontinuation of the daily watering whilst all the control plants appeared normal at 14 days. The five plants which did not receive additional watering after 8 days growth (and had been wilted for approximately 12 hours) were harvested and the total RNA was extracted from the top half of each root system as previously described (2.7.2.1).

There were 4 phosphate treatments (again see Table 6.1). The NM:P_{Add} treatment (plants without added phosphorous but receiving phosphate in solution after 12 days growth) was an attempt to 'mimic' the changes in phosphate physiology within the root which may be brought about by a sudden increase in phosphate availability due to the rapid and extensive formation of a functional mycorrhizal root system. All plants were harvested after 14 days growth and total RNA was extracted from the top half of each root system and the shoots of the M+:P_0 plants.

6.3.3 Expression Analysis

Two sets of hybridisation membranes were prepared using total RNA (15µg/lane) isolated from plants receiving each of the treaments described above.

These were then hybridised (2.7.2.6) with radio-labelled DNA probes prepared from either pBMR6-1 or pBMR78-1 (2.7.3.11).

6.3.4 Results

The results of the Northern hybridisation experiments are shown at Figures 6.1 and 6.2. Figure 6.1 shows that the accumulation of RNA transcripts detected by pBM6-1 and pBM78 (after 7 days exposure of the membranes) was higher in the roots of the mycorrhizal control plants than in the non-mycorrhizal controls. This agrees with the results previously obtained (see Chapter 5). The accumulation of these transcripts was not increased by infection with either of the *G. graminis* biotypes. It was concluded that the genes corresponding to BMR6 and BMR78 are unlikely to belong to the group of genes which are induced by many pathogens and environmental stresses, although water-stressing appeared to increase accumulation of both the BMR6-1 and BMR78 transcripts to a small degree. This does not appear to be a result of uneven loading because the ethidium bromide staining of the original gels and the methylene blue staining of the RNA after use of these two membranes (data not shown) showed no obvious differences between lanes. The apparent increase in expression of both clones in the water stressed roots may reflect changes in the ratio of the mRNA to ribosomal RNA fractions (which is nearly all of the total RNA) brought about by dehydration of the roots. As water-stress induction of the corresponding genes cannot be ruled out, more detailed investigations are warranted.

The hybridisation membranes prepared with RNA extracted from the phosphate treatments were exposed for 10 days. These membranes did not contain a M+:P_0 control lane (the RNA sample was degraded) so direct comparisons between them and the membranes prepared with RNA from the water-stressed or pathogen-colonised roots are not possible. However, it is clear that the accumulation of BMR6 and BMR78 detectable RNA transcripts was unaffected by the three phosphate treatments used (see Figure 6.2). The results give no support to the idea that increased accumulation of either transcript is a response to increased phosphate nutrition, rather than colonisation by the fungus itself.

It was concluded that the gene expression patterns observed in this experiment for BMR6 and BMR78, support the hypothesis that the genes corresponding to these two cDNA clones are up-regulated during mycorrhiza development. Further, these genes do not appear to be involved in the general plant defence responses to pathogen-colonisation and/or environmental stresses or in phosphate uptake and metabolism.

FIGURE 6.1

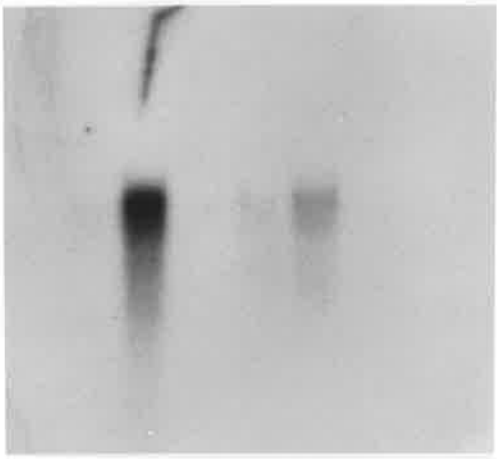
Expression analysis of BMR6 and BMR78: DNA:RNA hybridisations comparing the accumulation of mRNA transcripts in the roots or shoots of 14 day old *Hordeum vulgare* cv. 'Galleon' plants grown under various conditions.

Total RNA (15µg/lane), extracted from non-mycorrrhizal roots (lane 1), mycorrhizal roots (lane 2), avirulent*Gaeumannomyces graminis* - infected roots (lane 3), virulent *G graminis* - infected roots (lane 4), water-stressed roots (lane 5), and the shoots of mycorrhizal plants (lane 6), was size fractionated by gel electrophoresis under denaturing conditions and transferred to Hybond N+ membranes. Insert DNA was isolated from pBMR6-1 and pBMR78-1 and labelled by random priming using the Klenow fragment of DNA polymerase 1. The membranes were autoradiographed for 7 days at -80°C with intensifying screens.

The approximate sizes of the hybridising bands, shown at the right of the figure, were determined by comparing the distances of migration of the hybridising bands with those of the 28S and 18S ribosomal bands.

BM78

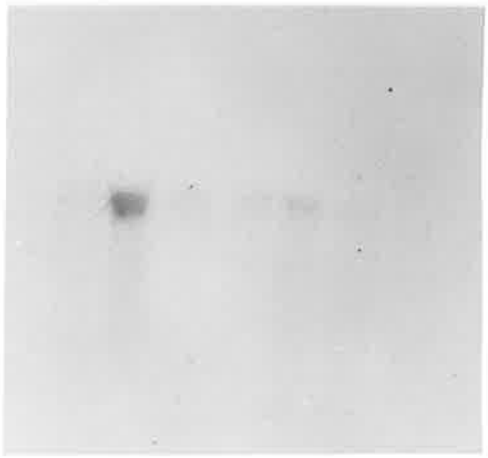
1 2 3 4 5 6



3.5 kb

BM6

1 2 3 4 5 6



1.3 kb

FIGURE 6.2

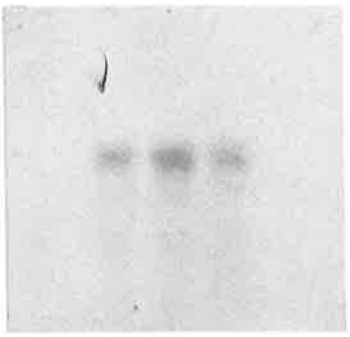
Influence of phosphate nutrition on the accumulation of mRNA transcripts detected by BMR6 and BMR78 in non-mycorrhizal roots of 14 day old *Hordeum vulgare* cv. 'Galleon'.

Total RNA (15µg/lane), extracted from the roots of plants grown without additional phosphate (P_0), grown with additional phosphate (0.5mmoles/kg) (P_1), and grown without additional phosphate but receiving phosphate after 12 days (0.5 mmoles/kg), was size fractionated by gel electrophoresis under denaturing conditions and transferred to Hybond N+ membranes. Insert DNA was isolated from pBMR6-1 and pBMR78-1 and labelled by random priming using the Klenow fragment of DNA polymerase 1. The membranes were autoradiographed for 7 days at -80°C with intensifying screens.

The approximate sizes of the hybridising bands, shown at the right of the figure, were determined by comparing the distances of migration of the hybridising bands with those of the 28S and 18S ribosomal bands.

BMR78

P_0 P_1 P_A



← 3.5 kb

BMR6

P_0 P_1 P_A

← 1.3 kb

6.4 Chromosomal Mapping of the cDNA Clones BMR6 and BMR78.

6.4.1 Aims

The aim of the series of experiments described in this section was to produce chromosome maps of barley showing the genetic positions of the genes corresponding to BMR6 and BMR78 in relation to other markers which had already been mapped. One of the long term aims of the Waite Institute cereal breeding program is to identify molecular markers linked to phenotypic traits because it is envisaged that this could reduce the time and cost involved in selecting breeding lines for further crossing and/or release.The genes encoding BMR6 and BMR78 were mapped as part of the ongoing mapping project and to further characterise these clones.

Three doubled haploid mapping populations had been generated by crossing the barley cultivars Galleon x Haruna Nijo, Clipper x Sahara 3771 and Chebec x Harrington (Langridge *et al.*, unpublished). Doubled haploids generated from these crosses (which constitute the mapping populations) were used to determine linkage groups for a large number of DNA sequences (marker probes); these marker probes were selected because they had been shown to reveal restriction fragment length polymorphisms (RFLP's), detectable by Southern hybridisation, between the parent cultivars of one or more of the above sets of doubled haploid populations. The probes were assigned to and ordered within linkage groups based on the segregation of these RFLP's in the doubled haploids. The software program MAPMAKER was used for this analysis. The ready availability of barley cv. 'Betzes' addition and ditelosomic lines (Islam *et al.*, 1981) also enabled the rapid assignment of many probes to a particular chromosome arm.

6.4.2 Genetic Mapping

The first step in localising the genes corresponding to BMR6 and BMR78 was to identify any RFLP's between the sets of parental lines which could be used for the

segregation analysis of the respective doubled haploid mapping populations. Accordingly, radio-labelled probe, prepared from either pBMR6-1 and pBMR78-1 was hybridised to duplicate membranes containing total DNA (10 μg) extracted from each of the parental lines digested with each of the following restriction endonuleases: *Eco* R1, *Dra* 1, *Hind* 111, *Bam* H1 and *Eco* RV.

Both clones detected a single band of moderate intensity in at least one of the restriction endonuclease/barley cultivar combinations that were tested (data not shown). BMR6 detected RFLP's in several of the combinations. The *Bam* H1 digestion of Clipper and Sahara 3771 genomic DNA produced an unambiguous RFLP which was chosen for the linkage analysis. BMR78 only produced one RFLP, in the *Dra* 1 digestion of 'Galleon' and Haruna Nijo. Fortunately, it was unambiguous and was selected for further analysis.

Radio-labelled probe prepared from pBMR6 was then hybridised to membranes containing *Bam* HI digested genomic DNA from each of the doubled haploids of the Clipper x Sahara 3771 cross. Similarly, radio-labelled probe prepared from pBMR78 was hybridised to *Dra* 1 digested total DNA extracted from each of the doubled haploids recovered from the Galleon x Haruna nijo cross. The autoradiograms of the membranes were then visually assessed to determine the segregation of the polymorphic bands in each doubled haploid plant. These data were then analysed using the MAPMAKER (Lander *et al.*, 1987) program.

The gene corresponding to the cDNA clone BMR78 appears to be located on the long arm of barley chromosome 2H (see Figure 6.3). The BMR78 RFLP co-segregated with many clones (not all are shown in Figure 6.3) that had been unambiguously mapped, using the wheat/barley chromosome addition and ditelosomic lines, to the long arm of chromosome 2. A similar chromosome 2H genetic map generated using the doubled haploids obtained from the Clipper x Sahara 3771 cross contained several markers (but not BMR78) which were common to both maps. A comparison of these two maps showed that the markers common to both maps were in the same linear

order and that the genetic distances between these loci were in good agreement. Moreover, BMR78 maps to a region of chromosome 2H which contains markers generated by two other mycorrhizal-root cDNA's.

The chromosomal position obtained for BMR6 was less reliable than that obtained for BMR78 because only two other probes were in the same linkage group as BMR6. This was primarily because the mapping project is being carried out in chromosome number order (ie. 1H-7H) and, at the time of this investigation, more markers had been mapped for chromosomes 1H-3H than chromosmes 4H-7H. Both of the markers linked to BMR6 generated multiple RFLP's which map to various chromosomes. However, one of the RFLP's generated by each of the linked markers do map to a common region. On this basis the gene corresponding to BMR6 was placed onto the long arm of chromosome 6H.

These data showed that the gene corresponding to BMR78 is almost certainly situated on the long arm of barley chromosome 2H and strongly suggested that the gene corresponding to BMR6 is located on the long arm of barley chromosome 6H. The locations were further investigated using the wheat/barley-chromosome addition lines generated by Islam, *et al.,* (1981). It was hoped that this would confirm the results obtained from the mapping experiments.

6.4.3 Addition Line Mapping

Total genomic DNA was extracted (2.7.3.1) from 'Chinese Spring' wheat, 'Betzes' barley and wheat barley/addition lines 1H-7H, digested with *Hind* III, and transfered to Hybond N+ hybridisation membrane. The membranes were hybridised with radio-labelled probe prepared from either pBMR6 or pBMR78.

The results of these hybridisations (see Figures 6.4 and 6.5) confirmed that the genomic sequences homologous to BMR6 and BMR78 are situated on barley chromosomes 6H, and 2H, respectively. The signal intensities of the strongly hybridising bands identified in this experiment and the mapping hybridisation

experiments indicated that these cDNA's represent the partial-sequences of single or low copy-number genes. Not surprisingly, hybridising bands were detected in the 'Chinese Spring' wheat DNA by both probes and probably represent heterologous genes present in this closely-related cereal species. Longer (12-14 day) exposure of each hydridisation membrane (not shown) identified several other bands in the digested barley DNA which weakly hybridised to both probes, indicating that the gene sequences detected by BMR6 and BMR78 may represent highly divergent multigene families.

BMR78 also detected a band in addition line 3H which did not correspond to any of the hybridising bands in 'Chinese Spring' or 'Betzes'. This band may represent the large (7.5 kb), strongly hybridising band in 'Chinese Spring' which is common to all addition lines but only weakly hybridising in addition line 3H. The polymorphism could indicate contamination of the seed stock used for genomic DNA preparation or a mutation in the 'Chinese Spring' genomic component of addition line 3H.

FIGURE 6.3

Genetic linkage maps of *Hordeum vulgare* chromosomes 2H (6.3a) and 6H (6.3b) showing the positions of BMR6 and BMR78.

The maps were generated by linkage analysis (using the MAPMAKER program) of doubled haploid populations derived from the crossing of Galleon x Haruna Nijo (for chromosome 2H) and Clipper x Sahara (for chromosome 6H).

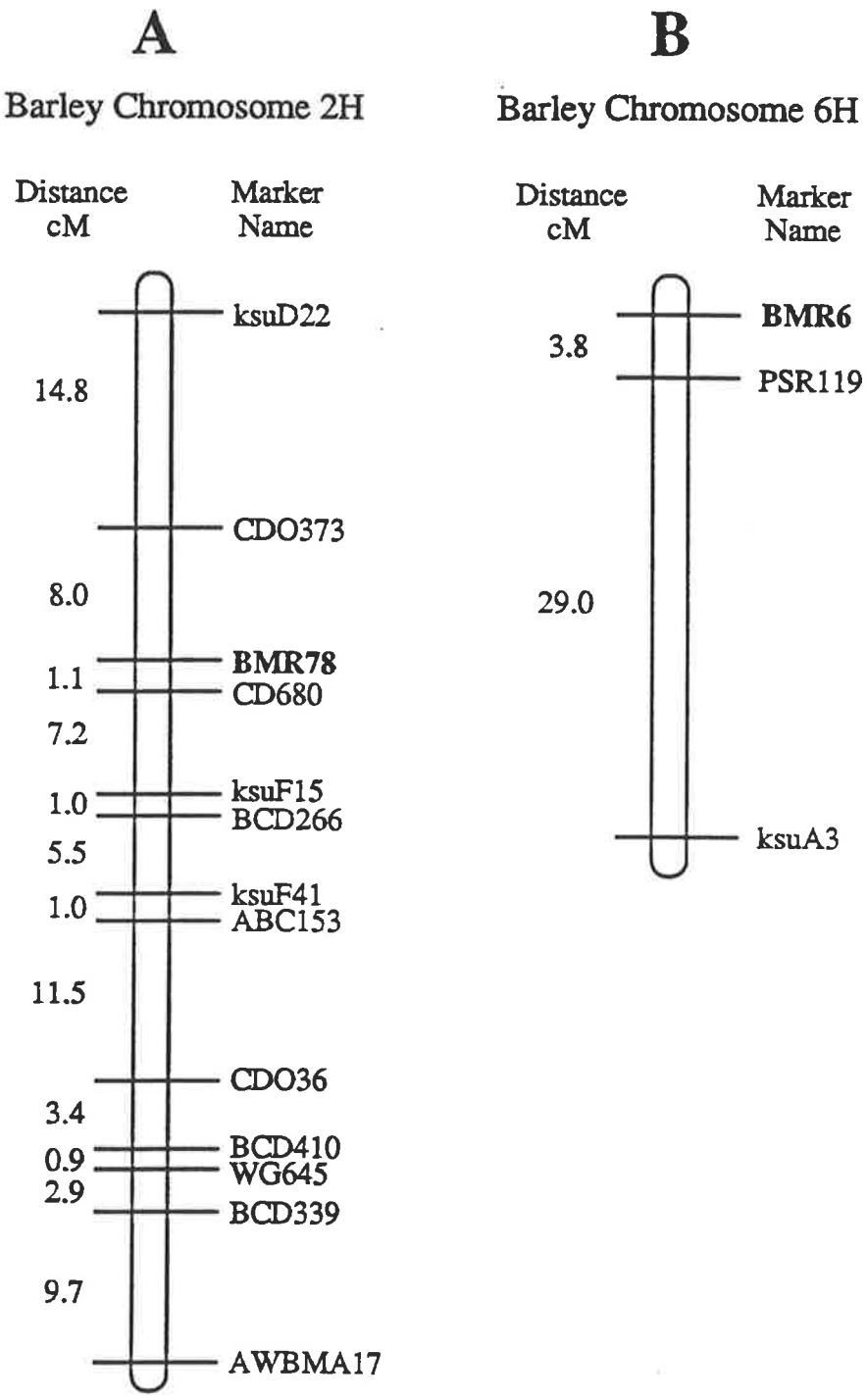

A
Barley Chromosome 2H
Distance cM
Marker Name
ksuD22
14.8
CDO373
8.0
BMR78
1.1
CD680
7.2
ksuF15
1.0
BCD266
5.5
ksuF41
1.0
ABC153
11.5
CDO36
3.4
BCD410
0.9
WG645
2.9
BCD339
9.7
AWBMA17
B
Barley Chromosome 6H
Distance cM
Marker Name
BMR6
3.8
PSR119
29.0
ksuA3

Figure 6.3

FIGURE 6.4

Chromosome mapping of BMR6 using wheat/barley addition lines 1H - 7H (Islam *et al.*, 1981).

Plant genomic DNA (10μg) from *Triticum aestivum* cv. 'Chinese Spring' (CS), *Hordeum vulgare* cv. 'Betzes' (B), and wheat/barley addition lines 1H-7H (lanes marked 1-7) was digested to completion with *Hind* III, size fractionated by gel electrophoresis (1% agarose) and transferred to Hybond N+ hybridisation membranes. The membranes were hybridised overnight with radiolabelled probes generated by random-primer labelling of insert derived from phage BMR6 by polymerase chain reaction amplification. The membranes were washed and autoradiographed for 7 days at -80°C with intensifying screens.

The hybridising bands at the far right of the figure are from the marker DNA, *Hind* 111 digested λDNA. The positions of the 6742bp and 580bp bands are indicated on the right hand side of the figure.

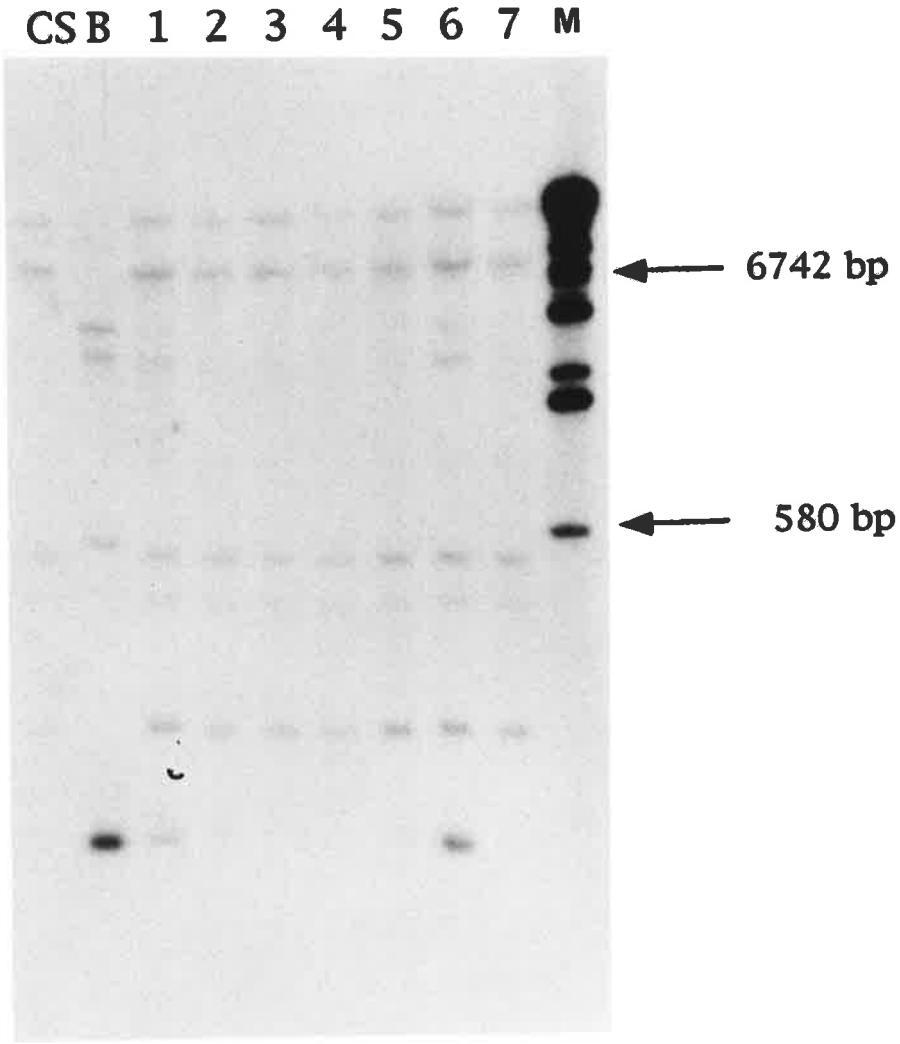
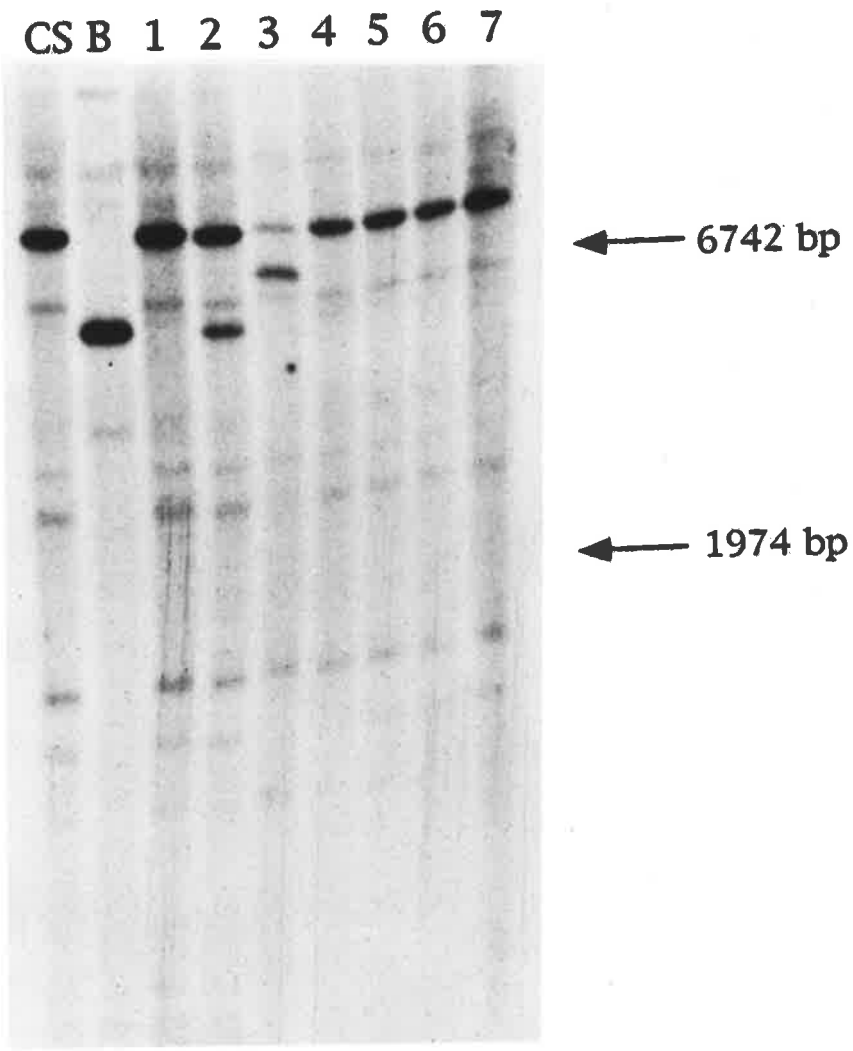

CS B 1 2 3 4 5 6 7 M
6742 bp
580 bp

FIGURE 6.5

Genetic mapping of BMR78 using wheat/barley addition lines 1H - 7H (Islam *et al.*, 1981).

Plant genomic DNA (10µg) from *Triticum aestivum* cv. 'Chinese Spring' (CS), *Hordeum vulgare* cv. 'Betzes' (B), and wheat/barley addition lines 1H-7H (lanes marked 1-7) was digested to completion with *Hind* III, size fractionated by gel electrophoresis (1% agarose) and transferred to Hybond N+ hybridisation membranes. The membranes were hybridised overnight with radiolabelled probes generated by random-primer labelling of pBMR78-1 insert DNA. The membranes were washed and autoradiographed for 7 days at -80°C with intensifying screens.

The positions of the 6742bp and 580bp bands of the marker DNA, *Hind* 111-digested λDNA, are indicated on the right hand side of the figure.


CS B 1 2 3 4 5 6 7
6742 bp
1974 bp

6.5 Sequencing of the cDNA Clones BMR6 and BMR78

6.5.1 Introduction

The aims of these experiments were to determine the DNA sequences of the cDNA clones BMR6 and BMR78. The DNA sequences and the putative protein sequences deduced from the DNA sequences could then be compared to the existing data banks. Regions of these clones with high or even moderate homology to other, better characterised genes, could provide some evidence of the biological function of the genes which correspond to BMR6 and BMR78.

6.5.2 Sequencing Reactions

The phage inserts, BMR6 and BMR78, were cloned into the plasmid vector pTZ18U (see section 6.1) primarily for ease of handling (ie. DNA replication, isolation and storage) but also because it is suitable for PCR-assisted sequencing (based on the di-deoxy method of Sanger *et al.*, 1977) using commercially available primers. The restriction maps and strategies used for sequencing (2.7.3.13) each clone are shown in Figure 6.6 and the deduced DNA sequences are shown in Figure 6.7.

6.5.3 Sequence Analyses of BMR6 and BMR78

The BLAST program (Altschul *et al.,* 1990; Gish and States, 1993) was used to compare the DNA and predicted protein sequences of BMR6 and BMR78 with existing data bases containing reported DNA and protein sequences. By comparing these sequences with known sequences, it was hoped that putative biological functions could be ascribed to each of these clones.

The first 250 bases of BMR78 proved to be highly homologous (over 80%) at the DNA level to the 3' coding sequences of several reported proton (H+) ATPases (Harper *et al.*, 1989; Pardot and Serrano, 1989; Ewing *et al.*, 1989; Harper *et al.*, 1990 and Wada *et al.*, 1992) The predicted protein sequence of this DNA region (approximately 80 residues) showed even higher homologies with known H+-

ATPases (Figure 6.8). The sequence 3' distal to this region of high homology appears to be non-coding. It contains an in-frame stop codon and the protein homology between the sequence and the reported H+-ATPases abruptly stops at this point .

The BLAST search did not locate any homology between the DNA sequence of BMR6 and any well characterised genes. However, two regions of the putative protein encoded by this cDNA show homology (55-65% positives) to a scenescence-related protein from radish, *din* 1 and a stress-related protein from *E. coli* , *psp* E, both of which are induced under a number of environmental conditions (Azumi and Watanabe, 1991; Brissette *et al.*, 1991).

FIGURE 6.6

Restriction mapping and sequencing strategies for BMR6 and BMR78.

The phage inserts were cloned into the *Eco* R1 site of pTZ18U. Restriction maps were prepared by endonuclease-digestion analysis of pBMR6 and pBMR78. The inserts of the original clones and sub-clones containing various restriction fragments were sequenced as indicated. The arrows indicate the direction and extent of each sequencing reaction.

E = Eco R1, R = Rsa 1, P = Pst 1, S = Sac 1 and X = Xba 1.

Figure 6.6

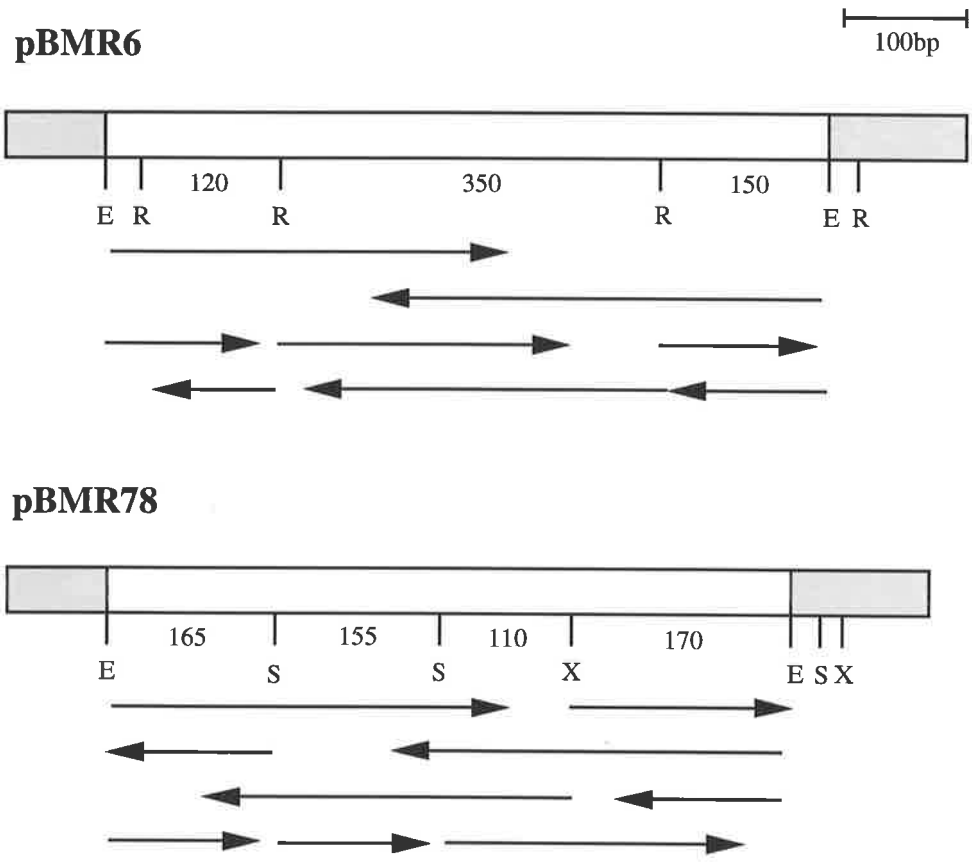

pBMR6
100bp
120
350
150
E R
R
R
E R
pBMR78
165
155
110
170
E
S
S
X
E S X

FIGURE 6.7

The nucleic acid and predicted protein sequences of BMR6 and BMR78.

The predicted protein sequence is shown under the open reading frame for each clone and the stop codon in each sequence is marked with an asterisk (*). Putative poly-adenylation signal sequences (AATAA) in the untranslated 3' regions of each clone are underlined as is a putative C-terminal phosphorylation site (see Harper *et al.*, 1990) in the predicted protein sequence of BMR78.

BMR6 (657 bp)

```
CCT CTG GAG AGC ACC ACA TAC GGA ACC GGA AGC TCT TTC CTC CCC GGT
P   L   E   S   T   T   Y   G   T   G   S   S   F   L   P   G

GCT CAA TCC TCC ATA GAT GGG CTC CCT GCG AAG ACA GTG AGC ACG GCG
A   Q   S   S   I   D   G   L   P   A   K   T   V   S   T   A

GTG GAC AGC GTG GAC GCG GAG GCG GCG TGC GCA CTG CTG GCC TTG GAG
V   D   S   V   D   A   E   A   A   C   A   L   L   A   L   E

CAG TAC GGG TAC GTG GAC GTG CGG ATG TGG GAG GAC TTC GAA AAG GGT
Q   Y   G   Y   V   D   V   R   M   W   E   D   F   E   K   G

CAC GTT GCC GGT GCA CGC AAC GTG CCC TAC TAC CTG TCC GTC AGC GCC
H   V   A   G   A   R   N   V   P   Y   Y   L   S   V   S   A

CCA TGG CAA GGA GCG CAA CCC GGA CTT CGG TCG ATC AGG TTG CGG CGC
P   W   Q   G   A   Q   P   G   L   R   S   I   R   L   R   R

TCC ACT CCA AGG AGG ACC GGT TCC TTG TTG GTT GTC GCT CTG GGG TCC
S   T   P   R   R   T   G   S   L   L   V   V   A   L   G   S

GGT CCA GGC TCG CCA CCG CAG ACC TTG TCG CCG CTG GAT TCG CAA ACG
G   P   G   S   P   P   Q   T   L   S   P   L   D   S   Q   T

TGA AGA ACC TCG AAG GCG GCT ACC TCT CCT TGC TCA AGA GCG CAA GTT
*

ACC AAG CAG CCA GCC ACC GAT GAT CCA TCA ATT GAT TCT TTG GAG TTT
GGA AAG TTT GTT AGT GAC CTG TTT GTG TAC TAT GTC TCT GTC ATC CTT
CGC CTT ATG AAG AAG AAG AAG AAG AAG CAA GTC CTT TAG CTG AGG TGC
ACG GCT GGG ATT GAA TGT AGT TCA AGG GGA AAA CAA GGC ATT ATG TAT
GTA GGT ACG AAT AAT GGA ACT GCG GAA AAA AAA A
```

BMR78 (570 bp)

```
AAA GGT GAG AGG GAG GCA CAG TGG GCT ACC GCA CAG AGA ACA CTT CAT
K   G   E   R   E   A   Q   W   A   T   A   Q   R   T   L   H

GGC CTT CAA GCA CCT GAG CCG GCT TCC CAC ACA CTC TTC AAC GAC AAG
G   L   Q   A   P   E   P   A   S   H   T   L   F   N   D   K

AGC AGC TAC CGT GAG CTT TCT GAG ATC GCT GAG CAA GCC AAG AGA AGA
S   S   Y   R   E   L   S   E   I   A   E   Q   A   K   R   R

GCT GAG ATT GCA AGG TTG AGG GAG CTC AAC ACA CTC AAG GGG CAC GTT
A   E   I   A   R   L   R   E   L   N   T   L   K   G   H   V

GAG TCC GTG GTG AAG CTC AAG GGC CTT GAC ATC GAC ACC ATC AAC CAA
E   S   V   V   K   L   K   G   L   D   I   D   T   I   N   Q

AAC TAC ACC GTG TGA GGA ATT TGG GCA TTG GAC CAA ACA ACA TGA CAT
N   Y   T   V   *

CCG TAT TGG AGC GAG ATT GGA ATG GAG CTC CGC TTT AAG ATA TTT CCG
GTG CAT GAA TGA AAG AAA CAT GGC TGA TCT GAC CAG CCC CTA GTT GTA
AAG TGC TAC CAA ATA TAT GGT TTC GGT TGT CCC TCT AGA GAG GTT TTA
ATG TCT AGT GGA GTT ATA CCG TGT TTG GAG CAC GCG TGG TGT AAT CGG
TTT ACC CTT TCC CCT AAC TAG TCG GTG ATC TTG CGG AAG CAA GAC GAG
AAA TAA ACA CTG CAA ATT TTC TGC CAA AAA AAA AAA AAA AAA
```

FIGURE 6.8

Alignments and summary of homologies with the predicted proteins of the BMR78 (Figure 6.8a) and BMR6 (Figure 6.8b) cDNA sequences.

The translations, alignments and retrieval of homologous sequences were carried out using the BLAST (Altschul *et al.*, 1990) program. The predicted protein sequence of BMR78 shows 80-90% homology with the C-terminal region of all known plant ATPases. BMR6 has two regions which have homologies (55-65%) to genes from *E.coli* and radish.

Figure 6.8a

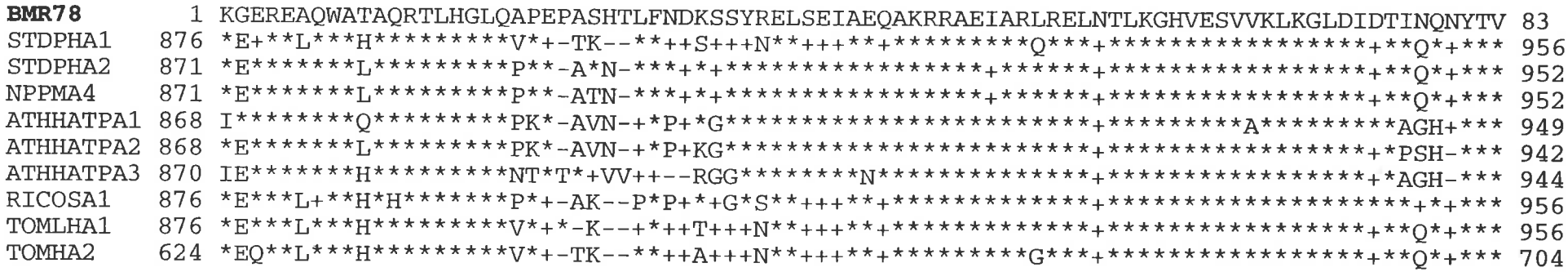
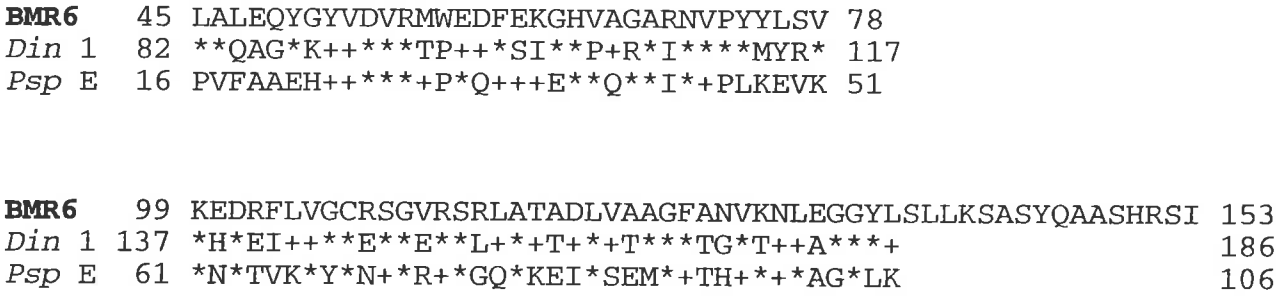


Figure 6.8b



6.6 Discussion

These results provide evidence that the cDNA clones BMR6 and BMR78 detect root-specific, low-copy plant genes which are specifically upregulated during the establishment of the mycorrhizal symbiosis with *G. intraradices* and not by general biotic or abiotic conditions.

The doubled haploid and addition line mapping experiments have placed the genes represented by BMR6 and BMR78 on barley chromosomes 6H and 2H respectively. BMR78 and AWBM21 (another RFLP marker which was isolated from the M+ barley root cDNA library) map to a region of 2H near the *Ha* 2 gene (A. Karakousis *et al.*, unpublished data) which confers resistance to a cereal cyst nematode (CCN). At a purely applied level, these two markers could prove useful in experiments aimed at isolating the resistance gene. At the very least these cDNA clones provide further coverage of the barley genetic map and could represent useful markers for CCN resistance.

There seems little doubt that BMR78 represents the partial sequence of a H+-ATPase and is the first such sequence isolated from *H. vulgare*. This clone detects root-specific mRNA transcripts which increase in abundance during mycorrhizal development. These results support the data provided by enzymatic and immunolabelling experiments which show that membrane-bound ATPase activity is increased in the host plant-arbuscule interface (Marx *et al.*, 1982; Gianninazzi-Pearson *et al.*, 1991) and add further weight to the argument that this interface is involved in the active transport of solutes between the symbiotic partners (see Smith and Smith, 1990) and as such, may play a specific role in the development and/or maintenance of the symbiosis.

Analyses of the complete-gene sequences for proton ATPases from *Nicotiana plumbaginofolia, Lycopersicon esculentum* and *Arabidopsis thaliana,* show them to be multigene families which are highly conserved both within and between species

(Boutry *et al.,* 1989; Ewing *et al.*, 1990; Harper *et al.*, 1990). *A. thaliana,* the best characterised of the H+-ATPase families, contains at least 10 isoforms of the gene, some of which are tissue specific (Harper *et al.,* 1994; Houlné and Boutry, 1994). The mapping experiments provide some evidence that the gene corresponding to BMR78 may be a member of a multigenic family in which case the specificity of expression of this gene (and any other isoform) needs to be more completely assessed.

Reprobing 20000 clones from the M+ library (as for the differential screening described in Chapter 5) with radiolabelled probe made from BMR78, identified 12 strongly hybridising phage clones and 7 weakly hybridising phage clones. PCR amplification and subsequent *Xba* 1 restriction analysis of six phage from each group indicated that the twelve strongly hybridising phage were other clones of or very similar to BMR78 whilst the seven weakly hybridising phage were a different sequence (results not shown). These results remain inconclusive in the absence of a more detailed analysis of these clones but nevertheless indicate that at least two types of proton ATPases may be expressed in the roots of mycorrhizal barley roots. This raises the question of whether BMR78 represents an isoform of the gene which is specifically upregulated during the development of mycorrhizas. There is also a possibility that the constitutive expression observed in the original Northern hybridisations may reflect cross-hybridisation between BMR78 and different root H+-ATPase genes, all of which produce mRNA transcripts of approximately the same length. These are both important and interesting questions which warrant further detailed and more specific attention.

The characterisation of BMR6 yielded far less information. It has two virtually continuous regions of homology with genes isolated from radish and *E. coli* which can be induced by a range of stress-like conditions. These two regions from BMR6 are separated by 21 amino acid residues. Similarly, the regions of homology identified in the *din* 1 and *psp* E genes are only separated by 2O and 10 amino acid residues, respectively (see Figure 6.8). As water-stress conditions and pathogen-infection did

not greatly induce accumulation of the transcripts detected by this cDNA sequence (see Figure 6.1), the gene BMR6 represents is not thought to be a general stress-induced or defence-related gene. As such, these two regions of homology may indicate a common secondary or tertiary structural feature of the three gene products rather than a common functional region.

The abundance of gene transcripts detected by BMR6 does not appear to greatly increase in response to either pathogen infection or water-stress. It is possible, indeed likely, that BM6 detects the RNA transcripts of a gene which is induced purely in response to the mycorrhiza colonisation process and that it is not a symbiosis-related gene actively involved in the developmental process itself. Further and more extensive expression analyses using RNA extracted from various barley tissues grown under a range of environmental conditions may provide better evidence of the biological function of the gene product. The biological significance of the gene remains unclear.

Chapter 7

General Discussion

7.1 Model plant-fungus systems for investigating mycorrhizal symbioses.

Mycorrhizal symbioses are the culmination of a complex sequence of events which are genetically determined by both the plant and fungal partners. Elucidating the molecular genetic events which result in the formation of a compatible (ie. mutualistic) mycorrhizal association would enrich our understanding of these symbioses.

VA mycorrhizal fungi are obligate symbionts and efforts to culture them *in vitro* have been only partially succesful. At present these interactions can only be studied using model plant/fungus systems grown in some type of pot culture. The VA mycorrhizal system developed during the course of this work used *Hordeum vulgare* (barley) cv. Galleon as the host plant and *Glomus intraradices* as the fungal partner.

Molecular analyses are extremely sensitive in comparison with many biochemical and enzymatic methods but still have finite limits of detection. This work (and that of Baon *et al.*, 1994) showed that considerable phenotypic variation exists between local South Australian barley cultivars with respect to the rate and extent of root colonisation by Glomales species. This is in agreement with published data for several other plant species (see Smith, Robson and Abbott, 1992) and was an important consideration because the success of the approach described in this work was dependant upon being able to reliably produce substantial quantities of heavily-colonised mycorrhizal root tissue. This was difficult to achieve over the whole barley root-system, but root tissues 'enriched' for the early mycorrhizal colonisation stages

were obtainable by manipulating the levels of inoculum in the pot system and studying the time-course and distribution of colonisation. Some recent work by Rosewarne *et al.* (pers. comm.) with tomato seedlings indicates that a 'nurse plant' pot culture system may be an easier and more effective means of producing large amounts of early-stage colonised VA mycorrhizas.

Barley has a fast growing root system which produces sufficient quantities of RNA for the types of analyses undertaken in this work. The extent of colonisation using this model system was highly reproducible within any given batch of inoculum. However, variation did exist between batches of inoculum. Some produced poor levels of colonisation in 14 day old barley seedlings and were immediately rejected to be used only for inoculum production. The wastage of several inoculum batches was not a problem because a large amount of inoculum was available (courtesy of Dr. S. Smith and S. Dixon) but does need to be considered if the resources available are more limited.

7.2 Towards identifying *Myc* mutants.

The importance of identifying and characterising plant phenotypes defective in mycorrhiza formation or function cannot be overstated (see Koornneef, 1991 and Kyla, 1994). They are the key to elucidating many of the developmental events, at both the molecular and physiological levels, that lead to a successful mutualistic interaction. Considerable research effort has gone into developing strategies (such as gene-tagging) which simplify either the generation of mutations or more importantly, the subsequent isolation of mutant genes from plants. A major problem still remains (and it is emphasised by this work; obtaining sufficient resources (chiefly human) to produce and analyse the large number of plants required to have a high probability of success.

Screening for barley-mycorrhiza mutants was unsuccessful largely because only 1800 plants from the two mutagenised 'Galleon' populations were screened. Nutritional screening for non-functional mycorrhizal status was attempted but was

unsuccessful. A large percentage of plants from all the treatment groups (ie. with or without added phosphorus and/or mycorrhizal inoculum) showed P deficiency-like symptoms which remained till the time of harvest (3-4 weeks). It is presumed that the fast growth rate of barley roots (and thus requirement for P) outstripped the extra P made available by having mycorrhizal roots. As a consequence, the roots of each seedling had to be individually assessed for extent and type of mycorrhizal colonisation using a microscope.

Many of the mycorrhizal and phosphate-amended plants grown in pots containing 1kg of soil medium (see Chapter 1) did recover their normal colour after initially displaying P deficiency-like chlorosis. However, large-scale nutritional screening for *myc-* mutants under these conditions would require several tonnes of soil medium for each experiment as well as large areas of growth room space for an extended period of time (4-6 weeks). The resources required to set up and manage screening experiments on this scale are not readily available.

Individual screening of a large number of plants for mycorrhizal mutant phenotypes is obviously expensive in terms of time and resources and is unlikely to be directly funded. Therefore, it is difficult to see much progress being made in this area of mycorrhizal research other than by serendipidy (much like the *nod- /myc-* mutations described by Duc *et al.*, 1989) unless a quick and simple assay (possibly nutritional) can be devised which allows the screening of many thousands of plants at once. In hindsight, barley may not be the best plant for this type of approach. A slower-growing host plant, suitable for genetic manipulation and analysis, coupled with an aggressive *Glomales* species may provide a more amenable model system for the detection of plant mutants defective in mycorrhiza development and/or function.

7.3 Molecular approaches to cloning functional mycorrhiza genes

These experiments have shown that it is practicable to use molecular approaches to investigate the mycorrhizal symbiosis. Over 1000 putative clones were detected after

the initial diffrential screening, this was subsequently reduced to 121 after a second round of screening. DNA:RNA hybridisation analysis of 46 of these clones identified four cDNA clones that showed changes in expression patterns which correlated with colonisation of roots by *Glomus intraradices*. Two of these mRNA species are down-regulated (decreased accumulation) and two are up-regulated (increased accumulation) during mycorrhizal development.

The sequence analysis of the two upregulated clones indicates that the putative protein of BMR6 may be a stress-related protein and that BMR78 is highly homologous to a multigenic family of proton ATPases isolated from a range of plant species. These results are not surprising as there is considerable physiological and biochemical evidence which shows plant pathogenic reactions and increased ATPase activity occurs in response to mycorrhizal colonisation. Whether these clones are essential to the formation and maintenance of a fuctional mycorrhiza is not known but they may prove useful in monitoring and defining certain stages of colonisation.

A clearer picture of the temporal and spatial expression of the two genes that BMR6 and BMR7 represent may emerge if the clones are used for *in situ* hybridisation experiments. Similarly, purification of the proteins (in an *Escherechia coli* expression system) and preparation of antibodies should allow precise localisation of the proteins within root sections. It may also be possible to use transgenic approaches to 'knockout' or overexpress genes in barley and assess the effects on plant/VA mycorrhiza phenotype.

More extensive cloning and screening should yield additional sequences of interest. A targeted approach, using cloned sequences from other organisms as heterologous probes, should also provide further basic information on the interaction as well as producing additional probes for monitoring developmental stages.

7.4 Concluding statements

This is the first time an untargeted cloning approach has been used to investigate the VA mycorrhizal symbiosis at the molecular level. Whilst the mutagenesis

experiment failed to identify any obviously useful phenotypes, it nevertheless demonstrated that screening for *myc-* mutations is feasable as it appears that a large number of genes may have an effect on the development of mycorrhizas. Principally, this work has provided good molecular-genetic evidence that plant-root gene expression is altered during the ontogeny of these specialised structures. The barley/*Glomus* model system which was developed could provide a basis for future studies of this interaction using other plant-VA mycorrhizal fungus combinations.

Mycorrhizas appear to be important in the nutritional dynamics of nearly all ecosystems. Studying the symbiosis in the intimate detail afforded by molecular analyses may eventually allow manipulation of VA mycorrhizal fungi at the agricultural field level. More importantly though, it may give us considerable insight into the evolution of plant-micobial interactions and the mechanisms underlying cellular communication. This work represents a small but important first step.

References

Abbott L.K., Robson A.D. and de Boer G. (1984). The effect of phosphorus on the formation of hyphae in the soil by the vesicular-mycorrhizal fungus *Glomus fasiculatum* . New Phytol. 97: 437-446

Abu-Abied M. and Holland D. (1994). The gene *c-ino1* from *Citrus paradisi* is highly homologous to *tur1* and *ino1* from yeast and *Spirodela* encoding for *myo*-inositol phosphate synthase. Plant Physiol. 106: 1689

Alexander T., Toth R., Meier R. and Weber H.C. (1989). Dynamics of arbuscule development and degeneration in onion, bean and tomato with reference to vesicular-arbuscular mycorrhizae with grasses. Can. J. Bot. 67: 2505-2513

Allen M.F. (1982). Influence of vesicular-arbuscular mycorrhizae on water movement through *Bouteloua gracilis* (HBK) Lag x Steud. New Phytol. 91: 191-196

Allen M.F., Moore T.S. and Christensen M. (1980). Phytohormone changes in *Bouteloua gracilis* infected by vesicular-arbuscular mycorrhizae. I. Cytokinin increases in the host plant. Can. J. Bot. 58: 371-374

Allen M.F., Smith W.K., Moore T; and Christensen M. (1981). Comparative water relations and photosynthesis of mycorrhizal and non-mycorrhizal *Bouteloua gracilis* (HBK) Lag x Steud. New Phytol. 88: 683-693

Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. (1990). Basic local alignment search tool. J. Mol. Biol. 215: 403-410

Ames R.N., Porter L., St. John T.V. and Reid C.P.P. (1984). Nitrogen sources and 'A' values for vesicular-arbuscular and non-mycorrhizal sorghum grown at three rates of ^{15}-N ammonium sulphate. New Phytol. 97: 269-276

Ames R.N., Reid C.P.P., Porter L. and Cambardella C. (1983). Hyphal uptake and transport of nitrogen from two ^{15}-N-labelled sources by *Glomus mosseae*, a vesicular-arbuscular fungus. New Phytol. 95: 381-396

Amijee F., Tinker P.B. and Stribley D.P. (1989). The development of endomychorrizal root systems: VII. A detailed study of effects of soil phosphorous on colonisation. New Phytol. 111: 435-446

Anderson A.J. (1988). Mycorrhizae-host specificity and recognition. Phytopath. 78: 375-378

Arnheim N. and Erlich H. (1992). Polymerase chain reaction strategy. Ann. Rev. Biochem. 52: 131-155

Azcon R. and Ocampo J.A. (1981). Factors affecting the vesicular-arbuscular infection and mycorrhizal dependency of thirteen wheat cultivars. New Phytol. 87: 677-685

Azcon R., Marin A.D. and Barea J.M. (1978). Comparative role of phosphate in soil or inside the host on the formation and effects of endomycorrhiza. Plant Soil 49: 561-567

Azumi Y. and Watanabe A. (1991). Evidence for a scenescence-associated gene induced by darkness. Plant Physiol. 95: 577-583

Baker B., Schell J., Lorz H. and Federoff N. (1986). Transposition of the maize controlling element "Activator" in tobacco. Proc. Natl. Acad. Sci. (USA) 83: 4844-4848

Balcells B., Swinburne J., Coupland G. (1991). Transposons as tools for the isolation of plant genes. Trends in Biotech. 9: 31-37

Baon J.B., Smith S.E. and Alston A.M and Wheeler R.D. (1992). Phosphorus efficiency of three cereals as related to indigenous mycorrhizal infection. Aust. J. Agric. Res. 43: 479-491

Baon J.B., Smith S.E. and Alston A.M. (1993). Mycorrhizal responses of barley cultivars differing in P efficiency. Plant and Soil 157: 97-105

Bartchi H., Gianinazzi-Pearson V. and Vegh I. (1981). Vesicular-arbuscular mycorrhiza formation and root rot disease (*Phytophthora cinnamoni*) development in *Chaemaecyparis lawsoniana.* Phytopath. Z. 102: 213-218

Bauer W.D. (1981). Infection of legumes by rhizobia. Ann. Rev. Plant Physiol. 32: 407-449

Bécard G. and Piché Y. (1989). New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus *Gigaspora margarita.* New Phytol. 112: 77-83

Bécard G. and Piché Y. (1990). Physiological factors determining vesicular-arbuscular mycorrhizal formation in host and nonhost Ri T-DNA transformed roots. Can. J. Bot. 68: 1260-1264

Bécard G. Douds D.D. and Pfeffer P.E. (1992). Extensive in vitro hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO2 and flavanols. Appl. Envir. Microbiol. 58: 821-825

Beckman J.S. and Soller M. (1990). Toward a unified approach to genetic mapping of eukaryotes based on sequence tagged microsatellite sites. Bio/Technology 8: 930-932

Bel Rhid R., Chabot S., Piché Y. and Chênevert R. (1993). Isolation and identification of flavanoids from Ri T-DNA-transformed roots (*Daucus carota*) and their significance in vesicular-arbuscular mycorrhiza. Phytochem. 33: 1369-1371

Berta G., Sgorbati S., Soler V., Fusconi A., Trotta A., Citterio A., Bottone M.G., Sparvoli E. and Scannerini S. (1990). Variations in chromatin structure in host nuclei of a vesicular arbuscular mycorrhiza. New Phytol. 114: 199-205

Bevege D.I. and Bowen G.D. (1975). *Endogone* strain and host plant differences in development of of vesicular-arbuscular mycorrhizas. In: Endomycorrhizas; Sanders F.E., Mosse B. and Tinker P.B. (eds.), Academic Press, London and New York. pp 77-86

Bhuvaneswari T.V. and Solheim B. (1985). Root hair deformations in the white clover / *Rhizobium trifolii* symbiosis. Physiol. Plant. 63: 25-34

Bhuvaneswari T.V., Turgeon G.B. and Bauer W.D. (1981). Early events in the infection of soybeans (*Glycine max* L. Mer). Plant Physiol. 66: 1027-1031

Bieleski R.L. and Ferguson I.B. (1983). Physiology and metabolism of phosphate and its compounds. In: Encyclopaedia of Plant Physiology, Vol. 15A: Inorganic Plant Nutrition; Lauchli A. and Bielesky R.L. (eds.), Springer Verlag, Berlin. pp 422-449

Bolton G.W., Nester E.W. and Gordan M.P. (1986). Plant phenolic compounds induce the expression of the *Agrobacterium tumefaciens* loci needed for virulence. Science 232: 983-985

Bonfante-Fasolo P. (1987). Vesicular-arbuscular mycorrhizae: Fungus-plant interactions at the cellular level. Symbiosis 3: 249-268

Bonfante-Fasolo P. (1988). The role of the cell wall as a signal in mycorrhizal associations. In: Cell to Cell Signals in Plant, Animal and Microbial Symbioses; S. Scannerini, D. Smith, P. Bonfante-Fasolo, V. Gianinazzi-Pearson (eds.), Springer-Verlag, Berlin, Heidelberg. pp 64-78

Bonfante-Fasolo P. (1988). The role of the cell wall as a signal in mycorrhizal associations. In: Cell to Cell Signals in Plant, Animal and Microbial Symbiosis; Springer-Verlag Berlin Heidelberg. pp 219-235

Bonfante-Fasolo P. and Grippiolo R. (1982). Ultrastructural and cytological changes in the wall of a vesicular-arbuscular mycorrhizal fungus during symbiosis. Can. J. Bot. 60: 2303-2312

Bonfante-Fasolo P., Dexheimer J., Gianinazzi-Pearson V. and Scannerini S. (1981). Cytochemical modifications in the host-fungus interface during intracellular interactions in vesicular-arbuscular mycorrhizae. Plant Sci. Lett. 22: 13-21

Bonfante-Fasolo P., Vian B., Perotto S., Faccio A. and Knox J.P. (1990). Cellulose and pectin localisation in roots of mycorrhizal *Allium porum*: labelling between the host cell wall and interfacial material. Planta 180: 537-547

Bonfante-Fasoslo P. and Spanu P. (1992). Pathogenic and endomycorrhizal associations. In: Methods in Microbiology Volume 24. Academic Press Ltd. London. pp 141-169

Bonnet H.T., Djurberg I., Fajardo M. and Glimelius K. (1993). A mutation causing variegation and abnormal development in tobacco is associated with an altered mitochondrial DNA. Plant J. 3: 519-525

Borojevic K., Gottschallk W. and Micke A. (1977). Factors influencing the mutant spectrum and quality of mutants. In: Manual on Mutation Breeding, IAEA, Vienna. pp. 146-150

Boutry M., Michelet B. and Goffeau A. (1989). Molecular cloning of the family of plant genes encoding a protein homologous to plasma membrane H+-translocating ATPases. Biochem. Biophys. Res. Commun. 162: 567-574

Bowles D.J. (1990). Defense-related proteins in higher plants. Ann.Rev. Biochem. 59: 873-907

Bowman J.L., Smyth D.R. and Meyerowitz E.M. (1989). Genes directing flower development in *Arabidopsis.* Plant Cell 1: 37-52

Boyetchko S.M. and Tewari J.P. (1990). Root colonisation of different hosts by the vesicular-arbuscular mycorrhizal fungus *Glomus dimorphicum.* Plant Soil 129: 131-136

Bradbury S.M., Peterson R.L. and Bowley S.R. (1991). Interactions between three alfalfa nodulation genotypes and two *Glomus* species. New Phytol. 119: 115-120

Briggs R.W. and Constantin M.J. (1977). Radiation types and radiation sources. In: Manual on Mutation Breeding, IAEA, Vienna, pp. 7-21

Brissette J.L., Weiner L., Ripmaster T.L. and Model P. (1991). Characterisation and sequence of the *Escherichia coli* stress-induced *psp* operon. J. Mol. Biol. 220: 35-48

Brown T.A. (1991). Molecular Biology: Labfax. Bios Scientific Publishers, Blackwell Scientific Publications, Oxford.

Brundrett M.C., Piché Y. and Peterson R.L. (1985). A developmental study of the early stages in vesicular-arbuscular mycorrhizal formation. Can. J. Bot. 63: 184-194

Bryla D.R. and Koide R.T. (1990). Role of mycorrhizal infection in the gowth and reproduction of wild vs.cultivated plants II. Eight wild accessions and two cultivars of *Lycopersicon esculentum* Mill. Oecologia 84: 82-92

Cabrera y Poch H.L., Peto C.A. and Chory J. (1993). A mutation on the *Aribidopsis DET3* gene uncouples photoregulated leaf development from gene expression and chloroplast biogenesis. Plant J. 4: 671-682

Caetano-Anollés G. and Gresshoff P.M. (1992). Plant genetic suppression of the non-nodulating phenotype of *Rhizobium meliloti* host range nodH mutants: gene-for-gene interaction in the alfalfa-*Rhizobium* symbiosis? Theor. App. Genet. 84: 624-632

Carling D.R., Riehle W.G., Brown M.F. and Johnson D.R. (1978). Effects of a vesicular-arbuscular mycorrhizal fungus on nitrate reductase and nitrogenase activities in nodulating and non-nodulating legumes. Phytopath. 68: 1590-1596

Carr G.R., Hinkley M.A., Le Tacon F., Hepper C.M., Jones M.G.K. and Thomas E. (1985). Improved hyphal growth of two species of vesicular-arbuscular mycorrrhizal fungi in the presence of suspension cultured plant cells. New Phytol. 101: 417-426

Cervone F., Castoria R., Spanu P. and Bonfante-Fasolo P. (1988). Pectinolytic activity in some ericoid mycorrhizal fungi. Trans. Br. Mycol. Soc. 91: 537-539

Chen J. and Varner J.E. (1985). Isolation and characterisation of cDNA clones for carrot extensin and a proline-rich 33-kDa protein. Proc. Natl. Acad. Sci. (USA) 82: 4399-4403

Christou P., Ford T.L. and Kofron M. (1992). The development of a variety-independant gene-transfer method for rice. Trends in Biotech. 10: 239-246

Collinge D.B. and Slusarenko A.J. (1987). Plant gene expression in response to pathogens. Plant Mol. Biol. 9: 389-410

Cooke R.J. (1988). The use of electrophoresis for the distinctness testing of varieties of autogamous species. Plant Var. Seeds 2:3-13

Cooper K. (1985). Physiology of VA mycorrhizal associations. In: VA Mycorrhizas; C.L. Powell and D.J. Bagyaraj (eds.), CRC Press, Boca Raton, Florida. pp 165-183

Cordignola A., Verotta L., Spanu P., Maffei M., Scannerini S. and Bonfante-Fasolo P. (1989). Cell wall bound-phenols in roots of vesicular-arbuscular mycorrhizal plants. New Phytol. 112: 221-228

Cove D.J. (1993). Mutant analysis, a key tool for the study of metabolism and development. Plant J. 3: 303-308

Cox G. and Sanders F.E. (1974). Ultrastructure of the host-fungus interface in vesicular-arbuscular mycorrhiza. New Phytol. 73: 901-912

Cox G. and Tinker P.B. (1976). Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. I. The arbuscule and phosphorus transfer: a quantitative ultrastructural study. New Phytol. 77: 371-378

Cress W.A., Throneberry G.O. and Lindsey D.L. (1979). Kinetics of phosphate absorption by mycorrhizal and non-mycorrhizal tomato roots. Plant Physiol. 64: 484-487

Crush J.R. and Carradus J.R (1980). Effect of mycorrhizas on growth of some white clovers. N.Z. J. Agric. Res. 23: 233-237

Daniels B.A. and Graham S.O. (1976). Effects of nutrition and soil extracts on germination of *Glomus mosseae* spores. Mycologia 68: 108-116

Darlington C.D. and McLeish J. (1951). Action of maleic hydrazide on the cell. Nature (London) 167: 407-408

Darnell J.E. (1982). Variety in th level of gene control in eukaryotic cells. Nature (London) 285: 365-371

Davidson A.D., Manners J.M., Simpson R.S. and Scott K.J. (1987). cDNA cloning of mRNAs induced in resistant barley during infection by *Erysiphe graminis* f.sp. *Hordei.* Plant Mol. Biol. 8: 77-85

Davies E. and Schuster A. (1981). Intercellular communication in plants: Evidence for a rapidly generated, bidirectionally transmitted wound signal. Proc. N. Acad. Sci. (USA) 78: 2422-2426

De Pater S., Hensgens L.A.M. and Schilperoort R.A. (1990). Structure and expression of a light-inducible shoot-specific rice gene. Plant Mol. Biol. 15: 399-406

Dénaré J., Debellé F. and Rosenberg C. (1990). Signalling and host range variation in nodulation. Ann. Rev. Microbiol. 46: 497-531

Dexheimer J., Gianinazzi S. and Gianinazzi-Pearson V. (1979). Ultrastructural cytochemistry of the host-fungus interface in the endomycorrhizal association *Glomus mosseae/Allium cepa.* Z. Pflanzenphysiol. 92: 191-206

Dixon R.A. and Lamb C.J. (1990). Molecular communication in interactions between plants and microbial partners. Ann. Rev. Plant Physiol. Plant Mol. Biol. 41: 339-367

Dixon R.A., Harrison M.J. and Lamb C.J. (1994). Early events in the activation of plant defence responses. Ann. Rev. Phytopath. 32: 479-501

Djordjevic M.A., Gabriel, D.W. and Rolfe, B.G. (1987). Rhizobium - the refined parasite of legumes. Ann. Rev. Phytopath. 25: 145-168

Duc G., Trouvelet A., Gianinazzi-Pearson V. and Gianinazzi S. (1989). First report of non-mycorrhizal plant mutants (Myc-) obtained in Pea (*Pisum sativa* L.) and Fababean (*Vicia faba* L.). Plant Science 60: 215-222

Dumas E., Gianinazzi-Pearson V. and Gianinazzi S. (1989). Production of new soluble proteins during VA mycorrhiza formation. Agric. Eco. Env. 29: 111-114

Dumas-Gaudot E. Furlan V. Grenier J. and Asselin A. (1992). New acid chitinase isoforms induced in tobacco roots by vesicular-arbuscular mycorrhizal fungi. Mycorrhiza 1: 133-136

Elias K.S. and Safir G.R. (1987). Hyphal elongation of Glomus fasciculatus in response to root exudates. Appl. Env. Microbiol. 53: 1928-1933

Ellingboe A.H. (1981). Changing concepts in host-pathogen genetics. Ann. Rev. Phytopath. 19: 125-143

Ellis J.G., Lawrence G.J., Peacock W.J. and Pryor A.J. (1988). Approaches to cloning plant genes conferring resistance to fungal pathogens. Ann. Rev. Phytopath. 26: 245-263

Ellis R.J. (1990). Molecular chaperones: the plant connection. Science 250: 954-959

Estaun V., Calvert C. and Hayman D.S. (1987). Influence of plant genotype on mycorrhizal infection: Response of three pea cultivars. Plant and Soil 103: 295-298

Evans C.T., Sumegi B., Srere P.A., Sherry A.D. and Malloy C.R. (1993). (^{13}C) propionate oxidation in wild-type and citrate synthase mutant *Escherichia coli* : evidence for multiple pathways of propionate utilisation. Biochem. J. 291: 927-932

Ewing N.N., Wimmers L.E., Meyer D.J., Chetelat R.T. and Bennett A.B. (1990). Molecular cloning of tomato plasma membrane H+-ATPase. Plant Physiol. 94: 1874-1881

Federoff N.V., Furtek D.B. and Nelson O.E. Jn. (1984). Cloning of the *bronze* locus in maize by a simple and generalisable procedure using the transposable element *Activator* (*Ac*). Proc. Natl. Acad. Sci. (USA) 81: 3825-3829

Feldmann K.A. (1991). T-DNA insertion mutagenesis in *Arabidopsis* : mutational spectrum. Plant J. 1: 71-82

Flor H.H. (1971). Current status of the gene-for-gene concept. Ann. Rev. Plant Path. 9: 275-296

Folch J.L., Antaramian A., Rodriguez L., Brava A. and Brunner G. (1989). Isolation and characterisation of a *Saccharomyces cerevisiae* mutant with impaired glutamate synthase activity. J. Bacteriol. 171: 6776-6781

Franken P. and Gnadinger F. (1994). Analysis of parsley arbuscular mycorrhiza: Infection develpment and mRNA levels of defense-related genes. Mol. Plant-Mic. Inter. 7: 612-620

Friese C.F. and Allen M.F. (1991). The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture. Mycologia 83: 409-418

Garbaye J. (1994). Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phyt. 128: 197-210

Garcia-Romero I., Garcia-Garrido J.M. and Ocampo J.A. (1991). Pectinase activity in vesicular-arbuscular mycorrhiza during colonisation of lettuce. Symbiosis 12: 189-198

Garriock M.L., Peterson R.L. and Ackerly C.A. (1989). Early stages in colonisation of *Allium porum* (leek) roots by the vesicular-arbuscular mycorrhizal fungus, *Glomus versiforme*. New Phytol. 112: 85-92

Gaul H., Frimmel G., Gichner T. and Ulonska E. (1972). Efficiency of mutagenesis. In: Induced Mutations and Plant Improvement. IAEA, Vienna. pp 121-139

Gianinazzi-Pearson V. and Gianinazzi S. (1983). The physiology of vesicular-arbuscular mycorrhizal roots. Plant and Soil 71: 197-209

Gianinazzi-Pearson V. and Gianinazzi S. (1989). Cellular and genetical aspects of interactions between hosts and fungal symbionts in mycorrhizae. Genome 31: 336-341

Gianinazzi-Pearson V. and Gianinazzi S. (1992). Influence of intergeneric grafts between host and non-host legumes on formation of vesicular-arbuscular mycorrhiza. New Phytol. 120: 505-508

Gianinazzi-Pearson V., Brazanti B. and Gianinazzi S. (1989). In vitro enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host-root exudates and plant flavanoids. Symbiosis 7: 234-255

Gianinazzi-Pearson V., Smith S.E., Gianinazzi, S. and Smith F.A. (1991). Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhizas: V. Is H+ - ATPase a component of ATP-hydrolysing enzyme activities in plant-fungus membranes. New Phytol. 117: 61-74

Gianinazzi-Pearson V., Tahiri-Alaoui A., Antoniw J.F., Gianinazzi S. and Dumas E. (1990) Weak expression of the pathogenesis related PR-b1 gene and localisation of related protein during symbiotic endomycorrhizal interactions in tobacco roots. Endocyto. Cell Res. 8: 177-185

Gibson S. and Somerville C. (1993). Isolating plant genes. Trends in Biotech. 11: 306-313

Gilmore A.E. (1971). The influence of mycorrhizae on the growth of peach seedlings. J. Am. Soc. Hort. Sci. 96: 35-38

Giltrap N.J. and Lewis D.H. (1982). Catabolic repression of the synthesis of pectin degrading enzymes of *Suillis luteus* (L. ex Fr) S.F. Gray and *Hebeloma oculatum* Bruchet. New Phytol. 90: 485-490

Giovannetti M. and Mosse B. (1980). An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytol. 84: 489-500.

Giovannetti M., Avio L., Sbrana C. and Citernesi A.S. (1993). Factors affecting appressorium development in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe. New Phytol. 123: 115-122

Giovannetti M., Sbrana C. and Logi C. (1994a). Early processes involved in host recognition by arbuscular mycorrhizal fungi. New Phytol. 127: 703-709

Giovannetti M., Sbrana C., Avio L., Citernesi A.S., Avio L., Gollotte A., Gianinazzi-Pearson V. and Gianinazzi S. (1994b). Recognition and infection process, basis for host specificity of arbuscular mycorrhizal fungi. In: Impact of Mycorrhizas on Sustainable Agriculture and Natural Ecosystems. Gianinazzi S. and Schuepp H. (eds.), Birkhauser Verlag, Basel/Switzerland. pp 61-72

Gish W. and States D. (1993). Identification of protein coding regions by database similarity search. Nat. Genet. 3: 266-272

Glenn M.G., Chew F.S. and Williams P.H. (1985). Hyphal penetration of *Brassica* (Cruciferae) roots by a vesicular arbuscular-mycorrhizal fungus. New Phytol. 99: 463-472

Glenn M.G., Chew F.S. and Williams P.H. (1988). Influence of glucosinolate content of *Brassica* (Cruciferae) roots on growth of vesicular-arbuscular mycorrhizal fungi. New Phytol. 110: 217-225

Gollotte A., Gianinazzi-Pearson V., Giovannetti M., Sbrana C., Alvio.L and Gianinazzi S. (1993). Cellular localisation and cytochemical probing of resistance reactions to arbuscular mycorrhizal fungi in a 'locus a' myc- mutant of *Pisum sativum* L. Planta 191: 112-122

Gottschalk W. and Wolff G. (1983). Induced Mutations in Plant Breeding. Springer-Verlag Berlin Heidelberg, pp.128-144

Govers F., Nap J-P., Moerman M., Franssen H.K., van Kammen A. and Bisseling T. (1987). cDNA cloning and developmental expression of pea nodulin genes. Plant Mol. Biol. 8: 425-435

Graham J.H. (1982). Effects of citrus root exudates on germination of chlamydospores of the vesicular-arbuscular mycorrhizal fungus *Glomus epigaeus*. Mycologia 74: 831-835

Gurr S.J., McPherson M.J., Scollan C., Atkinson H.J. and Bowles D.J. (1991). Gene expression in nematode-infected plant roots. Mol. Gen. Genet. 226: 361-366

Guttenberger M. and Hampp R. (1992). Ectomycorrhizins - Symbiosis-specific or artifactual polypeptides from ectomycorrhizas? Planta 188: 129-136

Hall I.R. (1978). Effect of vesicular-arbuscular mycorrhizas on two varieties of maize and sweetcorn. N.Z. J. Agric. Res. 21: 517-519

Hardie K. and Leyton L. (1981). the influence of vesicular-arbuscular mycorrhiza on growth and water relations of red clover. I. In phosphate deficient soil. New Phytol. 89: 599-608

Harley J. L. and Smith S.E. (1983). Mycorrhizal Symbiosis. London, New York: Academic Press.

Harper J.F., Manney L. and Sussman M.R. (1994). The plasma membrane H+ - ATPase gene family in *Arabidopsis* : genomic sequence of AHA10 which is primarily expressed in developing seeds. Mol. Gen. Genet. 244: 572-587

Harper J.F., Manney L., DeWitt N.D., Yoo M.H. and Sussman M.R. (1990). The *Arabidopsis thaliana* plasma membrane H+-ATPase multigene family. J. Biol. Chem. 265 : 13601-13608

Harper J.F., Surowy T.K. and Sussman M.R. (1989). Molecular cloning and sequence of cDNA encoding the plasma membrane proton pump (H+-ATPase) of *Arabidopsis thaliana.* Proc. Nat. Acad. Sci. (USA) 86: 1234-1238

Harrison M.J. and Dixon R.A. (1994). Spacial patterns of expression of flavanoid/isoflavanoid pathway genes during interactions between roots of *Medicago trunculata* and the mycorrhizal fungus *Glomus versiforme.* Plant J. 6: 9-20

Hayman D.S. (1983). The physiology of vesicular-arbuscular endomycorrhizal symbiosis. Can. J. of Bot. 61: 944-963

Heckman J.R. and Angle J.S. (1987). Variation between soybean cultivars in vesicular-arbuscular mycorrhizal fungi colonisation. Agron. J. 79: 428-430

Hepper C.M. (1984a). Inorganic sulfur nutrition of the vesicular-arbuscular mycorrhizal fungus Glomus caledonius. Soil Biol. Biochem. 16: 669-671

Hepper C.M. (1984b). Isolation and culture of VA mycorrhizal (VAM) fungi. In: VA mycorrhiza. Powell C.L. and Bagyaraj D.J. (eds.), CRC, Boca Raton. pp 95-112

Hepper C.M. (1979). Germination and growth of *Glomus caledonius* spores: the effects of inhibitors and nutrients. Soil Biol. Biochem. 11: 269-277

Heslot H. (1977). Review of main mutagenic compounds. In: Manual on Mutation Breeding, IAEA, Vienna. pp. 51-59

Hirrell M.C., Mehravaran H. and Gerdemann J.W. (1978). Vesicular-arbuscular mycorrhiza in Chenopodiaceae and Cruciferae, do they occur? Can.J. Bot. 56: 2813-2817

Holley J.D. and Peterson R.L. (1979). Development of a vesicular-arbuscular mycorrhiza in bean roots. Can. J. Bot. 57: 1960-1978

Houlné G. and Boutry M. (1994). Identification of an *Arabidopsis thaliana* gene encoding a plasma membrane H+ - ATPase whose expression is restricted to anther tissue. Plant J. 5: 311-317

Huynh T.V., Young R.A. and Davis R.W. (1984). Constructing and screening cDNA libraries in lgt10 and lgt11. In: DNA Cloning: A Practical Approach. Glover D. (ed.), IRL, Oxford. pp. 49-78

Innes R.W., Kuempel P.L., PLazinski J., Canter-Cremers H. and Rolfe B.G. (1985). Plant factors induce expression of nodulation and host-range genes in *Rhizobium meliloti*. Mol. and Gen. Genetics 201: 426-432

Islam A.K.M.R., Shepherd K.W. and Sparrow D.H.B. (1981). Isolation and characterisation of euplasmic wheat-barley chromosome addition lines. Heredity 46: 161-174

Jabaji-Hare S.H., Thérien J. and Charest P.M. (1990). High resolution cytochemical study of the vesicular-arbuscular mycorrhizal association, *Glomus clarum* x *Allium porrum*. New Phytol. 114: 481-496

Jacobs M. (1964). Test de l'activité mutagénique de la 5-Bromodeoxyuridine et de la 5-iodo-deoxyuridine chez une plante supérieure *Arabidopsis thaliana* L., cultivée in vitro. Lejeunia 235: 1-7

James Jr. D.W. and Dooner H.K. (1990). Isolation of EMS-induced mutants in *Arabidopsis* altered in seed fatty acid composition. Theor. App. Genet. 80: 241-245

Jeanmaire C., Dexheimer J., Marx C;, Gianinazzi S. and Gianinazzi-Pearson V. (1985). Effect of vesicular-arbuscular mycorrhizal infection on the distribution of neutral phosphatase activities in root cortical cells. J. Plant Physiol. 119: 285-293

Jende-Strid B. (1991). Gene-enzyme relations in the pathway of flavanoid biosynthesis in barley. Theor. App. Genet. 81: 668-674

Jende-Strid B. and Moller B.L. (1981). Analysis of proanthocyanidins in wild-type and mutant barley (*Hordeum vulgare* L.). Carlsberg Res. Comm. 46: 53-64

Jensen H.A. and Nittler L.W. (1971). Varietal differences among barley seedlings grown with different nutrient solutions. Agron. J. 63: 714-717

John M.E. and Crow L.J. (1992). Gene expresion in cotton (Gossypium hirsutam) fibre: cloning of the mRNAs. Proc. Nat. Acad. Sci. (USA) 89: 5769-5773

Katz D.B., Sussman M.R., Mierzwa R.J., Evert R.W. (1988). Cytochemical localisation of ATPase activity in oat roots localises a plasma mebrane-associated soluble phosphatase, not the proton pump. Plant Physiol. 86: 841-847

Kerr A. (1987). The impact of molecular genetics on plant pathology. Ann. Rev. Phytopath. 25: 87-110

Khan A.H. (1974). The occurence of mycorhhizas in halophytes and xerophytes and of *Endogone* spores in adjacent soils. J. Gen. Microbiol. 81: 7-14

Kinden D.A. and Brown M.R. (1975). Electron microscopy of vesicular-arbuscular mycorrhizae of yellow poplar. II: Intracellular hyphae and vesicles. Can. J. Microbiol. 21: 1768-1780

Kleinhofs A., Sander C., Nilan R.A. and Konzac C.F. (1974). Azide mutagenicity-mechanism and nature of mutants involved. In: Polyploidy and Induced Mutations in Plant Breeding. IAEA, Vienna, pp. 195-199

Kleinschmidt G.D. and Gerdemann J.W. (1972). Stunting of citrus seedlings in fumigated nursery soils related to the absence of endomycorrhizae. Phytopath. 62: 1447-1453

Koide R.T. (1991). Nutrient supply, nutrient demand and plant response to mycorrhizal infection. New Phytol. 117: 365-386

Koide R.T. and Li M. (1990). On host regulation of the vesicular-arbuscular mycorrhizal symbiosis. New Phytol. 114: 59-74

Koide R.T. and Schreiner R.P. (1992). Regulation of the vesicular-arbuscular symbiosis. Ann. Rev. Plant Physiol. Mol. Biol. 43: 557-81

Koncz C., Martini N., Mayerhofer R., Koncz-Kalman Z., Korber H., Redei G.P. and Schell J. (1989). High-frequency T-DNA-mediated gene tagging in plants. Proc. Natl. Acad. Sci. (USA) 86: 8467-8471

Koncz C., Mayerhofer R., Koncz-Kalman Z., Nawrath C. Reiss B., Redei G.P. and Schell J. (1990). Isolation of a gene encoding a novel chloroplast protein by T-DNA tagging in *Arabidopsis thaliana*. EMBO J. 9: 1337-1346

Koornneef M. (1991). Isolation of higher plant developmental mutants. Symposia of the Society for Experimental Biology 45. Molecular Biology of Plant Development. CBL, University of Cambridge. pp 1-19

Krishna K.R., Shetty K.G., Dart P.J. and Andrews D.J. (1985). Genotype dependant variation in mycorrhizal colonisation and response to inoculation to of pearl millet. Plant and Soil 86: 113-125

Kucey R.M.N. and Paul E.A. (1982). Carbon flow, photosynthesis and Né fixation in mycorrhizal and nodulated faba beans (*Vicia faba* L.). Soil Biol. Biochem. 14: 407-412

Lamb C.J. (1994). Plant disease resistance genes in signal perception and transduction. Cell 76: 419-422

Lamb C.J., Lawton M.A., Dron M. and Dixon R.A. (1989). Signals and transduction mechanisms for activation of plant defences against microbial attack. Cell 56: 215-224

Lambert D.H., Baker D.F. aand Cole H. (1979). The role of mycorrhizae in the interactions of phosphorus with zinc, copper and other elements. Soil Sci. J. 43: 976-980

Lamont B. (1982). Mechanisms for enhancing nutrient uptake in plants with particular reference to mediterranean South Africa and Western Australia. Bot. Rev. 48: 597-689

Lamport D.T.A. (1977). Structure, biosynthesis and significance of cell wall glycoproteins. Rec. Adv. Phytochem 11: 79-115

Lander E.S., Green P., Abrahamson J., Baarlow A and Daly M.J. (1987). MAPMAKER: an interactive package for constructing primary genetic linkage maps of experimental and natural populations. Genome 1: 174-181

Legocki R.P. and Verma D.P.S. (1980). Identification of "nodule-specific" host proteins (nodulins) in soybean involved in the development of *Rhizobium* - legume symbiosis. Cell 20: 153-163

Leong S.A and Holden D.W. (1989). Molecular genetic approaches to the study of fungal pathogenisis. Ann. Rev. Phytopath. 27: 463-481

Levy Y. and Krikun J. (1980). Effect of vesicular-arbuscular mycorrhiza on *Citrus jambhiri* water relations. New Phytol. 85: 25-31

Lewis D.H. (1973). Concepts in fungal nutrition and the origen of biotrophy. Biol. Rev. 48: 261-278

Lewis D.H. (1975). Comparative aspects of the carbon nutrition of mycorrhizas. In: Endomycorrhizas; F.E. Sanders, B. Mosse and P.B. Tinker (eds.), Academic Press, London and New York. pp 119-148

Lindeberg G. and Lindeberg M. (1977). Pectinolytic ability of some mycorrhizal and saprophytic hymenomycetes. Arch. Mycrobiol. 115: 9-12

Linthorst H.J.B. (1991). Pathogenesis-related proteins of plants. Crit. Rev. Plant Sci. 10: 123-150

Litt M. and Luty J.A. (1989). A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am. J. Hum. Genet. 44: 388-396

Lobler M. and Hirsch A.M. (1993). A gene that encodes a proline-rich nodulin with limited homology to PsENOD12 is expressed the invasion zone of *Rhizobium meliloti* - induced alfalfa root nodules. Plant Physiol. 103: 21-30

Long S.R. (1989a). Rhizobium genetics. Ann. Rev. of Gen. 23: 483-506

Long S.R. (1989b). Rhizobium-Legume Nodulation: Life together in the underground. Cell: 203-214

Luo D., Coen E.S., Doyle S. and Carpenter R. (1991). Pigmentation mutants produced by transposon mutagenesis in *Antirrhinum majus*. Plant J. 1: 59-69

Maniatis T., Fritsch E.F. and Sambrook J. (1982). In: Molecular Cloning. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Manners J.M., Davidson A.D., JutidamrongphanW., Mackinnon G.M. and Scott K.J. (1988). mRNA induction in cereals by fungal pathogens. J. Cell. Biochem. Suppl. 12c: 284

Mansfield J.W. (1983). Antimicrobial compounds. In: Biochemical Plant Pathology; J.A. Callow (ed.), John Wiley and Sons, New York. pp 237-265

Manske G.G.B. (1989). Genetical analysis of the efficiency of VA mycorrhiza with spring wheat. Ag. Ecosys. Envir. 29: 273-280

Mantis N.J. and Winans S.C. (1993). The chromosome response regulatory gene *chv* 1 of *Agrobacterium tumefaciens* complements an *Escherichia coli pho* B mutant and is required for virulence. J. Bacteriol. 175: 6626-6636

Marks M.D. and Feldmann K.A. (1989). Trichome development in *Arabidopsis thaliana*. &. T-DNA tagging of the *GLABROUS1* gene. Plant Cell 1: 1043-1050

Marx C., Dexheimer J., Gianinazzi-Pearson V. and Gianinazzi S. (1982). Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhizas: iv. Ultracytoenzymological evidence (ATPase) for active transfer processes in the host-arbuscle interface. New Phytol. 90: 37-43

McGee P (1985). Lack of spread of endomycorrhizas of *Centaurium* (Gentianaceae). New Phytol. 115: 451-458

McGonigle T.P., Miller M.H., Evans D.G., Fairchild G.L. and Swan J.A. (1990). A new method which gives an objective measure of colonisation of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. 115: 495-501

Menge J.A., Steirle D., Bagyaraj D.J., Johnson E.L. and Leonard R.T. (1978). Phosphorus concentrations in plants responsible for inhibition of mycorrhizal infection. New Phytol. 80: 575-581

Mercy M.A., Shivashankar G. and Bagyaraj D.J. (1990). Mycorrhizal colonisation in cowpea is host dependant and heritable. Plant and Soil 121: 292-294

Miao G-H and Verma D.P.S. (1993). Soybean Nodulin-26 gene encoding a channel protein is expressed only in the infected cells of nodules and is regulated differently in roots of homologous and heterologous plants. Plant Cell 5: 781-794

Monke G. and Sonnewald U. (1995). Elevated mRNA levels of the ribosomal protein L19 and a calmodulin-like protein in assimilate-accumulating transgenic tobacco plants. Plant Physiol. 107: 1451-1452

Morandi D., Bailey J.A. and Gianinazzi-Pearson V. (1984). Isoflavanoid accumulation on soybean roots infected with vesicular-arbuscular mycorrhizal fungi. Physiol. Plant Path. 24: 357-364

Morandi D., Brazanti B. and Gianinazzi-Pearson V. (1992). Effect of some plant flavanoids on *in vitro* behaviour of an arbuscular mycorrhizal fungus. Agronomie 12: 811-816

Moreley C.D. and Mosse B. (1976). Abnormal vesicular-arbuscular mycorrhizal infections in white clover induced by lupin. Trans. B. Mycol. Soc. 67: 510-513

Morgante M. and Olivieri A.M. (1993). PCR-amplified microsatellites as markers in plant genetics. Plant J. 3: 175-182

Morton J.B. and Benny G.L. (1992). Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): A new order, Glomales, two new suborders, Glominae and Gigasporinae, and two new families, Acaulosporaceae and Gigasporaceae. Mycotaxon 37: 471-491

Mosse B. (1959). The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. Trans. Brit. Mycol. Soc. 42: 273-286

Mosse B. (1973). Plant growth responses to vesicular-arbuscular mycorrhiza IV. In soil given additional phosphate. New Phytol. 72: 127-136

Mosse B. (1988). Some studies related to "independent" growth of vesicular-arbuscular endophytes. Can. J. Bot. 66: 2533-2540

Muller K.J., Romano N., Gerstner O., Garcio-Maroto F., Pozzi C., Salamini F. and Rohde W. (1995). The barley *Hooded* mutation caused by a duplication in a homeobox gene intron. Nature 374: 727-730

Mullis K.B. and Faloona F.A. (1987). Specific synthesis of DNA in vitro via polymerase-catalysed chain reaction. Meth. in Enzym. 155: 335-350

Murai N., Li Z., Kawagoe Y. and Hayashimoto A. (1991). Transposition of maize activator element in transgenic rice plants. Nuc. Acids Res. 19: 617-622

Nair M.G., Safir G.R. and Siqueira J.O. (1991). Isolation and identification of vesicular-arbuscular mycorrhiza-stimulatory compounds from clover (*Trifolium repens*) roots. App. Envir. Micro. 57: 434-439

Nambura E., Naito S. and McCourt P. (1992). A mutant of Arabidopsis which is defective in seed development and storage protein accumulation is a new abi 3 allele. Plant J. 6: 435-441

Nap J-P. and Bisseling T. (1990). The roots of nodulins. Physiol. Plant. 79: 407-414

Nemec S. and Meredith F.I. (1981). Amino-acid content of leaves in mycorrhizal and non-mycorrhizal citrus rootstocks. Ann. Bot. 47: 351-358

Nester E.W., Gordon M.P., Amasino R.M. and Yanofsky M.F. (1984). Crown Gall: A molecular and physiological analysis. Ann. Rev. Plant Physiol. 35: 387-413

Neumann P.M., Azaizeh H. and Leon D. (1994). Hardening of root cell walls: a growth response to salinity stress. Plant Cell Envir. 17: 303-309

Nilan R.A. (1987). Trends in barley mutagenesis. In Barley Genetics V; Proceedings of the Fifth International Barley Genetics Symposium. Okayama, Japan, Eds. Yasuda S. and Konishi T (eds). Springer-Verlag, Berlin. pp 241-249

Nilan R.A., Kleinhofs A. and Fonzak C.F. (1981). The role of induced mutation in supplementing natural genetic variability. Ann. New York Acad. Sci. 287: 367-384

Nye P. and Tinker P.B. (1977). Solute Movement in the Root System, Oxford:Blackwell Scientific, U.K..

Olivier A.J., Smith S.E., Nicholas D.J.D., Wallace W. and Smith F.A. (1983). Activity of nitrate reductase in *Trifolium subterraneum:* effects of mycorrhizal infection and phosphate nutrition. New Phytol. 94: 63-79

Olsen O., Jakobsen K.S. and Schmelzer E. (1990). Development of barley aleurone cells: temporal and spacial patterns of accumulation and cell-specific mRNA's. Planta 181: 462-466

Pacovski R.S. (1989). Carbohydrate, protein and amino acid status of *Glycine - Glomus - Bradyrhizobium* symbioses. Physiol. Plant. 75: 346-354

Pardo J.M. and Serrano R. (1989). Structure of a plasma membrane H+-ATPase gene from the plant *Arabidopsis thaliana.* J. Biol. Chem. 264 (15) : 8557-8562

Passioura J.B. and Fry S.C. (1992). Turgor and cell expansion: beyond the Lockhart equation. Aust. J. Plant Physiol. 19: 565-576

Paula M.A. and Sequeira J.O. (1990). Stimulation of hyphal growth of the VA mycorrhizal fungus *Gigaspora margarita* by suspension cultured *Pueraria phaseoloides* cells and cell products. New Phytol. 115: 69-75

Pear J.R., Ridge N., Rasmussen R., Rose R.E. and Houck C.M. (1989). Isolation and characterisation of a fruit-specific cDNA and the corresponding genomic clone from tomato. Plant Mol. Biol. 13: 639-651

Peters N.K. and Verma D.P.S. (1990). Phenolic compounds as regulators of gene-expression in plant-microbe interactions. Mol. Plant-Microbe Int. 3: 4-8

Peterson R.L., Ashford A.E. and Allaway W.G. (1985). Vesicular-arbuscular mycorrhizal associations of vascular plants on Heron Island, a great barrier reef coral cay. Aust. J. Bot. 33: 669-676

Phillips D.A. and Tsai S.M. (1992). Flavanoids as plant signals to rhizosphere microbes. Mycorrhiza 1: 55-58

Phillips J.M. and Hayman D.S. (1970). Improved procedures for clearing roots and staining parasitic and VA mycorrhizal fungi for rapid assessment of infection. Trans. Brit. Mycol. Soc. 55: 543-552.

Powell C.L. (1976). Development of mycorrhizal infections from *Endogone* spores and infected root segments. Trans. Brit. Mycol. Soc. 66: 439-445

Rafalski J.A. and Tingey S.V. (1993). Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. Trends in Genet. 9: 275-279

Rajapakse S. and Miller J.C. (1987). Intraspecific variability for V.A. mycorrhizal symbiosis in cowpea (*Vigna unguiculata*). In: Genetic Aspects of Plant Mineral

Nutrition; W.H. Gabelman and B.C. Loughman (Eds.), Martinus Nijhoff, Dordrecht, Netherlands. pp 165-173

Reddy A.S.N., Jena P.K., Mukherjee S.K. and Poovaiah B.W. (1990). Molecular cloning of cDNAs for auxin-induced mRNAs and develomental expression of the auxin-inducible genes. Plant Mol. Biol. 14: 643-653

Redmond J.W., Batley M., Djordjevic M.A., Innes R.W., Kuempel P.L. and Rolfe B.G. (1986). Flavones induce expression of the nodulation genes in *Rhizobium.* Nature 323: 632-635

Riggleman R.C., Fristensky B. and Hadwiger L.A. (1985). The disease resistance response is associated with increased levels of specific mRNA's. Plant Mol. Biol. 4: 81-86

Rolfe B.G. (1988). Flavones and isoflavones as inducing substances of nodulation. Biofactors 1: 3-10

Rolfe B.G. and Gresshoff P. (1988). Genetic analysis of legume nodule initiation. Ann. Rev. Physiol. Plant Mol. Biol. 39: 297-319

Rossen L., Davis E.O. and Johnston A.W.B. (1987). Plant-induced expression of *Rhizobium* genes involved in host specificity and early stages of nodulation. Trends Biochem. Sci. 12: 430-433

Rossen L., Shearmen C.A., Johnston A.W.B. and Downie J.A. (1985). The *nod* D gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate ionduces the *nod* A, B, C genes. EMBO J. 4: 3369-3373

Roth L.E. and Stacy G. (1989). Cytoplastic membrane systems involved in bacterium release into soybean nodule cells as stuidied with two *Bradyrhizobium japonicum* mutant strains. Eur. J. Cell Biol. 49: 24-32

Rozycka M., Khan S., Lopez I., Greenland A.J. and Hussey P.J. (1995). A Zea mays pollen cDNA encoding aputative actin-depolymerizing factor. Plant Physiol. 107: 1011-1012

Safir G.R., Boyer J.S. and Gerdemann J.W. (1971). Mycorrhizal enhancement of water transport in soybean. Science 172: 581-583

Saif S.R. and Khan A.G. (1977). The effect of vesicular-arbuscular mycorrhizal associations on growth of cereals III. Effects on barley growth. Plant and Soil 47: 17-26

Saiki R.K., Gelfand D.H., Stoffel S., Scharf S.J., Higuchi R., Horn G.T., Mullis K.B. and Erlich H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491

Salganik R.I. and Dianov G.L. (1992). Molecular mechanisms of the formation of DNA double-strandes breaks and the induction of genomic rearrangements. Mutation Res. 266: 163-170

Sanders F.E. (1975). The effect of foliar-applied phosphate on the mycorrhizal infections of onion roots. In: Endomycorrhizas; F.E. Sanders, B. Mosse and P.B. Tinker (eds.), Academic Press, London and New York. pp 261-276

Sanders F.E. and Tinker P.B. (1973). Phosphate flow into mycorrhizal roots. Pestic. Sci. 4: 385-395

Sanger F., Nickler S. and Coulson A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. (USA) 74: 5463-5467

Scannerini S. and Bonfante-Fasolo P. (1979). Ultrastructural cytochemical demonstration of polysaccharides and proteins within the host-arbuscule interfacial matrix in an endomycorrhiza. New Phytol. 83: 87-94

Scannerini S., Bonfante-Fasolo P. and Fontana A. (1975). An ultrastructural model for the host-symbiont interaction in the endotrophic mycorrhizae of *Ornithogalum umbellatum.* In: Endomycorrhizas; Sanders F.E., Mosse B. and Tinker P.B. (eds.), Academic Press, London and New York. pp 313-324

Schenck N.C., Kinloch R.A. and Dickson D.W. (1975). Interaction of endomycorrhizal fungi and root-knot nematode on soybean. In: Endomycorrhizas; Sanders F.E., Mosse B. and Tinker P.B.(eds.), Academic Press, London and New York. pp 605-617

Schenck N.C., Kinloch R.A. and Dixon D.W. (1974). Interaction of endomychorrhizal fungi and root-knot nematode of soybean. Proc. Am. Phytopath. Soc. 69: 1974

Scheres B., van Engelen F., van der Knaap van de Weil C., vab Kammen A. and Bisseling T. (1990). Sequential induction of nodulin gene expression in the developing pea nodule. Plant Cell 2: 687-700

Sequeira L. (1984). Recognition systems in plant-pathogen interactions. Biological Cell 51: 281-286

Serrano R. (1989). Structure and function of plasma membrane ATPase. Annu. Rev. Plant Mol. Biol. 40: 40-61

Sheahan J.J., Ribeiro-Neto L. and Sussman M.R. (1993). Cesium-insensitive mutants Arabidopsis thaliana. Plant J. 3: 647-656

Sigurbjornsson B. (1975). Improvement of barley through induced mutation. Proc. 3rd Int. Barley Genetics Symposium, Garching. pp 145-149

Siqueira J.O., Safir G.R. and Nair M.G. (1991). Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavanoid compounds. New Phytol. 118: 87-93

Skipper H.D. and Smith G.W. (1979). Influence of soil pH on the soybean: endomycorrhizal symbiosis. Plant and Soil 53: 559-563a

Skolnick M.H. and Wallace R.B. (1988). Simultaneous analysis of multiple polymorphic loci using amplified sequence polymorphisms (ASPs). Genomics 2: 273-279

Smith H.H. (1961). The reactor as a tool for research in plant sciences and agriculture. In: Programming and Utilisisation of Research reactors, Acedemic Press, London. pp 425-438

Smith S.E. (1980). Mycorrhizas of autotrophic higher plants. Biol. Rev. 55: 475-510

Smith S.E. (1995). Discoveries, discussions and directions in mycorrhizal research. In: Mycorrhiza; Varma A. and Hock B. (eds.), Springer-Verlag, Berlin Heidelberg New York. pp 3-24

Smith S.E. and Gianinazzi-Pearson V. (1988). Physiological interactions between symbionts in vesicular-arbuscular mycorrhizal plants. Ann. Rev. Plant Physiol. 39: 221-244

Smith S.E. and Smith F.A. (1990). Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. New Phytol. 114: 1-38

Smith S.E., Dickson S. and Walker N.A. (1992). Distribution of VA mycorrhizal entry points near the root apex: Is there a uninfectible zone at the root tip of leek or clover? New Phytol. 122: 469-477

Smith S.E., Robson A.D. and Abbott K. (1992). The involvement of mycorrhizas in assessment of genetically dependant efficiency of nutrient uptake and use. Plant and Soil 146: 169-179

Smith S.E., St. John B.J., Smith F.A. and Bromley J.-L. (1986). Effect of mycorrhizal infection on plant growth nitrogen and phosphate nutrition of glasshouse-grown *Allium cepa* L.. New Phytol. 103: 359-373

Smith S.E., St. John B.J., Smith F.A. and Nicholas D.J.D. (1985). Activity of glutamine synthase and glutamate dehydrogenase in *Trifolium subterraneum* L. and *Allium cepa* L.: effects of mycorrhizal infection and phosphate nutrition. New Phytol. 99: 211-227

Snellgrove R.C., Splittstosser W.E., Stribley D.P. and Tinker P.B. (1982). The distribution of carbon and the demand of the fungal symbiont in leek plants with vesicular-arbuscular mycorrhizas. New Phytol. 92: 75-87

Snellgrove R.C., Stribley D.P., Tinker P.B. and Lawlor D.W. (1986). the effect of vesicular-arbuscular mycorrhizal infection on photosynthesis and carbon distribution in leek plants. In: Physiological and Genetical Aspects of Mycorrhizae; Gianinazzi-Pearson V. and Gianinazzi S. (eds.), INRA, Paris. pp 421-424

Son C.L. and Smith S.E. (1988). Mycorrhizal growth responses: interactions between photon irradiance and phosphorus nutrition. New Phytol. 108: 305-314

Southern E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517

Spanu P. and Bonfante-Fasolo P. (1988). Cell-wall-bound peroxidase activity in roots of mycorrhizal *Allium porrum.* New Phytol. 109: 119-124

Spanu P., Boller T., Ludwig A., Wiemken A., Faccio A, and Bonfante-Fasolo P. (1989). Chitinase in roots of mycorrhizal *Allium porrum* : regulation and localisation. Planta 177: 447-455

Sprent J.I. (1989). Which steps are essential for the formation of functional legume nodules? New Phytol. 111: 129-153

Sreenivasa M.N. and Rajashekhara E. (1992). Influence of the stage of host development and genotypes on mycorrhizal colonisation of field grown wheat. Z. Mikrob. 144: 381-384

Stachel S.E., Messens E., Van Montagu M. and Zambryski P. (1985). Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens.* Nature 318: 624-629

Staskawicz B.J., Dahlbeck D. and Keen N.T. (1984). Cloned avirulence gene of *Pseudomonas syringae* pv. *Glycinea* determines race-specific incompatibility on *Glycine max* (L) Merr. Proc. Natl. Acad. Sci. (USA) 81: 6024-6028

Stegemann H. (1984). Retrospect on 25 years of cutivar identification by protein pattern and prospects for the future. In: Biochemical tests for cultivar identification; Draper S.R. and Cooke R.J. (eds.), International Seed-testing Association. pp 20-31

Sussman M.R. and Harper J.F. (1989). Molecular biology of the plasma membrane of higher plants. Plant Cell 1: 953-960

Sutton J.C. (1973). Development of vesicular-arbuscular mycorrhiza in crop plants. Can. J. Bot. 51: 2487-2493

Sutton J.C. and Barron G.L. (1972). Population dynamics of *Endogone* spores in soil. Can J. Bot. 50: 1909-

Swegle M., Huang J-K., Lee G. and Muthukrishnan S. (1989). Identification of an endochitinase cDNA from barley aulerone cells. Plant Mol. Biol. 12: 403-412

Tagu D., Python P., Crétin C. and Martin F. (1993). Cloning symbiosis-related cDNA's from eucalyptus mycorrhiza by PCR-assisted differential screening. New Phytol. 125: 339-343

Tester M., Smith S.E. and Smith F.A. (1987). The phenomenon of 'nonmycorrhizal' plants. Can. J. Bot. 65: 419-431

Timmis J.M., Whisson D.L., Binns A.M., Mayo M.J. and Mayo G.M.E. (1990). Deletion mutation as ameans of isolating avirulence genes in flax rust. Theor. Appl. Genet. 79: 411-416

Tinker P.B. (1975a). Effects of vesicular-arbuscular mycorrhias on higher plants. Symp. Soc. Exper. Biol. 29: 325- 371

Tinker P.B. (1975b). Soil chemistry of phosphorus and mycorrhizal effects on plant growth. In: Endomycorrhizas; F.E. Sanders, B. Mosse and P.B. Tinker (eds.), Academic Press, London. pp 353-371.

Tommerup I.C. and Abbott L.K. (1981). Prolonged survival and viability of V-A mycorrhhizal hyphae after root death. Soil Biol. Biochem. 13: 431-433

Torp J. and Jorgensen J.H.. (1986). Modification of barley powdery mildew resistance gene Ml-a12 by induced mutation. Can. J. Genet. 28: 725-731

Toth R. and Miller R.M. (1984). Dynamics of arbuscule development and degeneration in a Zea mays mycorrhiza. Am. J. Bot. 71: 449-460

Toth R., Page T. and Castleberry R. (1984). Differences in mycorrhizal colonisation of maize selections for high and low ear leaf phosphorus. Crop Sci. 24: 994-996

Toth R., Toth D., Starke D. and Smith D.R. (1900). Vesicular-arbuscular mycorrhizal colonisation in *Zea mays* affected by breeding for resistance to fungal pathogens. Can. J. Bot. 68: 1039-1044

Trinick M.J. (1977). Vesicular-arbuscular infection and soil phosphorus utilisation in *Lupinus* spp. New Phytol. 78: 297-304

Van Kammen (1984). Suggested nomeclature for plant genes involved in nodulation and symbiosis. Plant Mol. Biol. Rep. 2: 43-45

Van Loon L.C., Gerritsen Y.A.M. and Ritter C.E. (1987). Identification, purification and characterisation of pathogenesis-related proteins from virus-infected Samsun NN tobacco leaves. Plant Mol. Biol. 9: 593-598

Van Sluys M.A., Tempé J. and Federoff N. (1987). Studies on the introduction and mobility of the maize *Activator* element in *Arabidopsis thaliana* and *Daucus carotus.* EMBO J. 6: 3881-3889

Vierheilig H. and Ocampo J.A. (1991). Receptivity of various wheat cultivars to infection by VA mycorrhizal fungi as influenced by inoculum potential and the relation of VAM-effectiveness to succinic hydrogenase activity of the mycelium in roots. Plant and Soil 133: 291-296

Wada M., Takano M. and Kasamo K. (1992). Nucleotide sequence of a complementary DNA encoding plasma membrane H+-ATPase from rice (*Oryza sativa* L.). Plant Physiol. 9: 794-795

Walbot V. (1992). Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. Ann. Rev. Plant Physiol.Plant Mol. Biol. 43: 49-82

Walden R., Hayashi H. and Schell J. (1991). T-DNA as a gene tag. Plant J. 1: 281-288

Weining S. and Langridge P. (1991). Identification and mapping of polymorphisms in cereals based on polymerase chain reaction. Theor. Appl. Genet. 82: 209-216

Westenberg B., Boller T. and Wiemken A. (1989). Lack of arginine-storage and polyphosphate-storage pools in a vacuole-deficient mutant (end1) of *Sacchoromyces cerevisiae.* FEBS Letters 254: 133-136

Winnacker E.L. (1987). From Genes to Clones: Introduction to gene cloning. VCH Weinheim. pp149-177

Woloshuk, C.P. and Kolattukudy, P.E. (1986). Mechanism by which contact with plant cuticle triggers cutinase gene expression in the spores of *Fusarium solani* f. sp. pisi. Proc. Nat. Acad. Sci. (USA) 83: 1704-08

Wyss P., Boller T. and Wiemken A. (1990b). Vesicular-Arbuscular mycorrhizas of wild-type soybean and non-nodulating mutants with *Glomus mosseae* contain symbiosis-specific polypeptides (mycorrhizins) immunologically cross-reactive with nodulins. Planta 182: 22-26

Wyss P., Boller T. and Wiemken A. (1991). Phytoalexin response is elicited by a pathogen (*Rhizoctonia solani*) but not by a mycorrhizal fungus (*Glomus mosseae*) in soybean roots. Experientia 47: 395-401

Yanisch-Perron C., Veira J. and Messing J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119

Yoder J.I. (1990). A genetic analysis of mutations recovered from tomato following *Agrobacterium*- mediated transformation with the maize transposable elements *Activator* and *Dissociation.* Theor. Appl. Genet. 79: 657-662

Young J.L., Davis E.A. and rose S.L. (1985). Endomycorrhizal fungi in breeder wheats and *Triticale* cultivars field grown in fertile soils. Agron. 77: 219-224

Zehetner G., Frischauf A. and Lehrach H. (1987). Approaches to restriction map determination. In: Nucleic Acid and Protein Sequence Analysis: A Practical Approach. Bishop M.A. and Rawlings C.J. (eds.), IRL Press Ltd., Oxford Washington D.C.. pp 147-164

Zidan I., Shavav A., Ravina I., and Neumann P.M. (1992). Does salinity inhibit maize leaf growth by reducing tissues concentration of essential mineral nutrients. J. Plant Nutrit. 15: 1407-1419

Appendix 1

Sources or Composition of Materials and Solutions

Escherichia coli strains	Source
HB101	: Professor A. Kerr.
DH5α	: Ms. Jan Nield.
ER1647	: New England Biolabs.
C600 and C600*hfl*	: Promega.

Enzymes	Source
Alkaline Phosphatase	: Promega
E. coli DNA Polymerase 1	: Pharmacia
Klenow DNA Polymerase	: Boehringer Mannheim
DNase 1	: Promega
T4 DNA Ligase	: Boehringer Mannheim
Restriction Endonucleases	: Boehringer Mannheim and Promega
RNase inhibitor	: Promega
RNase A	: Boehringer Mannheim
RNase H	: Pharmacia
Lysozyme	: Sigma
Proteinase K	: Sigma
Reverse Transcriptases:	
Superscript RT H^-	: BRL
M-MLV RT	: Promega.
Taq Polymerase	: Promega

Radio-Nucleotides

Radio-nucleotides (α–^{32}P-dCTP) were supplied by Amersham.

Buffers, Solutions, Microbial Growth Media and Electrophoresis Gels

The buffers etc. used for these investigations are listed hereunder in alphabetical order along with their compositions.

Agarose gels	: 0.7-3g/L of agarose (as indicated) in either TAE or TBE buffer.
Alkali lysing solution	: 0.5M NaOH : 1.5M NaCl
Chloroform/IAA	: Chloroform/Isoamyl alcohol (24:1 v/v)
Denaturing solution	: 1.5M NaCl : 0.5M NaOH
Denhardts Solution	: 2% Polyvinylpyrolidone : 2% Ficoll 400 : 2% Bovine Serum Albumin
Depurination solution	: 0.25N HCl
Formamide/Formaldehyde	: 89µl Formaldehyde (37%) : 250µl deionised Formamide
Hybridisation solutions:	
Solution 1	: Prehybridisation Solution 1 plus 200µl denatured salmon sperm (5 mg/ml), denatured radioactive probe and 3ml of Dextran Sulphate (25% w:w)
Solution 2	: Prehybridisation Solution 2 plus 200µl of denatured salmon sperm (5 mg/ml), denatured radioactive probe and 1ml of Dextran Sulphate (25% w:w)
Ligase buffer	: as supplied by Promega.

LB complete medium : 10g Tryptone
: 5g Yeast Extract
: 10g NaCl
(Medium was adjusted to pH 6.8 before autoclaving.)

MOPS/EDTA Buffer (10x) : 0.5M MOPS
: 10mM EDTA
(adjusted to pH 7.0 before autoclaving)

Neutralisation buffer : 1.0M Tris-HCl (pH 6.5)
: 2.0M NaCl
(adjusted to pH 5.0)

Phage Buffer : 20mM Tris-HCl (pH 7.4)
: 100mM NaCl
: 10mM $MgSO_4$

Plant Nutrient Solutions (5 or 10 times concentration as indicated)

Solution A (10X) : 0.6M K_2SO_4
: 0.2M $MgCl_2$
: 12mM $MnSO_4$
: 10mM $ZnSO_4$
: 6mM $CuSO_4$
: 1.5mM $CoSO_4$

Solution B (10X) : 3mM H_3BO_3
: 1.5mM Na_2MoO_4

Solution C (5X) : 0.7M $Ca(NO_3)_2$
: 1.4M KNO_3

Solution D (5X) : 16mM $FeSO_4$

Solution E (5X) : 2M NH4

Phosphate Solution : 1M NaH_2PO_4

(All solutions were made using npH_2O and stored at 4°C. Solutions A-E were diluted to the correct concentrations when required)

Prehybridisation Solutions

Solution 1 : 3ml Denhardts Solution
: 6ml 5x HSB
: 18ml nanopure H_20

(The solution was made up fresh for each hybridisation and warmed to 65°C. Three ml of freshly boiled salmon sperm DNA (10 mg/ml) was added just before the prehybridisation began)

Solution 2 : 5ml Deionised formamide
: 5ml 20x SSPE
: 2ml Denhardts solution
: 1ml 10% SDS
: 6ml nanopure H_2O

(The solution was made up and stored as 19ml aliquots at -20°C. The solution was thawed on ice and 1ml of freshly boiled salmon sperm DNA was added just before the prehybridisation began)

Phenol : Phenol equilibrated in 1 x TE and 0.1% hydroxy-quinoline

Phenol/Chloroform : 50% Phenol
: 50% Chloroform/Isoamyl alcohol

RNA buffer A : 294µl MOPS/EDTA (10x) buffer
: 706µl nanopure H_2O

RNA denaturing gel : 0.5g Agarose
: 36ml nanopure H_20
: 5ml MOPS/EDTA buffer
: 9ml formaldehyde (37%)

(The agarose was dissolved and cooled to 60°C before addition of the formaldehyde)

RNA loading buffer	: 322µl RNA buffer A : 178µl formaldehyde (37%) : 500µl deionised formamide : 3mg xylene cyanol : 3mg bromocresol green : 250mg sucrose
SDS (10%)	: 100g SDS : 900ml nanopure H_2O
SM buffer	: Phage buffer plus 0.1% gelatin
SSC (20 x)	: 6.0M NaCl : 0.6M Na Citrate
TAE buffer	: 400mM Tris-HCl (pH7.5) : 10mM EDTA : 50mM Na Acetate
TBE buffer	: 500mM Tris-HCl (pH 7.5) : 10mM EDTA : 500mM Boric acid
TE buffer	: 10mM Tris-HCl (pH 7.5) : 1mM EDTA
Wash buffer 1 (to 2L)	: 200ml SSC (20x) : 200ml SDS (10%) : 1600ml nanopure H_2O
Wash buffer 2 (to 2L)	: 100ml SSC (20x) : 20ml SDS (10%) : 1880ml nanopure H_2O
Wash buffer 3 (to 2L)	: 20ml SSC (20x) : 20ml SDS (20x) : 1960ml nanopure H_2O

Miscellaneous materials.

'Geneclean' plasmid DNA purification kits were purchased from BIO 101 Inc.

'Hybaid' oven and hybridisation bottles were provided by Dr. Peter Langridge, Dept of Plant Sciences.

Appendix 2

DNA Isolations from Plant Roots and Fungal Spores

This method uses a high salt buffer containing cationic detergent cetyltrimethylammonium bromide. Use wide-bore (ie. cut-off blue) tips or glass pipettes for transfering all DNA preparations as this is a major source of DNA shearing. Gloves must be worn (phenol and β-mercaptoethanol are used in this procedure).

1. Freeze tissue (50mg-0.25g) in liquid N_2 and grind to a fine powder with a suitably sized mortar and pestle.

2. Add 0.5-5ml of the extraction buffer (see below) and regrind for a further 1-2 minutes.

3. Transfer the slurry to a 15ml Corex tube (use a 2ml Eppendorf tube for volumes less than 1ml) and incubate at 65°C in a water bath for 20 minutes.

4. Extract the proteins with an equal volume of Phenol/Chloroform/IAA solution

5. Separate the phases by centrifugation (5 minutes/12000g) and transfer the aqueous (upper) phase to a clean tube.

6. Re-extract with an equal volume of Chloroform/IAA.

7. Separate the phases by centrifugation (5 minutes/12000g) and transfer the aqueous (upper) phase to a clean tube taking care not to transfer any of the material deposited at the interphase of the two phases.

8. Ethanol precipitate the DNA (see 2.7.3.5) and recover by centrifugation (10 minutes/12000g).

9. Wash the pellet 2-3 times with a 70% ethanol solution (1ml) to remove contaminating salts. Take care to remove as much of the residual ethanol as possible before drying under vacuum for 10 minutes.

9. Resuspend the pellet in an appropriate volume (20-100µl) of RNAse solution (40µg/ml in TE).

EXTRACTION BUFFER

100mM Tris-HCl (pH7.8)
50mM EDTA
500mM NaCl
1% cetyltrimethylammonium bromide
3 µl of 98% β-mercaptoethanol for each 5ml of buffer (add just prior to use)

Appendix 3

DNA Isolations from Plant Leaves

This protocol is based on the method used by Guidet *et al.* (1991) and uses a high-salt buffer to reduce enzyme activity and the detergent 'Sarcosyl' to disrupt the cell nuclear membranes during the grinding procedure. It is important to work quickly and at the coldest temperature possible in order to minimise oxidative reactions. However, do not add too much liquid nitrogen at the start or the 'Sarcosyl' will solidify and take 5-10 minutes to re-liquify. Use wide-bore (ie. cut-off blue) tips or glass pipettes for transfering all DNA preparations as this is the major source of DNA shearing. Gloves must be worn (phenol and β-mecaptoethanol are used in the procedure).

1. Grind 1-1.5 g of plant leaf tissue in liquid nitrogen; regrind 2 or 3 times by adding a little extra liquid nitrogen.

2. Add 45 ml of extraction buffer (see below) and 1/2 weight (ie.150-750mg) of insoluble Polyvinylpirolidine and mix for a further 1 minute while still cold.

3. Transfer the slurry into two 30 ml Corex tubes and centrifuge (10 minutes/10000g/4°C) to pellet cell debris.

4. Transfer the supernatant (about 20ml) to fresh Corex tubes and extract with phenol/chloroform/IAA (see appendix 1) for at least 10 minutes. Centrifuge (5 minutes/10000g/4°C) to separate phases and transfer aqueous (upper) phases to a new set of tubes.

5. Chloroform/IAA (see appendix 1) extract for 5 minutes to remove residual phenol. Centrifuge as above.

6. Add a 1/10th volume of 3M NaAcetate to each tube then precipate the DNA by adding an equal volume of iso-propanol.

7. Allow the mixture to stand at room temperature for about 5 minutes then pellet the DNA by centrifugation (10 minutes/10000g/4°C). Wash the pellet thrice with 70% ice-cold ethanol (about 10 ml). Try and remove as much of the ethanol as possible using a fine pipette.

8. Resuspend the DNA in 320 µl of npH_2O, add 80 µl of 4MNaCl and 400 µl of 13% PEG solution.

9. Leave on ice for at least 30 minutes (the longer the better) and then pellet the DNA by centrifugation. Wash with 70% ethanol as above and then resuspend in an appropriate volume of RNase solution (40µg/ml in TE).

EXTRACTION BUFFER

100mM Tris-HCl (pH7.8)
50mM EDTA
100mM NaCl
4% Sarcosyl
35 µl of 98% β-mercaptoethanol for each 50ml of buffer (add just prior to use).

Appendix 4

Plasmid DNA Isolations

1. Pick a single bacterial colony from plate and grow overnight in 5ml of LB buffer containing appropriate antibiotic.

2. Decant 1.5ml of culture into Eppendorf tube and centrifuge (2 minutes/4000g)to pellet cells.

3. Add 100µl of Solution 1 and thoroughly resuspend the cells by vortexing. Incubate at room temperature for 5 minutes.

4. Add 200µl of Solution 2 and mix by gentle inversion of the tube. Incubate on ice for 5 minutes.

5. Add 150µl of Solution 3 and vortex briefly to mix the contents of the tube. Incubate on ice for 5 minutes.

6. Centrifuge (5 minutes/12000g) the tube and transfer the aqueous (upper) phase into a fresh tube containing 450µl of phenol/chloroform/IAA (see Appendix 1).

7. Extract for 5 minutes by forming an emulsion every minute (gently invert the tube).

8. Centrifuge (5 minutes/12000g) the tube and transfer the aqueous (upper) phase into a fresh tube containing 200µl of chloroform/IAA (see Appendix 1).

9. Ethanol precipitate the DNA (see 2.7.3.5) and recover by centrifugation (10 minutes/12000g).

9. Wash the pellet 2-3 times with a 70% ethanol solution (0.5ml) to remove contaminating salts. Take care to remove as much of the residual ethanol as possible before drying under vacuum for 10 minutes.

9. Resuspend the pellet in an appropriate volume (50-100µl) of RNAse solution (40µg/ml in TE).

PLASMID-ISOLATION SOLUTIONS

Solution 1:
50mM Glucose
25mM Tris-HCl (pH 8.0)
10mM EDTA

Solution 2: (freshly prepared for each plasmid preparation)
0.2N NaOH
1% SDS

Solution 3:
3.0M K Acetate (Prepared by adding 11.5ml glacial acetic acid and 28.5ml H_2O to 60ml of 5M potasium acetate)
Adjust to final pH 4.8 with galacial acetic acid.

Appendix 5

Phage DNA Isolations

1. Core out phage plaque with a sterile Pasteur pipette and expel into 200µl of SM buffer. Allow the phage to diffuse into the buffer for at least 2 hours at 4°C.

2. Add 100µl of the phage solution to 400ml of competent C600 *hfl* cells in a large (50ml) test tube

3. Allow the phage to adsorb to the cells for 20 minutes then add 5ml of LB (+5mM $CaCl_2$) and shake vigorously (200rpm) at 37°C until the culture lyses (usually 5-6 hours).

4. Transfer the lysate to a 10ml plastic centrifuge tube and spin down the cellular debris (10 minutes/5000g).

5. Remove the supernatant, add DNAse J and RNAse A to a final concentration of 1µg/ml and incubate at 37°C for 30 minutes.

6. Add 5ml of PEG solution (see below) and allow to precipitate on ice for at least 1 hour.

7. Pellet the phage by centrifugation (15 minutes/10000g), pour off the supernatant and invert the tubes (on absorbant paper) and allow to drain for 15 minutes.

8. Resuspend the phage pellet in 700µl of LB and transfer to a 1.5ml Eppendorf tube containing 700µl of DEAE-52. Gently invert the tubes 20-30 times.

9. Centrifuge (5 minutes/12000g) to spin down the resin and transfer the supernatant to a fresh tube. Add 13µl of Proteinase K (0.1mg/ml) and 32µl of 10% SDS. Mix and incubate at room temperature for at least 10 minutes.

10. Add 130µl of 3M potasium acetate solution (see Appendix 4) and incubate at 88)C for 20 minutes then on ice for 10 minutes.

11. Centrifuge (5 minutes/12000g) to pellet the denatured proteins and transfer the supernatant to a fresh Eppendorf tube. A phenol/chloroform/IAA extraction can be incorporated at this point but it is not necessary and will reduce the final yield of phage DNA.

12. Add an equal volume of isopropanol to precipitate the DNA. the tubes can placed on ice for 1 hour or snap frozen and left in liquid N_2 for 5 minutes.

13. Centrifuge (10 minutes/12000g) to pellet the DNA, wash 2-3 times with 70% ethanol and resuspend the pellet in DNAse-free H_2O or TE (30-50µl).

PHAGE PRECIPITATION (PEG) SOLUTION

20% polyethyleneglycol (w:v)
2M NaCl in SM buffer

Appendix 6

PCR-Primer Sequences

Lambda gt10 Primers	5' - AGCAAGTTCAGCCTGGTTAAGT - 3'
	5' - CTTATGAGTATTTCTTCCAGGGTA -3'
AT Primers	5' - ATGGCTTCGGTTACTTTC - 3'
	5' - AGACGATGGAAACGAACT - 3'
CP Primers	5' - AAGTACACCCTCAAAAGC - 3'
	5' - TTGCCAGAAACAATAACC - 3'
LA Primers	5' - GGACGTTGTAGAACTTGT - 3'
	5' - ACTGTTCCACTATTCGTT - 3'
ST Primers	5' - CAGCTCAACAAGTGGTAA - 3'
	5' - ACAAAAAGGGGAATCTAC - 3'
R1 Primer	5' - TCGTCGCTGACTTAGCTG - 3'
E2 Primer	5' - GAATTCCAGGTAAGT - 3'
E4 Primer	5' - GGAATTCCACCTGCA - 3'