Functional characterisation of phosphorus uptake pathways in a non-responsive arbuscular mycorrhizal host

by

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Abstract

AM plants acquire P_i via two pathways; the direct uptake pathway via plant roots and the AM pathway via external fungal hyphae and colonised cortical cells. It has been assumed that these two pathways are additive and therefore in non-responsive plants the AM pathway is often considered to be non-functional. However, data from ^{32}P uptake studies indicates that the AM pathway is functional in many non-responsive symbioses and in some instances supplies the majority of plant P. In recent years the high-affinity P_i transporters involved in both direct and AM P_i uptake pathways have been identified. They are expressed at the root epidermis and the symbiotic interface of colonised cortical cells and respond to the P and AM status of the plant. The overall objective of the work described in this thesis was to characterise P_i uptake via the AM pathway in barley, a non-responsive AM host, using an approach which integrated physiological measurements of plant responsiveness and AM contribution with investigations of gene expression and functional characterisation of the plant P_i transporters.

A preliminary survey of field-grown barley demonstrated the persistence of AM colonisation under commercial cropping regimes in southern Australia and highlighted the relevance of AM studies to commercial agriculture. Under glasshouse conditions AM colonisation of barley induced depressions in growth and P uptake compared to NM controls. Growth depressions were unrelated to percent colonisation by two AM fungal species and could not readily be explained by fungal C demand; the strong correlation between growth and P content suggested that P was the limiting factor in these experiments. However, a compartmented pot system incorporating ^{32}P-labelling demonstrated that the AM pathway is functional in colonised barley and, in the interaction with *G. intraradices,* contributed 48% of total P. This suggested that P flux via the direct uptake pathway is decreased in AM barley.

The expression of three P_i transporters, *HvPT1*, *HvPT2* and *HvPT8* was investigated in colonised roots. *HvPT1* and *HvPT2* have previously been localised to the root epidermis and root hairs and are involved in P_i uptake via the direct pathway whilst *HvPT8* is an AM-inducible P_i transporter which was localised by *in-situ* hybridisation to colonised cortical cells. Using promoter::GFP gene fusions the localisation of *HvPT8* to arbuscule-containing cortical cells was confirmed in living roots from transgenic barley. Quantitative real-time PCR analysis of the expression of these three P_i transporters indicated that *HvPT1* and *HvPT2* were expressed constantly, under all conditions regardless of AM colonisation status and indicated that decreased P flux via the direct pathway is

not related to expression of these transporters. *HvPT8* was induced in AM colonised roots. However, the level of expression was not related to flux via the AM pathway or arbuscular colonisation.

The *HvPT8* transporter was further characterised by constitutive over-expression in transgenic barley. ^{32}P uptake assays in excised roots demonstrated increased P_i uptake from low P solution compared to wild-type roots and confirmed that *HvPT8* is a functional P_i transporter with high-affinity transport properties. This is the first report of characterisation of an AM-inducible P_i transporter *in planta*. When these transgenic plants were grown in solution culture there was no increase in growth or P uptake relative to wild-type or transgenic controls and growth in soil and AM colonisation were also unaffected in these transgenic lines.

The data presented in this thesis highlights the importance of combined physiological and molecular approaches to characterising plant AM interactions. The persistence of AM colonisation in barley in the field indicates the importance of improving our understanding of symbiotic function in non-responsive plants. Future efforts should be directed towards understanding the signals which regulate P flux via both the direct and AM pathways with the ultimate aim of enhancing AM responsiveness of non-responsive species. Making the direct and AM pathways additive in non-responsive species should be a key aim of future research.

Declaration

This work 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 has been made in the text.

I give consent for this copy of my thesis, when deposited in the University Library, being made available in all forms of media, now or hereafter known.

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Date

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Publications arising from this thesis

Book Chapter: Invited Publication

Grace EJ, FA Smith, SE Smith (*submitted*) Deciphering the arbuscular mycorrhizal pathway of P uptake in non-responsive hosts. In: Azcón-Aguilar C, Barea JM, Gianinazzi S, Gianinazzi-Pearson V, Eds. *Mycorrhizas: functional processes and ecological impact*, Springer.

Conference Abstracts: Oral presentations

Grace EJ (2007) Mycorrhizal symbiosis in barley: Secret deals and insider trading, Royal Society of South Australia, Adelaide, Australia.

Grace EJ, O Cotsaftis, M Tester, FA Smith, SE Smith (2006) Characterising phosphate transport in barley colonised by arbuscular mycorrhizal fungi, 8th International Congress of Plant Molecular Biology, Adelaide, Australia.

Grace EJ, O Cotsaftis, M Tester, FA Smith, SE Smith (2006) Phosphorous uptake in a non-responsive host plant: deciphering the mycorrhizal pathway, 5th International Conference on Mycorrhiza, Granada, Spain.

Conference Abstracts: Poster presentations

Grace EJ, D Glassop, O Cotsaftis, M Tester, FA Smith, SE Smith (2005) Arbuscular mycorrhizal fungi influence phosphorus uptake by barley, ComBio Conference, Adelaide, Australia.

Grace EJ, SE Smith, FA Smith, M Tester (2005) The influence of arbuscular mycorrhizal fungi on phosphate uptake by barley, Genomics in the Barossa Conference, Adelaide, Australia.

Abbreviations & Symbols

% RLC	percent root length colonised
%	percent
~	approximately
^{o}C	degrees celcius
AM	arbuscular mycorrhiza
ATP, ADP	adenosine triphosphate, adenosine diphosphate
bp	base pairs, of nucleic acids
C	carbon
cDNA	complementary DNA
cv.	cultivar
d, h, min, s	day, hour, minute, second
DNA, RNA	deoxyribo, ribonucleic acid
DTT	dithiothreitol
EC	electrical conductivity
EDTA	ethylenediaminetetraaceticacid
EST	expressed sequence tag
EtOH	ethanol
gDNA	genomic DNA
GDP	gross domestic product
GFP	green fluorescent protein
GOI	gene-of-interest
ha	hectare
kg, mg, µg, ng	kilo, milli, micro, nanogram
K_m	Michaelis constant; affinity of an enzyme for a substrate
L, mL, µL	Litre, millilitre, microlitre
LSCM	laser scanning confocal microscope
m, cm, µm, nm	metre, centimetre, micrometre, nanometre
M	molar
mol	mole
N	nitrogen
NM	non-mycorrhizal
P	phosphorus

P	probability
PCR (RT)	polymerase chain reaction; reverse transcription
P_i	inorganic orthophosphate
Q PCR	quantitative real-time PCR
RE	restriction enzyme
RO	reverse osmosis
rpm	revolutions per minute
RT	room temperature
S/N	supernatant
UV	ultraviolet
V	volt
V_{max}	maximum velocity of an enzyme mediated reaction
W	watt
w/v, w/w	weight per volume; weight per weight

Chapter 1

Introduction and Review of the Literature

The overall aim of the work described in this thesis was to investigate the contribution of arbuscular mycorrhizal fungi to plant P uptake in a non-responsive host using a combination of physiological and molecular techniques. This chapter reviews relevant literature and sets out the specific aims of the thesis. Since the project began in 2004 a number of key findings have been published; those publications up to the time of writing will be included in the following discussion.

1.1 Introduction

Arbuscular mycorrhizas (AM) are ancient symbioses which occur when fungi of the phylum Glomeromycota colonise the roots of host plants (Smith & Read, 1997; Schüßler *et al.*, 2001). Earliest evidence of AM dates to 400 million years ago and it has been proposed that AM fungi may have colonised primitive and poorly developed roots enabling the colonisation of the land by early plants (Nicolson, 1975; Remy *et al.*, 1994). In modern times AM symbioses are widespread, occurring in both agricultural and natural ecosystems and it has been estimated that ~80% of land plants are capable of forming AM associations (Smith & Read, 1997).

The basis for the symbiosis is bi-directional nutrient transfer. AM fungi are obligate symbionts which rely upon host plants as sole sources of carbon (C) in the form of hexose. In return plants are supplied with nutrients such as phosphate (P_i) and nitrogen (N) which are taken up from the soil by external hyphae of the fungi (Smith & Read, 1997). The evolutionary conservation of this symbiosis and its widespread occurrence are a testament to the importance of AM in plant function. Indeed AM symbioses have been demonstrated to improve disease tolerance, increase drought resistance and decrease the accumulation of heavy metals, although it has been suggested that these benefits have evolved relatively recently (Fitter, 2006). Improved P nutrition is still considered the primary benefit of AM symbioses and total plant P and growth response are the most commonly reported measures of AM function. Plant responses to AM colonisation are diverse, ranging from large positive increases in growth and P uptake to growth depressions (Tawaraya, 2003). This diversity in responsiveness is considered to reflect the diversity in function of different plant-AM fungal combinations. This study focuses on the role of AM fungi in plant nutrient uptake of non-responsive plant species, with particular emphasis on phosphorus (P).

1.2 Phosphorus – an essential plant nutrient

Phosphorus is one of the 14 mineral nutrients essential for the growth of higher plants. Although it accounts for a relatively small percentage of total plant biomass, 0.2% in grasslands and crop species and as little as 0.025% of forest biomass (Smil, 2000), it plays a significant role in biological processes central to the function of all living organisms. At the molecular level phosphodiester bonds form the backbone of DNA and RNA, phospholipids form the membrane boundaries of cells and the cleavage and formation of phosphoanhydride bonds of adenosine phosphates (ATP, ADP) are the major energy currency of all living organisms. In agronomic terms P supply is important for new growth, flowering, fruiting and seed production.

1.3 Phosphorus in agriculture and the environment

Low P availability results in one of the most widespread mineral nutrient deficiencies limiting agricultural productivity in the world and certainly in Australia (Holford, 1997). In order to overcome P deficiencies and achieve high levels of productivity, P-based fertilizers are applied in most agricultural systems. Whilst overcoming problems of P limitation this creates a new set of problems. The immediate recovery of P from fertilizers by plants is estimated to be as low as 10-20%, with the remainder being immobilised in the soil or lost to waterways where it is a significant contributor to eutrophication of lakes and streams (Holford, 1997; Miyasaka & Habte, 2001; Richardson, 2001). In addition, with the decline in readily available phosphate rock reserves, current P fertilisation practices cannot be sustained indefinitely (Richardson, 2001). Thus there is increasing emphasis on the efficient use of soil P in agricultural systems. Considerable resources have been invested into improving plant growth and nutrition under P-limiting conditions, through increased uptake and improved resource allocation via traditional plant breeding and modern technologies such as genetic engineering. In order to appropriately target such programs it is essential to first understand the mechanisms of plant P uptake.

1.4 Plant-available soil P

Plants acquire P in the form of inorganic orthophosphate (P_i), predominantly $H_2PO_4^-$, which they actively take up from the soil solution (Schachtman *et al.*, 1998; Miyasaka & Habte, 2001). Whilst the total quantity of P in soils can be quite high, as little as 1% of this occurs in solution where the concentration rarely exceeds 10 µM (Bieleski, 1973). The concentration of P_i in the soil solution is

governed by cycling between two forms, organic and inorganic P, and chemical equilibria associated with the adsorption and precipitation of the inorganic form (Figure 1.1).

Phosphate is extremely chemically reactive. In the soil it is adsorbed to the surface of clay minerals and forms insoluble phosphates, predominantly Fe and Al precipitates under acid conditions, and Ca and Mg complexes under alkaline or neutral conditions (Holford, 1997). The low solubility of these compounds and the tendency of equilibria to favour the solid phase results in the low concentration of P_i in the soil solution. In addition, some 20-80% of P in soils occurs in organic form and is only available to plants indirectly after mineralisation by soil microorganisms (Richardson, 1994). Mineralisation releases P_i into solution where it is subject to inorganic P equilibria (Figure 1.1). However, soil microorganisms are also responsible for the immobilisation of soil solution P_i.
Whether microorganisms act as a net source of P within the rhizosphere or as a temporary (or longer term) sink is unknown (Richardson, 2001).

Figure 1.1 Soil P pools and plant acquisition. Adapted from Schachtman *et al.* (1998)

Rapid removal of nutrients from the soil solution by plant roots results in a localised decrease in concentration at the root surface. The ability of soil to maintain nutrient concentrations at the root surface is therefore an important factor governing the rate of plant uptake. For the more soluble, and thus more mobile nutrients such as NO_3^-, NH_4^+, K^+ and SO_4^{2-}, supply to plant roots is maintained via the transpiration-driven process of mass flow, whereas P_i is moved mainly by diffusion (Marschner, 1995). The slow rate of diffusion of P_i in soil (10^{-12}-10^{-15} m^2 s^{-1}, Schachtman *et al.*, 1998) compared with high plant uptake rates (0.2-4.4 nmol g^{-1} FW root min^{-1} calculated from Elliot *et al.* (1984) and Clarkson *et al.* (1978) for maize and barley) results in the formation of a depletion zone immediately adjacent to the root surface. P_i uptake is thus limited by the rate of

diffusive movement of P_i anions into this depletion zone and/or the ability of the plant to extend into un-depleted patches of soil P.

1.5 Plant adaptations to low P

Plants have evolved a range of physical, biochemical and symbiotic adaptations to increase access to P under growth-limiting conditions. These include alterations to root morphology such as increasing total root length, degree of root branching and abundance and distribution of root hairs which increase the absorptive surface area of roots and extend P_i uptake beyond depletion zones at the root surface (Marschner, 1998). In some plant species P deficiency results in the formation of specialised roots (proteoid or cluster roots) which concentrate root exudates in localised patches and increase acquisition of both P and other immobile nutrients; for review see Dinkelaker *et al.* (1995), Marschner (1995), Neumann *et al.* (2000) and Vance *et al.* (2003). Biochemical adaptations include increased production of extracellular phosphatases and root exudates such as protons and/or organic anions which either directly, or indirectly through the stimulation of microbial activity, increase P_i availability in the rhizosphere (Miyasaka & Habte, 2001; Richardson, 2001).

As outlined at the beginning of this chapter, AM symbioses are perhaps the earliest adaptation of plants to the acquisition of scarcely available nutrients from soil. The AM fungus colonises host roots and the external mycelium extends beyond nutrient depletion zones, exploiting soil volumes which would otherwise be unavailable to the plant. Although AM are not the only type of mycorrhiza formed in plant communities they are the most widespread and are the predominant form amongst economically important crop species (Smith & Read, 1997); thus they are the focus of the current work.

1.6 Arbuscular mycorrhizal symbioses

1.6.1 Establishment of colonisation

Colonisation of host roots is initiated from established fungal mycelium, colonised root fragments and/or fungal spores. Spore germination utilises storage compounds from within the spore, allowing for host-independent germ tube development for up to two weeks. However, if a host is not encountered in this time the spore will re-enter a state of dormancy. This represents a considerable hurdle in the study of AM fungi which, despite repeated attempts, are unable to be cultured axenically (Bago *et al.*, 2000) in the absence of living plant roots.

Upon encountering a host root, penetration by fungal hyphae is aided by the formation of appressoria on the root surface. Fungal hyphae spread both inter- and intra-cellularly within the root cortex. Two distinct colonisation patterns, *Arum*- and *Paris*-types, were first recognised by Gallaud (1905) (Figure 1.2).

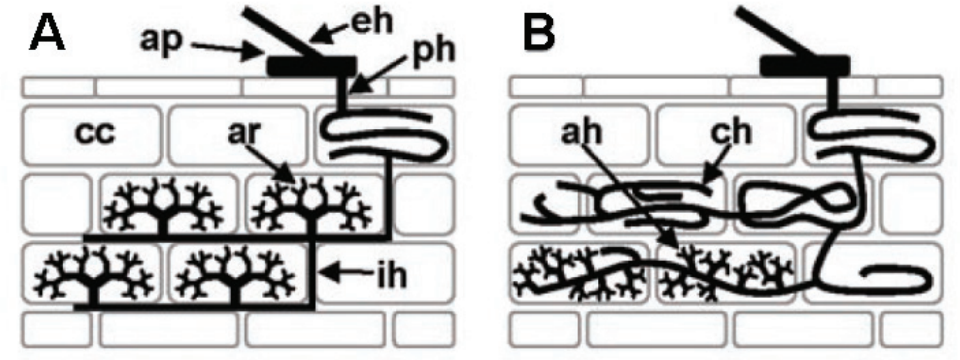


Figure 1.2 Features of *Arum*- (A) and *Paris*- (B) types of AM colonisation; (eh) extraradical hyphae, (ap) appressoria, (ph) penetration hyphae, (ih) intercellular hyphae, (cc) cortical cells and (ar) branched arbuscules of *Arum*-type; (ch) thick coiled intracellular hyphae and (ah) fine branched arbusculate hyphae of *Paris*-type. Intermediate type AM combine features of *Arum*- and *Paris*-types. Reproduced from Karandashov *et al.* (2004).

In *Arum*-type symbioses, hyphae extend rapidly through the intercellular spaces of the root cortex. Upon reaching the inner cortex short side branches penetrate cortical cell walls, invaginating the plasma membrane and forming dichotomously branched structures known as arbuscules (Figure 1.2 A). The extension of host plasma membrane surrounding the growing arbuscule represents a 3.7-fold increase in surface area compared with non-colonised cortical cells (Alexander *et al.*, 1989). The formation of these extensive and intimate interfaces led early researchers to hypothesise that arbuscules formed the primary site of nutrient transfer in AM symbioses.

Arbuscules are relatively short-lived structures; mature arbuscules last 3-4 d before they collapse and cortical cells return to their pre-colonised state (Alexander *et al.*, 1988). In comparison, the intercellular hyphae are relatively long-lived and capable of extending and continuing colonisation as roots lengthen. Thus a single root can contain colonisation units at all stages of AM development.

Although *Paris*-type AM are more widespread in natural ecosystems (Smith & Smith, 1997) the predominance of *Arum*-types in crops and many of the plant species used in laboratory investigations has led to a focus on this form (Harrison, 1999). It is only recently that the physiology and molecular biology of *Paris*-type AM has begun to be investigated.

In *Paris*-type symbioses there is very little, if any, intercellular growth. Spread of the fungus is much slower as it grows directly from cell to cell. On reaching the cortex, fungi forming *Paris*-type AM develop extensive intracellular coils known as hyphal coils which may then branch to form small protruding arbuscules (Figure 1.2 B). Both the intracellular hyphae and hyphal coils are relatively long-lived structures; as with arbuscules they are surrounded by host plasma membrane and remain in the apoplastic compartment of the root. Although the surface area of hyphal coils is equal to that of intracellular arbuscules (Dickson & Kolesik, 1999) these interfaces have been largely ignored as potential sites for nutrient transfer.

The morphology or colonisation type formed in AM symbioses was initially believed to be dependent on the host plant species, thus the *Arum*- and *Paris*-types derived their names from the plants in which they were first described (*Arum maculatum* and *Paris quadrifolia*) (Gallaud, 1905; Smith & Smith, 1997). However, experiments utilising a broader range of plant and fungal partners indicate that morphology is also influenced by fungal identity (Cavagnaro *et al.*, 2001) and as discussed in a recent review (Dickson *et al.*, 2007), environmental factors may also play a role. In addition, an extensive survey of 12 plant species colonised by 6 different fungal partners clearly demonstrated a continuum of mycorrhizal types ranging from true *Arum* colonisation patterns through Intermediate forms to *Paris*-types completely lacking arbuscules (Dickson, 2004). If symbiotic structures differ in their nutrient transfer abilities, the mycorrhizal types (*Arum*, *Paris* or Intermediate) may reflect differences in symbiotic efficiency. Whilst an extensive survey of AM types is beyond the scope of the current work it is important to note this diversity and to record the type of structures formed in different experimental host/fungus combinations.

1.6.2 Carbon metabolism and transfer between the symbionts

The C metabolism of the fungus has been investigated in a number of studies utilising ^{13}C isotopic labelling and Nuclear Magnetic Resonance spectroscopy (Shachar-Hill *et al.*, 1995; Pfeffer *et al.*, 1999; Bago *et al.*, 2000). These investigations demonstrated that the fungus was able to take up and utilise hexose from within the root. In contrast the extraradical mycelium was incapable of acquiring a range of C compounds from the external solution, including glucose, fructose, sucrose, mannitol and succinate (Pfeffer *et al.*, 1999; Bago *et al.*, 2000). Thus the AM fungus is entirely dependent on the host plant as its sole source of C. It has been estimated that C drain due to an AM fungus can account for 10 to 20 % of plant photosynthates (Jakobsen & Rosendahl, 1990).

The site of C transfer between the symbionts is uncertain; however, a growing body of evidence suggests that intercellular hyphae may be important sites for C uptake. The development of extraradical hyphae of the AM fungus has been used as an indicator of effective C transfer. Early observations by Mosse & Hepper (1975) and Hepper (1981) indicated growth of the external mycelium following appressorium formation and prior to the formation of arbuscules within plant cells. This is supported by a recent study with a reduced mycorrhizal colonisation mutant (*rmc*) of tomato (*Solanum lycopersicum*) (Barker *et al.*, 1998). Hyphae of the AM fungus *Scutellospora calospora* proliferated outside the root despite colonisation being blocked in the root hypodermis prior to arbuscule formation (Manjarrez-Martinez, 2007).

Whether C uptake by the AM fungus is mediated by active transport or concentration-dependent fluxes due to the rapid removal and conversion of hexose within fungal hyphae is unclear. As yet no AM fungal C transporters have been identified. The mechanism by which hexose is released from plant cells to the apoplast from where it is taken up by the fungus is also unknown, although a passive efflux mechanism stimulated by the presence of the fungus has been proposed (Woolhouse, 1975; Fitter, 2006). The identification of a plant hexose transporter that is up-regulated in colonised regions of mycorrhizal roots of *Medicago truncatula* (Mtst1) suggests that the plant is able to reabsorb hexose from the interfacial apoplast and thus compete with the AM fungus (Harrison, 1996). In the context of plant growth responses (whether positive or negative) the mechanisms regulating C transfer deserve increased attention.

1.6.3 Mineral uptake and transfer to the host plant

The external fungal mycelium extends beyond the nutrient depletion zone which develops around rapidly absorbing plant roots. The smaller diameter of fungal hyphae compared to plant roots gives them a greater surface area per unit volume (hence a smaller C investment) and enables access to smaller soil pores than plant roots. In addition fungal hyphae are better adapted to exploit patchy nutrients through rapid proliferation and competitive ability with soil microbes (Smith & Read, 1997). The extent and pattern of external mycelium is extremely variable and depends upon the AM fungal species. Hyphal lengths ranging from 1 to >30 m g^{-1} soil have been measured (Smith *et al.*, 2004). Compartmented pot systems utilising isotopically labelled or radiolabelled hyphal compartments have provided evidence for the translocation of P_i, Zn, NO_3^- and NH_4^+ from distances of up to 25 cm from plant roots (Johansen *et al.*, 1993; Pearson & Jakobsen, 1993; Tobar *et al.*, 1994; Smith *et al.*, 2000; Jansa *et al.*, 2003). An AM plant thus has two possible pathways for the acquisition of

nutrients from the soil; the direct uptake pathway via the root epidermis and root hairs and the AM pathway via external fungal hyphae and colonised cortical cells (Figure 1.3). The total nutrient content of an AM plant results from the combined operation of these pathways. In terms of plant P nutrition, the plant P_i uptake pathway has been studied extensively in model systems such as *Arabidopsis thaliana* (which is constitutively non-mycorrhizal [NM]). However, the prevalence of AM in most natural environments suggests that an understanding of the integration of the two pathways is essential to provide a realistic picture of plant P_i uptake. The following discussion focuses on the processes involved in P acquisition by AM plants.

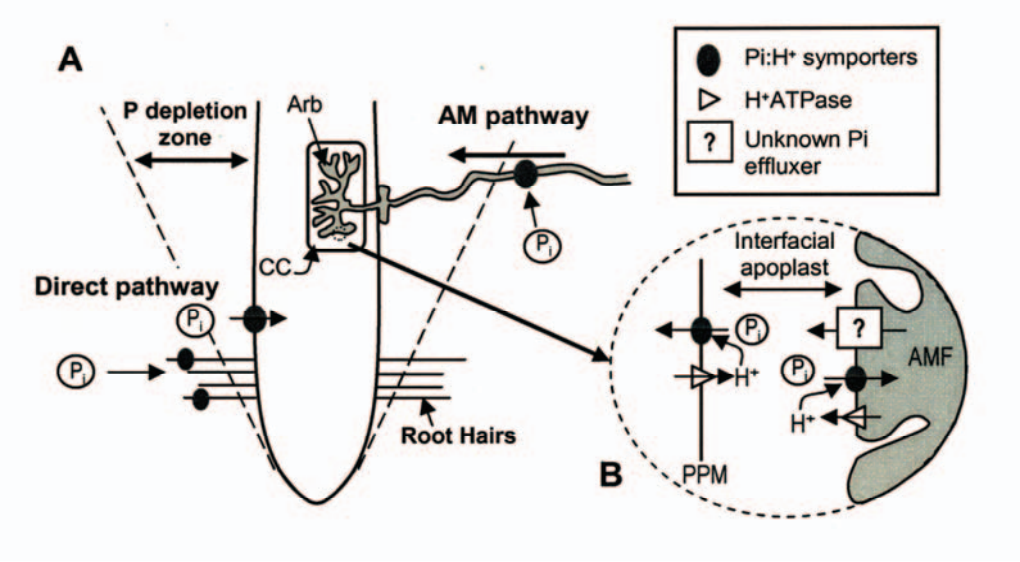


Figure 1.3 Schematic representation of P_i uptake in an AM root. A. P_i uptake from soil solution is mediated by plant and fungal P_i:H^+ symporters expressed in external hyphae or root epidermis and root hairs. Rapid uptake of P_i by roots leads to formation of a depletion zone, fungal hyphae extend beyond depletion zones taking up P_i and transporting it to intracellular arbuscules (Arb) in colonised cortical cells (CC). B. P_i taken up by the fungus is released into the interfacial apoplast via an unknown mechanism. Plant P_i uptake across the plant plasma membrane (PPM) at symbiotic interfaces is also mediated by P_i:H^+ symporters in competition with AM fungal retrieval.

1.7 The challenge of P_i uptake from soil

Both plants and AM fungi accumulate P_i against a considerable electrochemical gradient. Whilst soil solution concentrations rarely exceed 10 µM and are typically less than 2µM (Bieleski, 1973), cytosolic P_i concentrations are within the millimolar range (Mimura *et al.*, 1996; Smith *et al.*, 2001). In addition the P_i anion is accumulated against the negative potential difference that is generated across the plasma membrane of plant and fungal cells. Thus P_i accumulation requires an energy-driven, high-affinity transport process. This is consistent with observations in barley (*Hordeum vulgare*), potato (*Solanum tuberosum*) and tomato roots (Clarkson & Scattergood, 1982; Cogliatti &

Clarkson, 1983) and hyphal germ tubes of the AM fungus *Gigaspora margarita* (Thomson *et al.*, 1990), which demonstrated high-affinity kinetics when uptake was measured from ^{32}P-labelled media.

Plant P_i uptake has been extensively studied in the roots of higher plants and cultured cells. It has been proposed that P_i uptake occurs as an energy-mediated, co-transport process driven by the proton motive force generated by a plasma membrane H^+-ATPase. This hypothesis is supported by the observation of a transient decrease in cytoplasmic pH during short-term P_i uptake into roots of *Limnobium stoloniferum* and cells of *Catharanthus roseus* which is consistent with H^+:$H_2PO_4^-$ co-transport (Ullrich & Novacky, 1990; Sakano *et al.*, 1992). Furthermore, studies in *Lemna gibba* and *C. roseus* cells indicate that P_i uptake occurs with a stoichiometry of 2 to 4 $H^+/H_2PO_4^-$ transported, resulting in alkalisation of the media and transient membrane depolarisation (Ullrich-Eberius *et al.*, 1981; 1984; Sakano, 1990). In AM fungal hyphae a similar stoichiometry for H^+:$H_2PO_4^-$ co-transport has been calculated (Ayling *et al.*, 2000).

During the last decade the plant and fungal proteins involved in P_i uptake via both direct and AM fungal uptake pathways have been identified. These transporters share a high level of sequence similarity and together are members of the P_i:H^+ symporter family (Pao *et al.*, 1998). It is evident that these transporters play a pivotal role in plant and fungal P_i uptake.

1.8 The AM pathway of P_i uptake

Accumulation of P_i from soil solution by AM hyphae is governed by high-affinity P_i transporters. Three such transporters have been identified to date. The *Glomus versiforme* P_i transporter, *GvPT*, was identified by cross hybridisation to the *Saccharomyces cerevisiae* high-affinity P_i transporter *PHO84* (Harrison & van Buuren, 1995). GvPT was able to complement a *S. cerevisiae* mutant defective in the function of PHO84 with an apparent K_m of 18 µM. Expression analysis revealed that *GvPT* is predominantly expressed in the external mycelium, and provided the first evidence of differential regulation of gene expression between internal and external mycelium of the aseptate AM fungus. *In-vitro* split-plate analysis of a *GvPT* orthologue, *GiPT* from *G. intraradices*, revealed that these transporters respond to P concentrations in the environment and are sensitive to the overall P status of the fungus (Maldonado-Mendoza *et al.*, 2001). This was confirmed with the identification of *GmosPT* from *G. mosseae,* which also demonstrated reduced transcript abundance in extraradical mycelium with increasing external P concentration (Benedetto *et al.*, 2005). In

contrast to *GvPT*, expression of *GmosPT* was observed not only in external mycelium but also at high levels in AM roots. Using laser microdissection associated with gene expression analyses, Balestrini *et al.* (2007) demonstrated that the intraradical expression of *GmosPT* is localised to arbuscules in cortical cells of tomato. Expression of the AM fungal H^+-ATPase, *GmHA5* was also identified exclusively in arbuscules in this study. The expression of both a fungal P_i transporter and a fungal H^+-ATPase in arbuscules provides the first evidence for potential active transport of P by fungal structures at intracellular interfaces, and suggests that the fungal symbiont may compete with the plant for P_i uptake from the interfacial apoplast. If AM fungi have different abilities to reacquire P_i from the interfacial apoplast this may have significant implications for efficiency of symbiotic P transfer and hence the overall outcome of different plant-fungus combinations.

Following P_i uptake by extraradical hyphae, amounts in excess of fungal metabolic requirements are transferred to the vacuole where they are stored as polyphosphate (poly-P) (Rasmussen *et al.*, 2000). Numerous studies have investigated the dynamics of poly-P sequestration, storage and transport in AM hyphae. Cytoplasmic streaming and a motile tubular vacuole system have both been implicated in the subsequent movement of poly-P in the direction of the plant root (Cox *et al.*, 1980; Shepherd *et al.*, 1993). As poly-P enters the intraradical hyphae chain-lengths become shorter (Solaiman *et al.*, 1999) and poly-P is hydrolysed to P_i which is released to the plant cell. The identification of phosphatase activity in arbuscules and in the intraradical hyphae and hyphal coils of *Paris*-type AM and the localisation of plant P_i transporter expression to cortical cells containing arbuscules and hyphal coils (see Section 1.9.2), provides strong evidence for P_i transfer via intracellular interfaces (Harrison *et al.*, 2002; Karandashov *et al.*, 2004; Glassop *et al.*, 2005; van Aarle *et al.*, 2005). However, it is noteworthy that the fungus remains within an apoplastic compartment, with no cytoplasmic continuity between the symbionts. Transfer of P between the symbionts therefore requires both efflux from the fungus and uptake by the plant (Figure 1.3 B).

The mechanisms for efflux of P from AM hyphae to symbiotic plant interfaces remain obscure. Leakage of P_i across fungal plasma membranes in degenerating arbuscules seems unlikely to account for the considerable quantity of P transferred (Cox & Tinker, 1976); consequently the existence of specific efflux channels or transporters has been proposed (Ferrol *et al.*, 2002). However, no such transporters have been identified to date. Due to the electrochemical driving forces, P_i released by the fungus into the interfacial apoplast must be actively transported across the plasma membrane into the plant cell, presumably presenting a challenge similar to the uptake of P_i from the soil, although P concentrations in the apoplast have not been determined.

Localisation of plant H^+-ATPases to plant membranes surrounding arbuscules (the periarbuscular membrane) (Gianinazzi-Pearson *et al.*, 2000) and the expression of plant P_i transporters supports this model. The plant P_i transporters expressed at symbiotic interfaces are members of the same family as the root epidermal P_i transporters; insights from their expression and functional characterisation are discussed below.

1.9 Plant P_i uptake from soil and symbiotic interfaces

1.9.1 Plant high-affinity P_i transporters

The family of plant high-affinity P_i transporters responsible for uptake at the root:soil and symbiotic interfaces was first identified in *Arabidopsis* by EST similarities to P_i transporters from yeast, filamentous fungi and AM fungi (*GvPT*) (Muchhal *et al.*, 1996; Smith *et al.*, 1997). Soon afterwards orthologues were cloned from the roots of potato (*StPT1, StPT2, StPT3*) (Leggewie *et al.*, 1997; Rausch *et al.*, 2001), tomato (*LePT1, LePT2*) (Daram *et al.*, 1998; Liu *et al.*, 1998a; Rosewarne *et al.*, 1999) and *Medicago* (*MtPT1, MtPT2, MtPT4*) (Liu *et al.*, 1998b; Harrison *et al.*, 2002). Both StPT3 and MtPT4 are involved in plant P_i uptake via the AM pathway.

Phylogenetic analysis groups these transporters together in the PhT1 family (Bucher *et al.*, 2001), distinct from the P_i:H^+ symporters of the PhT2 and PhT3 families that have been cloned from plant organelles (Karandashov & Bucher, 2005). A sub-group of the PhT1 family is formed by the P_i transporters involved in P_i uptake via the AM pathway (see phylogenetic tree, Appendix 1). However, there are two notable exceptions; StPT3 of potato (Rausch *et al.*, 2001) and LjPT3 of *Lotus japonicus* (Maeda *et al.*, 2006) cluster with the main group of PhT1 transporters. It will be interesting to see whether these sub-groupings relate to real differences in transporter function.

Models of protein structure predict that PhT1 transporters are integral membrane proteins consisting of 12 membrane-spanning domains arranged in a 6+6 configuration separated by a long hydrophilic loop oriented towards the inner surface of the membrane. This is a common feature shared by a range of plant proteins involved in the transport of organic acids, amino acids, sugars and inorganic ions (Smith *et al.*, 2003a).

Those transporters with relevance to the current discussion are listed in Table 1.1. Eight closely related members of the PhT1 family have been isolated from barley (Smith *et al.*, 1999) and sequencing of the *Arabidopsis* and rice (*Oryza sativa*) genomes revealed nine and thirteen PhT1

transporters in each, respectively (Okumura *et al.*, 1998; Mudge *et al.*, 2002; Paszkowski *et al.*, 2002). The large number of genes found in these families suggests some functional overlap. However, it is also indicative of the pivotal role that P and hence its uptake and redistribution play in plant function.

Table 1.1 Details of plant and fungal P_i transporters referred to in the present discussion. The tissue localisation and response to AM colonisation of plant P_i transporters are reported. Adapted from Javot *et al.* (2007b).

Organism	Official nomenclature[a]	Other names[b]	Tissue localisation[c]	AM response[d]	Apparent K_m[e]	Reference[f]
Ascomycota (Saccharomycetaceae)						
Saccharomyces cerevisiae		PHO84			8 µM	1
Glomeromycota						
Glomus versiforme		GvPT			18 µM (yeast)	2
Glomus intraradices		GiPT				3
Glomus mosseae		GmosPT				4
Brassicaceae						
Arabidopsis thaliana	ARAth;Pht1;1	Pht1:1	St; Rt		110 µM (yeast) 3.1 µM (tobacco)	5, 6, 7, 8
	ARAth;Pht1;2	Pht1:2	Rt			5, 8
	ARAth;Pht1;3	Pht1:3	St; Rt			5
	ARAth;Pht1;4	Pht1:4	St; Rt		110 µM (yeast)	5, 7
	ARAth;Pht1;5	Pht1:5	St; Rt			5
	ARAth;Pht1;6	Pht1:6	St			5
	ARAth;Pht1;7	Pht1:7	St; Rt			5
	ARAth;Pht1;8	Pht1:8	Rt			5
	ARAth;Pht1;9	Pht1:9	Rt			5
Solanaceae						
Tomato (*Solanum lycopersicum)*	LYCes;Pht1;1	LePT1	St; Rt		31 µM (yeast)	9,10,11,12,
	LYCes;Pht1;2	LePT2	Rt			10,12
	LYCes;Pht1;3	LePT3	nd	AM +		12
	LYCes;Pht1;4	LePT4	Rt	AM S		12
	LYCes;Pht1;5	LePT5	Rt	AM +		12
Potato (*Solanum tuberosum*)	SOLtu;Pht1;1	StPT1	St; Rt	AM -	280 µM (yeast)	12,13,14
	SOLtu;Pht1;2	StPT2	Rt	AM -	130 µM (yeast)	12,14
	SOLtu;Pht1;3	StPT3	St; Rt	AM +	64 µM (yeast)	12, 14, 15
	SOLtu;Pht1;4	StPT4	Rt	AM S		12
	SOLtu;Pht1;5	StPT5	Rt	AM S		12
Fabaceae						
Medicago truncatula	MEDtr;Pht1;1	MtPT1	Rt	AM -	192 µM (yeast)	16,17, 18
	MEDtr;Pht1;2	MtPT2	Rt	AM -		16, 17
	MEDtr;Pht1;4	MtPT4	Rt	AM S	493–668 µM (yeast)	15, 18
Lotus japonicus	LOTja;Pht1;1	LjPT1	nd	AM -		19
	LOTja;Pht1;2	LjPT2	nd	AM -		19
	LOTja;Pht1;3	LjPT3	nd	AM +		19
Poaceae						
Barley (*Hordeum vulgare*)	HORvu;Pht1;1	HvPT1	Rt	AM -	9 µM (rice)	20, 21, 22
	HORvu;Pht1;2	HvPT2	Rt	AM -		20, 22
	HORvu;Pht1;3	HvPT3	Rt	nr		20, 22
	HORvu;Pht1;4					22
	HORvu;Pht1;5					22
	HORvu;Pht1;6		St		385 µM (rice)	21, 22
	HORvu;Pht1;7					22
	HORvu;Pht1;8		Rt	AM +		22

Continued over page…

Table 1.1 Continued...

Organism	Official nomenclature[a]	Other names[b]	Tissue localisation[c]	AM response[d]	Apparent K_m[e]	Reference[f]
Poaceae						
Rice (*Oryza sativa*)	ORYsa;Pht1;1	OSPT1		AM -		23
	ORYsa;Pht1;2	OSPT2	St; Rt	AM -		23
	ORYsa;Pht1;3	OSPT3		AM -		23
	ORYsa;Pht1;4	OSPT4				23
	ORYsa;Pht1;5	OSPT5				23
	ORYsa;Pht1;6	OSPT6		AM -		23
	ORYsa;Pht1;7	OSPT7				23
	ORYsa;Pht1;8	OSPT8				23
	ORYsa;Pht1;9	OSPT9		AM -		23
	ORYsa;Pht1;10	OSPT10		AM -		23
	ORYsa;Pht1;11	OSPT11	Rt	AM S		15, 23, 24
	ORYsa;Pht1;12	OSPT12				23
	ORYsa;Pht1;13	OSPT13		AM +		23, 24
Wheat *Triticum aestivum*	TRIae;Pht1;myc			AM S		22
Maize (*Zea mays*)	ZEAma;Pht1;1	ZmPT2	St; Rt	AM -		25, 26
	ZEAma;Pht1;2		St; Rt			26
	ZEAma;Pht1;3		St; Rt			26
	ZEAma;Pht1;4	ZmPT1	St; Rt	AM -		25, 26
	ZEAma;Pht1;5					26
	ZEAma;Pht1;6		St; Rt	AM +		22, 26
	*	ZmPT3		AM -		25

[a]Name of plant P_i transporters according to the official nomenclature (Karandashov & Bucher, 2005). *not yet assigned names following the official nomenclature.

[b]Name of plant P_i transporters as indicated in the original references and cited in this text.

[c]Tissue localisation of plant P_i transporters: Shoot expression (St); Root expression (Rt). Data are only listed for those transporters where both shoot and root tissues have been examined.

[d]Response to AM symbiosis: mycorrhiza specific (AM S), up-regulated (AM +), down-regulated (AM -) or no response (nr).

[e]The expression system used to determine the K_m is indicated in brackets. Heterologous expression in yeast (yeast), tobacco cell culture (tobacco) or rice cell culture (rice) has been reported.

[f]References: 1: Bun-Ya *et al.*, 1991; 2: Harrison & van Buuren, 1995; 3: Maldonado-Mendoza *et al.*, 2001; 4: Benedetto *et al.*, 2005; 5: Mudge *et al.*, 2002; 6: Mitsukawa *et al.*, 1997; 7: Muchhal *et al.*, 1996; 8: Smith *et al.*, 1997; 9: Daram *et al.*, 1998; 10: Liu *et al.*, 1998a; 11: Rosewarne *et al.*, 1999; 12: Nagy *et al.*, 2005; 13: Leggewie *et al.*, 1997; 14:Rausch *et al.*, 2001; 15: Karandashov *et al.*, 2004; 16: Liu *et al.*, 1998b; 17: Chiou *et al.*, 2001; 18: Harrison *et al.*, 2002; 19: Maeda *et al.*, 2006; 20: Smith *et al.*, 1999; 21: Rae *et al.*, 2003; 22: Glassop *et al.*, 2005; 23: Paszkowski *et al.*, 2002; 24: Güimil *et al.*, 2005; 25: Wright *et al.*, 2005; 26: Nagy *et al.*, 2006.

1.9.2 Localisation and expression analyses of plant P_i transporters

Whilst P_i transporters expressed in roots are the primary focus of the current work it should be noted that different PhT1 transporters are expressed in a cell-specific manner in tissues throughout the plant. Localisation of the *Arabidopsis* transporters using promoter::reporter gene fusions revealed precise spatial and temporal expression patterns for the nine PhT1 transporters (Mudge *et al.*, 2002). Although eight of these were expressed in roots, expression was also observed in senescing leaves, anthers, flower buds and pollen grains (Mudge *et al.*, 2002) and it has been proposed that the PhT1 transporters will play an important role in P_i mobilisation wherever

symplastic connections are absent (Smith *et al.*, 2003a). Such sites include the apoplastic interface of cortical cells colonised by AM fungi.

Root epidermal P_i transporters involved in the direct P_i uptake pathway

Four members of the *Arabidopsis* PhT1 family are likely to be involved in P_i uptake from the soil solution (Mudge *et al.*, 2002). Reporter gene expression driven by the promoters of *Pht1;1*, *Pht1;2*, *Pht1;3* and *Pht1;4* was observed in the root epidermis and was induced when roots were grown at low P. In addition *Pht1;1*, *Pht1;2* and *Pht1;3* were preferentially expressed in the trichoblast cells of the epidermis that produce root hairs. Selectivity of expression in trichoblast cells was also observed for the barley *HvPT1* and *HvPT2* transporters (Schunmann *et al.*, 2004). Utilising protein::reporter gene fusions and *in-situ* hybridisation, Chiou *et al.* (2001) demonstrated the localisation of *Medicago MtPT1* to the plasma membrane of both epidermal and root hair cells. This is consistent with expression patterns for *LePT1* and *LePT2* in tomato (Daram *et al.*, 1998; Liu *et al.*, 1998a) and *StPT1* and *StPT2* in potato (Leggewie *et al.*, 1997), although immunolocalisation of StPT2 revealed that its expression is restricted to the plasma membrane on the external surface of epidermal cells (Gordon-Weeks *et al.*, 2003). Polarised plasma membrane localisation was also observed for MtPT4 (Harrison *et al.*, 2002) (see below). These are the first examples of polarised membrane localisation of plant nutrient transporters to be reported and are consistent with a role in P_i uptake from the soil solution and symbiotic interfaces, respectively.

The up-regulation of gene transcripts under P-deficient conditions has been observed consistently for the root epidermal P_i transporters (Muchhal *et al.*, 1996; Leggewie *et al.*, 1997; Smith *et al.*, 1997; Liu *et al.*, 1998a; Liu *et al.*, 1998b; Rae *et al.*, 2003). Schunmann *et al.* (2004) reported a 2.5- to 3-fold increase in *HvPT1* and *HvPT2* expression in barley plants grown at zero P compared to plants grown at 0.2 mM P. In tomato this was shown to be a reversible response; *LePT1* and *LePT2* transcript abundance decreased to control levels within 24 h of the resupply of P (Liu *et al.*, 1998a). Analysis of LePT1 and MtPT1 protein abundance by western blots demonstrated that the observed increase in transcript under P starvation is mirrored by a concurrent increase in transporter protein, and that protein accumulation is also reversible upon resupply of P (Muchhal & Raghothama, 1999; Chiou *et al.*, 2001).

In addition to regulation in response to P, responsiveness of the root epidermal P_i transporters to AM colonisation has also been observed (see Table 1.1). In rice, six of the ten P_i transporters

expressed in roots were down-regulated by AM colonisation at low P supply (Paszkowski *et al.*, 2002). In *Medicago* a steady decline in expression of the epidermal transporters *MtPT1* and *MtPT2* was observed with increasing AM colonisation (Liu *et al.*, 1998b). Once again MtPT1 protein levels mirrored transcript levels in this response (Chiou *et al.*, 2001). Such concomitant changes in transcript and protein abundance provide evidence for transcriptional control of P_i transporter regulation.

It has been suggested that the down-regulation of epidermal P_i transporters upon AM colonisation is primarily a function of improved P status of the plant (Burleigh & Bechmann, 2002). However, few gene expression studies have included those physiological measurements that are necessary to provide further insight into this phenomenon. In a more extensive study of *Medicago* colonised by seven AM fungi, down-regulation of *MtPT2* varied depending on AM fungal species (Burleigh *et al.*, 2002). In this experiment a low level correlation was observed between shoot P concentration in AM plants and *MtPT2* expression. In barley, down-regulation of the root epidermal P_i transporters *HvPT1*, *HvPT2* and *HvPT3* was observed in conjunction with increases in tissue P content resulting from P fertilisation (Glassop *et al.*, 2005). Expression of *HvPT1* and *HvPT2* was also lower in roots of AM than NM plants grown at low P, despite similar shoot and root P concentrations, whereas *HvPT3* transcript levels remained quite high in AM roots. In contrast to data from *Medicago*, these results suggest an AM-specific signalling pathway involved in the down-regulation of epidermal P_i transporters that is independent of the P response pathways in the plant. If AM fungi have differential ability to directly regulate the expression of plant P_i transporters this may be pivotal to understanding the observed diversity in plant responses to AM colonisation as discussed in Section 1.10.

Plant P_i transporters at the arbuscular interface

The identification of a gene encoding a plant P_i transporter (*StPT3*) which was up-regulated in AM roots of potato provided the first insight into the molecular mechanism for transfer of P at the symbiotic interface of colonised cortical cells (Rausch *et al.*, 2001). Orthologues of *StPT3* have now been cloned from members of the Solanaceae (5 members), Fabaceae (2 members) and Poaceae (4 members) (Table 1.1 and Chen *et al.*, 2007). In members of the Fabaceae, a single AM-inducible P_i transporter has been identified, but multiple AM-inducible transporters have been identified in members of the Solanaceae and in rice (see Table 1.1 for references). AM-inducible P_i transporters fall into two categories based upon their expression patterns; AM-specific P_i transporters expressed

exclusively in mycorrhizal roots and AM-upregulated P_i transporters which also show low-level expression in NM roots and/or shoots.

RNA hybridisation and reporter-gene activity have been used to investigate expression patterns of the AM-inducible P_i transporters of potato, tomato, wheat (*Triticum aestivum*), rice, barley, maize (*Zea mays*) and *Lotus* (Rausch *et al.*, 2001; Paszkowski *et al.*, 2002; Glassop, 2004; Karandashov *et al.*, 2004; Glassop *et al.*, 2005; Nagy *et al.*, 2005; Maeda *et al.*, 2006). These studies consistently demonstrate that expression of the AM-inducible P_i transporters is confined to arbuscule-containing cells of the colonised root cortex and does not occur in nearby cells or cells adjacent to intercellular hyphae. In an elegant study of the AM-specific P_i transporter, MtPT4, immunolocalisation revealed expression of MtPT4 protein exclusively on the periarbuscular membrane of colonised cortical cells. Expression of MtPT4 protein was strongest around mature arbuscules and was coordinated with arbuscule development and decay (Harrison *et al.*, 2002).

Although most researchers have used *Arum*-type mycorrhizas, two recent investigations have demonstrated the localisation of AM-inducible P_i transporters around *Paris*-type structures. The AM-upregulated transporter, *StPT3*, was expressed in colonised cortical cells of potato hairy root cultures containing *Paris*-type coiled hyphae (Karandashov *et al.*, 2004) whilst *in-situ* hybridisation of colonised barley and wheat root sections revealed expression of *HvPT8* and *TRIae;Pht1;myc* in cells containing *Paris*-type arbusculate coils (Glassop *et al.*, 2005). This evidence suggests that intracellular interfaces in *Paris*-type AM are capable of P_i transfer and highlights the necessity for further investigation of *Paris*-type AM fungi in functional studies.

The root epidermal P_i transporter, LePT1, of tomato has also been implicated in P_i uptake from the symbiotic interface. Using *in-situ* hybridisation, Rosewarne *et al.* (1999) demonstrated a shift in localisation of *LePT1* transcripts in AM roots to cortical cells containing arbuscules. However, the high level of similarity to the recently identified AM-specific (LePT4) and AM-upregulated (LePT3, LePT5) P_i transporters in tomato suggests that this result may be due to cross-hybridisation of the labelled probe (Smith *et al.*, 2003a; Javot *et al.*, 2007b).

Recent investigations utilising plant mutants highlight significant differences between AM-inducible P_i transporters of different plant families. Nagy *et al.* (2005) used a transposon insertion mutant to investigate the mycorrhizal phenotype of tomato plants with loss-of-function of the AM-specific transporter, LePT4. Mycorrhizal colonisation and ^{33}P transfer via the AM pathway were unaffected

in *lept4-1* mutants, suggesting that LePT3 and LePT5 are able to compensate for loss of function of LePT4 and indicating functional overlap amongst these transporters. This is in stark contrast to recent reports of mutants of members of the Fabaceae. Partial RNA_i knockdown of the LjPT3 transporter of *Lotus* reduced both growth response and colonisation levels of mutant plants compared with vector control plants (Maeda *et al.*, 2006) whilst in *Medicago*, complete silencing of *MtPT4* using RNA_i resulted in total inhibition of the positive growth and P response usually observed for this highly responsive species (Javot *et al.*, 2007a). Detailed observation of colonisation patterns in the *MtPT4* RNA_i mutant and an MtPT4 loss-of-function mutant revealed the premature collapse and senescence of arbuscules (Javot *et al.*, 2007a). Both LjPT3 and MtPT4 appear to be crucial for transfer of P to the plant via symbiotic interfaces. In addition the data of Javot *et al.* (2007a) suggest that P transfer is essential for maintenance of a compatible AM interaction. These authors hypothesised that insufficient C transfer to the fungus may be responsible for arbuscule senescence; this was supported by the observation that fungal hyphae did not proliferate outside the root of the *mtpt4-1* mutant. The suggestion that P and C transfer may be intrinsically linked has been invoked by a number of authors (Woolhouse, 1975; Fitter, 2006). However, such linkage does not account for variations in P transfer via the AM pathway, and hence with the notion that some AM fungi 'cheat' their hosts by acquiring C without donating P (Johnson *et al.*, 1997; Kiers & van der Heijden, 2006).

1.9.3 Functional characterisation of plant P_i transporters

The functional characterisation of plant P_i transporters has been attempted in transport-defective mutants of the yeast, *S. cerevisiae*, and cell-suspension cultures of tobacco (*Nicotiana tabacum*) and rice. Yeast complementation has proved useful for the functional and kinetic analysis of a range of plant transporter proteins including sulphate, potassium and ammonium transporters (Smith *et al.*, 1997). Heterologous expression of plant cDNAs in transport-defective mutants results in complementation of the mutation leading to a restoration of transport capability. In addition these systems have enabled analysis of transporter kinetics.

Although a number of phosphate-defective yeast mutants are available, the heterologous expression of plant P_i transporters in these systems has met with limited success. In those instances where complementation has been successful, kinetic measurements are often higher than predicted from physiological data (Smith *et al.*, 2003a). It is now apparent that the yeast high-affinity transporter, *PHO84*, requires the interaction of several different proteins to form a functional

transporter (Bun-ya *et al.*, 1996) and heterologous expression of plant P_i transporters is suggested to interfere with the interactions of these sub-units (Leggewie *et al.*, 1997; Raghothama, 1999). The results of kinetic analyses of plant PhT1 transporters are summarised in Table 1.1. Complementation has been attempted in a *PHO84* mutant of *S. cerevisiae* and a second mutant, *PAM2,* defective in both *PHO84* and the Na^+-coupled P_i transporter, *PHO89*. Although kinetic estimates in *PAM2* are still higher than expected for high-affinity P_i transport, they are an order of magnitude lower than *PHO84* estimates.

In contrast to measurements in yeast, kinetic estimates based on over-expression of plant P_i transporters in plant cell-suspension cultures have proved significantly more realistic. Yeast complementation by Pht1:1 of *Arabidopsis* predicted a K_m of 110 μM (Muchhal *et al.*, 1996) compared with 3.1 μM for the same protein (Mitsukawa *et al.*, 1997) or 9 μM for barley HvPT1 (Rae *et al.*, 2003) determined in plant cell-suspension culture. These K_m values from plant cell-suspension culture are as predicted from physiological determination of high-affinity P_i uptake from soil solution (Clarkson & Scattergood, 1982; Cogliatti & Clarkson, 1983). The characterisation of HvPT6 from barley was also conducted in cell-suspension culture. Its low-affinity properties (385 μM) are consistent with its localisation to old leaves and flag leaves where it is predicted to play an important role in the remobilisation of P_i from senescing tissues (Rae *et al.*, 2003).

Early physiological investigations of the kinetics of plant P_i uptake in whole roots and cultured cells suggested a dual uptake system for soil P_i acquisition in plants (Barber, 1972; Epstein & Bloom, 2005). This is characterised by high-affinity transport (low K_m) at low external P concentrations and low-affinity transport (high K_m) at high external P concentrations and has been interpreted as resulting from the operation of two distinct transport systems. Similar observations and assumptions have been made for other mineral nutrients and for N were supported by the subsequent discovery of both high-affinity and low-affinity nitrate transporters (Tsay *et al.*, 1993; Trueman *et al.*, 1996; Huang *et al.*, 1999). However, a second possibility also exists. Dual affinity transporters which function in transport from both high and low external nutrient concentrations have been identified; AtKUP1, a K^+ transporter and CHL1, a nitrate transporter of *Arabidopsis* (Fu & Luan, 1998; Liu *et al.*, 1999). For CHL1 the switch between transport activities is controlled by phosphorylation of the transporter (Liu & Tsay, 2003). A similar dual-affinity mechanism for the uptake of P_i from external solution has been hypothesised for Pht1:1 and Pht1:4 of *Arabidopsis* (Shin *et al.*, 2004). Pht1:1 and Pht1:4 are high-affinity transporters involved in P_i acquisition under low P conditions. However, *Arabidopsis* mutants representing loss-of-function of Pht1:1 and/or

Pht1:4 also showed a significant reduction in P_i uptake capacity at high P. The utilisation of a single transporter over a range of P concentrations would enable the plant to switch rapidly between high-affinity and low-affinity transport without the need to initiate synthesis of additional proteins and may be a critical factor in competition for patchily-available soil P. The mechanisms behind this response warrant further investigation.

AM-inducible P_i transporters

The kinetic characterisation of AM-inducible P_i transporters has been attempted in yeast and has yielded contradictory results. The apparent K_m (64 µM) for the AM-upregulated transporter StPT3, suggests fairly high-affinity transport characteristics (Rausch *et al.*, 2001). In contrast the AM-specific transporter MtPT4, demonstrated low-affinity kinetics (493 & 685 µM) in two different yeast mutants (Harrison *et al.*, 2002). The occurrence of both high- and low-affinity AM-inducible P_i transporters suggests that P concentrations in the interfacial apoplast may be variable. This is conceivable if different AM fungi differ in efficiency of P release to the interfacial apoplast and/or reabsorption (see above) and is supported by observations that P retention time in fungal hyphae differs between AM species (Jakobsen *et al.*, 1992b; Smith *et al.*, 2000) and that some AM fungi accumulate poly-P in hyphae rather than releasing it directly to the plant (Shibata, 2007). Thus the plant transport system will need to be adaptable and capable of P_i uptake over a range of P concentrations. Such requirements may explain the existence of multiple AM-inducible P_i transporters in some plant species. It will be interesting to see whether these transporters differ in their uptake capacities or indeed are capable of dual-affinity transport, as proposed for *Arabidopsis* Pht1:1 and Pht1:4, and whether their expression is differentially regulated by different AM fungi or at different times during development of the symbiosis.

1.10 Functional diversity and plant responsiveness in the AM symbiosis

Whilst the arbuscular mycorrhizal condition is a widespread phenomenon amongst land plants, the response of plant species to fungal colonisation is highly variable (Smith & Read, 1997). With 400 million years of evolutionary history it is only to be expected that a mutualistic symbiosis will diversify to represent a continuum of interactions from true mutualism to near parasitism of host plants (Johnson *et al.*, 1997). Typically plants which respond positively to AM colonisation have limited capacity to grow at low P; AM colonisation improves P uptake and results in increased growth over a broad range of P concentrations. However, responses of plants to AM colonisation

are diverse, ranging from significant increases in growth and P nutrition to negligible changes in growth and even growth depressions (Khaliq & Sanders, 1998; Wilson & Hartnett, 1998; Tawaraya, 2003). This variation has been termed 'functional diversity' and is primarily attributed to perturbations in the balance between the benefit derived from increased access to growth-limiting nutrients and the cost of supplying C to the fungal symbiont. Plant growth responses have been shown to vary with the fungal genotype (Munkvold *et al.*, 2004), the host plant genotype or cultivar (Baon *et al.*, 1993; Ravnskov & Jakobsen, 1995; Singh *et al.*, 2002; Zhu *et al.*, 2003) and environmental conditions which influence the C and P status of the plant; primarily light, temperature, soil P and soil pH (Son & Smith, 1988; Jifon *et al.*, 2002; Heinemeyer & Fitter, 2004).

The term mycorrhizal responsiveness (MR), calculated according to Equation 1.1, has been developed to quantify AM-induced changes in plant growth (MGR) (Baon *et al.*, 1993) or P uptake (MPR) (Zhu *et al.*, 2001), where AM and NM refer to either dry matter or total plant P, respectively.

Equation 1.1 $$MR = \frac{(AM - NM)}{NM} \times 100$$

In the following discussion non-responsive plants are those which demonstrate negligible or negative growth responses to AM colonisation (zero or negative MR) even at low P supply. In an extensive literature survey of the growth responses of 250 AM plant species including field and forage crops, wild grasses and forbs, and trees Tawaraya (2003) showed that non-responsive species are less common than positively-responsive species, but that some were identified in all groups investigated.

Whilst calculation of MGR and MPR has proved useful for comparison of whole-plant responses to AM it is important to clearly distinguish MPR from estimates of the actual contribution of the AM pathway to P uptake. Most estimates of AM contribution have been based on the difference in P uptake between AM and NM plants (eg. Smith *et al.*, 1994) and rely on the assumption that AM colonisation has no effect on the direct uptake of nutrients into plant roots. As positive values are only obtained in plants that are positively responsive to AM, such calculations have led to the notion that the AM pathway is non-functional in terms of P_i uptake in non-responsive plants.

An increasing body of evidence from radiotracer experiments demonstrates that the contribution of the AM pathway is not necessarily related to plant responses and that the AM pathway is functional

even in non-responsive plants. Experimental designs using compartmented pot systems in which radiotracer is applied to a hyphal compartment (HC) accessible only to AM fungal hyphae, have been highly effective at demonstrating nutrient uptake and transfer to plants via the AM fungal pathway. AM transfer of ^{32}P from labelled HCs has been demonstrated for non-responsive AM interactions of cucumber (*Cucumis sativus*) (Ravnskov & Jakobsen, 1995), tomato (Smith *et al.*, 2004), wheat (Ravnskov & Jakobsen, 1995; Hetrick *et al.*, 1996) and barley (Zhu *et al.*, 2003). In addition, the magnitude of AM contribution is unrelated to responsiveness. In comparing ^{32}P uptake in AM wheat, Hetrick *et al.* (1996) observed that the modern, negatively-responsive cultivar, Newton, had a higher specific activity (kBq ^{32}P mg P^{-1}) than the positively-responsive landrace, Turkey, indicating that the contribution of the AM pathway was greater in the non-responsive Newton.

Although these early experiments with radiotracers demonstrated transfer of ^{32}P via the AM pathway and enabled comparison of relative transfer under particular experimental conditions, attempts to quantify the contribution of the AM pathway were confounded by large HCs distant from the plant roots, which favoured AM over NM plants and fungal symbionts with extensive hyphal networks (Li *et al.*, 1991; Pearson & Jakobsen, 1993). A recent advance, depending on determination of the specific activity of $^{32/33}P$ in the plant and in a small radiolabelled HC, and hyphal length densities in the main pot and HC, has largely overcome these issues (Smith *et al.*, 2003b, 2004). This experiment demonstrated significant differences in contribution of the AM pathway to *Medicago*, tomato and flax (*Linum usitatissimum*). The contribution also varied with fungal species and, as had been previously observed, was not related to percent colonisation, plant growth response or total P uptake. Of particular significance was the finding that *G. intraradices* contributed up to 100% of total plant P to both flax, which showed a positive growth response (MGR 1425%) and tomato, which showed a growth depression (MGR -18%). These results clearly demonstrate that the AM pathway can be functional in non-responsive plants.

Whilst reemphasising that responsiveness is not an appropriate measure of AM contribution to P uptake, these results also show that the direct, epidermal P_i uptake pathway can be suppressed during AM symbiosis in favour of the AM pathway. This provides a tantalising link with gene expression data which suggests that the root epidermal P_i transporters are down-regulated during AM colonisation and forms the basis of the current investigation.

1.11 Aims of the thesis

The aim of the current work was to characterise P_i uptake in a non-responsive AM plant using an approach which integrated plant physiological measurements of AM contribution and responsiveness with investigations of gene expression, particularly of the plant P_i transporters involved in P_i uptake via the direct and AM pathways.

Barley was chosen as a host plant because of the economic importance of cereals in Australian agriculture. In the Australian context, cropping accounts for 50% of agricultural GDP and barley is second only to wheat in terms of area of production and productivity (Brown *et al.*, 2007). As a model cereal barley provides an effective alternative to wheat. It has a diploid genome and efficient transformation protocols have been established in our research laboratories. In addition barley is generally considered to be non-responsive to AM colonisation (see Chapter 3 for relevant literature and discussion).

Thus my specific aims were to:

1. Determine the contribution of the AM pathway to total P content in barley, a non-responsive AM host;
2. Investigate the correlation between changes in expression of plant P_i transporters and contribution of the direct and AM pathways of P_i uptake;
3. Functionally characterise the plant P_i transporter, HvPT8, implicated in P_i uptake via the AM pathway in barley;
4. Investigate the potential for altering P_i uptake via the AM pathway through altered expression of HvPT8.

Two concurrent lines of investigation were followed. A physiology-based approach was used to investigate the relationship between the contribution of the AM pathway to plant P uptake and expression of plant P_i transporters and a transgenic approach was used to functionally characterise the AM-inducible P_i transporter, HvPT8 and investigate its role in P transfer via the AM pathway.

In the following thesis Chapter 2 outlines the general materials and methods commonly used during this work. Chapter 3 establishes the relevance of AM studies to field cropping of barley through a preliminary survey of AM colonisation in the field and investigates the AM responsiveness of barley under the experimental conditions used in this project. Chapter 4 assesses the contribution of the

AM pathway to total plant P and investigates the expression of three P_i transporters involved in P_i uptake via the AM and direct P_i uptake pathways. Chapters 5 and 6 are concerned with localisation and functional characterisation of the HvPT8 transporter in transgenic barley and Chapter 7 investigates the growth of these transgenic plants, which over-express an AM-inducible P_i transporter, in soil. Chapter 8 provides a general discussion of the findings of this research and highlights areas for further development.

Chapter 2

General Materials & Methods

This chapter describes the materials and methods commonly used in the following work. Further details and modifications relevant to particular experiments can be found in the appropriate chapters.

2.1 Plant species

All pot experiments and plant transformations were conducted with barley (*Hordeum vulgare* L.) cv. Golden Promise. Details concerning the choice of cultivar are provided in Chapter 3. Field investigations were performed during the 2004 cropping season on two commercial barley cultivars, Sloop and Keel. Cultures of AM fungi were maintained on leek (*Allium porrum* L. cv. Vertina).

2.2 Plant growth medium

2.2.1 Soil properties and treatment

Soil was obtained from an undisturbed site within the cemetery at Mallala, South Australia. The site is under native grasses and is neither fertilised nor irrigated. This soil is used extensively in our laboratories. It was chosen due to the low P concentration and alkaline pH suitable for culturing *Glomus* species of AM fungi which require neutral to alkaline soil conditions (Habte, 1995). Soil was collected from the top 0-20 cm and passed through a 5 mm sieve.

Mallala soil and fine quartz sand were sterilised by autoclaving twice over three d at 121^{o}C for 1 h. Soil and sand were subsequently dried at 110^{o}C then mixed in a cement mixer at a rate of 1 part soil to 9 parts sand. This unamended Mallala soil sand mixture was used for cultivating leek pot cultures and nurse pots as outlined in Section 2.3. The pH and plant-available P of the autoclaved Mallala soil and 1:9 soil sand mix are summarised in Table 2.1.

Table 2.1 Plant-available phosphorus and pH of Mallala soil and the 1:9 soil sand mix.

Soil	Olsen P (mg kg^{-1})	Resin P (mg kg^{-1})	pH (H_2O)	EC (dS cm^{-1})
100% Mallala soil	8.8	4.1	8.2	0.141
1:9 soil sand mix	2.7	1.3	8.2	nd

Soil analyses were performed according to Section 2.6. nd = not measured.

2.2.2 Nutrient addition

Pot experiments were designed to ensure that nutrients other than P were not limiting. Mineral nutrients were mixed thoroughly into the 1:9 soil sand mix at the following rates (mg kg^{-1} dry soil); NH_4NO_3, 85.7; $CaCl_2.2H_2O$, 75.0; K_2SO_4, 75.0; $MgSO_4.7H_2O$, 45.0; $MnSO_4.H_2O$, 10.5; $ZnSO_4.7H_2O$, 5.4; $CuSO_4.5H_2O$, 2.1; $CoSO_4.7H_2O$, 0.39; $NaMoO_4.2H_2O$, 0.18 (Jakobsen *et al.*, 1992a). Phosphorus was added as $CaHPO_4$ with thorough mixing. Two P application rates were used, 25 mg kg^{-1} (P1) or 50 mg kg^{-1} (P2) providing an additional 5.7 or 11.4 mg P kg^{-1}, respectively. Experimental pots received 1.4 kg of this Mallala growth medium.

2.3 Arbuscular mycorrhizal fungi

2.3.1 Selection of AM fungal species

The AM fungus, *Glomus intraradices* was selected for use in all experiments because this fungus is an 'aggressive' coloniser which colonises host roots rapidly and has shown high levels of colonisation in barley (Plenchette & Morel, 1996). Hyphal transfer of ^{32}P to barley has also been demonstrated for this fungus (Zhu *et al.*, 2003). The AM fungus *Glomus geosporum* was selected for comparison to *G. intraradices* in some experiments.

The AM fungi used in these experiments were from the collection of Prof. Sally Smith, Discipline of Soil and Land Systems, School of Earth and Environmental Sciences, The University of Adelaide. Details of fungal isolates and their authorities and isolate numbers are as follows:

Glomus intraradices Schenck & Smith, DAOM181602

Glomus geosporum (Nicolson & Gerdemann) Walker, BEG154

2.3.2 Inoculum production

Initial pot cultures were kindly supplied by Ms Debbie Miller (The University of Adelaide). Pot cultures were established by incorporating dry inoculum at a rate of 10% w/w with 1.4 kg unamended Mallala soil sand mixture. Five leek seedlings were planted in each pot and maintained in a glasshouse as outlined in Section 2.4.2 for at least 8 weeks. Pot cultures received 15 mL per week of half-strength Long Ashton solution (minus P). This solution contained (mM); $NaNO_3$, 8; $(NH_4)_2SO_4$, 4; $CaCl_2.2H_2O$, 3; K_2SO_4, 2; $MgSO_4.7H_2O$, 1.5; FeEDTA, 0.1; (mg L^{-1}) H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.22; $CuSO_4.5H_2O$, 0.08; $NaMoO_4.2H_2O$, 0.025. Prior to drying off, pot cultures were cored and checked qualitatively for the presence of spores and colonised root fragments; however, AM colonisation of pot cultures was not quantified.

2.3.3 Application of inoculum to experimental pots

Dried pot culture inoculum was mixed thoroughly with Mallala growth medium at a rate of 15% w/w in AM pots only. This inoculum contained soil sand mix, hyphae, spores and colonised root fragments. Non-mycorrhizal (NM) control pots did not receive any additional amendments.

2.3.4 Nurse pot preparation

A nurse pot system similar to that outlined by Rosewarne *at al*. (1997) was developed in order to establish rapid and synchronous colonisation in relatively young plant material. This method was used in both colonisation time-course experiments and in production of colonised material for laser scanning confocal microscope (LSCM) examination of gene expression in transgenic plants expressing the reporter gene GFP (Chapter 5). Briefly, nurse pots were established by the same method as pot cultures and grown for at least 8 weeks before being planted with germinated barley seed (4 per pot). Barley was harvested up to 2 weeks after planting and only roots remaining attached to the plant were used in subsequent analyses.

2.4 Plant propagation

2.4.1 Seed preparation

Seeds of barley and leek were surface sterilised by immersion in 70% ethanol for 3 min followed by 4% sodium hypochlorite for 10 min, then rinsed at least 4 times with sterile reverse osmosis (RO) water. Following sterilisation, barley seeds were soaked for at least 8 h or overnight in sterile RO

water with gentle agitation in order to induce rapid and synchronous germination. Seeds were then placed on moist filter paper in petri dishes, sealed with parafilm and maintained in an incubator at 24^{o}C with 16 h photoperiod (500 µmol m^{-2} s^{-1}). Germination usually occurred within 5 d and germinated seeds were then transplanted directly into experimental pots.

2.4.2 Glasshouse conditions and watering

Unless otherwise stated, experiments were conducted in a glasshouse with semi-controlled conditions on the Waite Campus of The University of Adelaide, South Australia. Plants were grown under Osram 1000 W growth lights. The light intensity ranged from 400-900 µmol m^{-2} s^{-1}. The average daytime temperature in the glasshouse ranged from 22-30^{o}C depending on season and weather conditions. Pots were watered to 10% w/w with RO water every 2 d.

2.5 Harvest and sampling

Plants were harvested at times specific to individual experiments. At harvest shoots were removed for determination of fresh and dry weights, P content and where applicable ^{32}P activity. Roots were gently shaken free from soil and washed over a fine mesh to remove remaining soil. Roots were then chopped into ~2 cm fragments and a weighed subsample of ~100 mg was quickly removed to liquid N_2 for gene expression studies. A second weighed subsample (~100-300 mg) was taken for determination of mycorrhizal colonisation. The remaining root system was treated in the same manner as plant shoots. Soil shaken from the root system was mixed thoroughly and at least 15 g was taken for determination of soil P and where applicable ^{32}P activity.

2.6 Soil analyses

2.6.1 Determination of plant-available soil P

Two methods were used to measure plant-available soil P as described below. In ^{32}P-labelling experiments soil extracts were also used for scintillation counting of ^{32}P activity as described in Section 2.8.

Olsen-extractable P

The most commonly used soil tests in Australia are the bicarbonate-extractable soil P tests; Colwell P (Colwell, 1963) and Olsen P (Olsen *et al.*, 1954). Colwell P differs from Olsen P in the greater

volume of extractant and longer shaking time. Although Colwell P shows a good correlation with the total exchangeable P fraction of a soil, Olsen P shows a better correlation with plant P response (Holford, 1997). Therefore the Olsen P method was chosen to estimate plant-available P in my experiments.

A weighed 1 g sample of moist soil was shaken with 20 mL 0.5 M $NaHCO_3$ (pH 8.5) for 30 min. Samples were centrifuged at 3000 rpm for 10 min to pellet soil debris and a 2.5 mL aliquot of supernatant (S/N) was neutralised with 2.5 mL HCl (0.5 M). After at least 30 min for debubbling, P content of extracts was measured colorimetrically (see below).

Resin-extractable P

Extraction of soil P by anion-exchange resin membranes is a relatively new technique for estimation of plant-available P. Although this method is not yet widely applied it is both more accurate at estimating plant-available P and more robust in terms of applicability to different soil types (van Raij, 1998). Therefore the Resin P extraction method was also used in my experiments. The method is as described by Kouno *et al.* (1995) with omission of the steps related to fumigation-extraction with chloroform. Anion-exchange resin membranes (#55164) were obtained from BDH Laboratory supplies, Poole, PH15 1TD, England. Resin membranes were cut into strips of 6x2 cm. Prior to soil P extraction resin membranes were brought into the bicarbonate form by shaking for 1 h in HCl (0.5 M) followed by two consecutive treatments with fresh $NaHCO_3$ (0.5M) shaking for 1 h each time. Strips were rinsed with RO water between each round of shaking and stored in RO water prior to use. A weighed 2 g sample of moist soil was placed in a falcon tube with 30 mL RO water and 1 resin strip then shaken for at least 16 h. The resin strip was removed and rinsed in RO water to remove adhering organic matter before placing in a clean tube with 30 mL NaCl:HCl (0.1M). After allowing 30 min for debubbling, strips were shaken for 2 h to elute P from the resin membrane. The concentration of P in the NaCl:HCl eluate was measured colorimetrically as outlined below.

Colorimetric determination of P

A 2.5 mL aliquot of soil extract was combined with 0.5 mL fresh Murphy & Riley colour reagent (Murphy & Riley, 1962) prepared as follows: the following components were combined in order, with swirling after each addition; 10 mL 2.5 M H_2SO_4; 3 mL ammonium molybdate (40 g per 1000 mL); 2 mL ascorbic acid (26.4 g per 500 mL); 1 mL antimony potassium tartrate (1.454 g per 100

mL); 4 mL H_2O. Following addition of colour reagent samples were left for 1 h to allow for colour development. The absorbance was measured on a Shimadzu UV-VIS spectrophotometer at 830 nm. P content was quantified by comparison to a standard curve with a concentration range of 0-0.5 μg P mL^{-1}.

2.6.2 Soil pH and EC measurements

The soil pH and EC were measured in water extracts; 20 g of soil was shaken with 100 mL RO water for 1 h in an end-over-end shaker. Samples were allowed to settle and pH and EC were measured.

2.7 Determination of P concentration in plant tissues

The concentration of P in shoots and roots was measured by digestion and subsequent colorimetric analysis. Dried, finely chopped shoot or root material (200-500 mg) was digested by standing overnight in 7 mL nitric-perchloric acid (6:1) followed by heating on a programmed Tecator R digestion block at 100^{o}C for 2 h then at 140^{o}C until the volume reduced to 1 mL. Digests were diluted to 20 mL with RO water. For determination of P content according to the phosphovanado-molybdate method (Hanson, 1950) a 0.5 mL aliquot of the tissue digest was combined with 1.7 mL RO water and 0.3 mL colour reagent; conc. nitric acid, 0.25% ammonium vanadate, 5% ammonium molybdate (1:1:1). After 30 min to allow for colour development the P concentration was determined by reading the absorbance on a Shimadzu UV-VIS spectrophotometer at 390 nm and comparison to a standard curve (0-8 μg P mL^{-1}). Where applicable plant tissue digests were also used to measure ^{32}P activity as outlined in Section 2.8.

2.8 Scintillation counting & calculation of specific activity

The activity of ^{32}P in a 2 mL volume derived from soil extracts or plant tissue digests was measured by Cerenkov ^{32}P counting in a 1215 Rackbeta II liquid scintillation counter and corrected for isotopic decay. The specific activity of soil or plant tissue extracts was calculated according to Equation 2.1, where ^{32}P is the measured counts in soil extracts or tissue digests and P is the plant-available P in soil or tissue P content, respectively.

Equation 2.1 $$\text{Specific activity} = \frac{^{32}P}{P}$$

2.9 Determination of AM colonisation

2.9.1 Clearing and staining of roots

Roots for staining were either harvested to 50% EtOH for storage or 10% KOH (w/v) for immediate clearing and staining. Roots in 50% EtOH were rinsed thoroughly in RO water then cleared in 10% KOH at 70°C for 40 min. Roots in 10% KOH were left at room temperature (RT) overnight then heated at 70°C for 15 min. Following clearing roots were rinsed in RO water and bleached in 0.1 M HCl for 5 min at RT (Phillips & Hayman, 1970). Roots were stained with 5% Schaeffer black ink in white vinegar, at 70°C for 1 h (Vierheilig *et al.*, 1998). Roots colonised by *G. geosporum* were heated for 2.5 h in ink and vinegar to achieve better staining of the fungal structures. Roots were then de-stained in 50% vinegar at RT for 20 min and stored in lactoglycerol (1:1).

2.9.2 Assessment of colonisation

Root fragments were mounted on slides in glycerol and observed at x100 magnification with a stereoscopic microscope. Measurements of the percentage colonisation were determined according to the magnified intersects method (McGonigle *et al.*, 1990). At least 150 intersections were observed for each root sample. The total number of intersects was recorded, as well as non-colonised intersects and intersects containing each of hyphae, arbuscules or vesicles. External AM hyphae closely associated with the roots were not recorded. Total colonisation is reported as percentage of total root length with internal hyphae, arbuscules or vesicles and was calculated according to Equation 2.2. The frequency of individual fungal structures, arbuscules or vesicles, was recorded as the percentage of colonised root only and was calculated according to Equation 2.3.

Equation 2.2 $$\text{Total colonisation} = \frac{\text{Total colonised intersects}}{\text{Total intersects}} \times 100$$

Equation 2.3 $$\text{arbuscular/vesicular colonisation} = \frac{\text{arbuscular/vesicular intersects}}{\text{Total colonised intersects}} \times 100$$

2.10 Statistical analyses

Data were analysed by two-way analysis of variance (ANOVA) using Genstat 8th Edition (Lawes Agricultural Trust), unless otherwise stated. Significant differences between means were tested using a post-hoc Tukey test at $P<0.05$.

2.11 Gene expression analyses

2.11.1 Extraction of total RNA

Tissue samples (~100 mg) for analysis of gene expression were ground in liquid N_2 with a mortar and pestle. RNA was extracted with the RNeasy Plant mini kit (#74904, QIAGEN, Doncaster, Australia) according to the manufacturer's instructions. Extracted RNA was treated to remove contaminating DNA with the Ambion DNA-*free* kit (#AM1906) supplied by Applied Biosystems (Scoresby, Australia) according to the manufacturer's instructions. RNA quantity and integrity was checked on a 1% agarose gel containing ethidium bromide (Section 2.12), and quantity and purity were confirmed on a ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA).

2.11.2 cDNA synthesis

cDNA was synthesised from DNA-free RNA as follows. The following components were combined to a total volume of 13 µL; 1 µg purified RNA, 1 µL oligo(dT) 20mer (50 µM), 1 µL dNTP (10 mM), sterile water to volume. The reaction mixture was heated at 65°C for 5 min to denature RNA then transferred to ice. A master mix (7 µL) was added to each reaction containing 20 units of RNaseOUT (#10777-019, Invitrogen, Mount Waverley, Australia), 4 µL first-strand synthesis buffer, 1 µL DTT (0.1 M) and 50 units of Superscript III RT (#18080-093, Invitrogen). Reactions were incubated at 50°C for 1 h followed by 15 min at 70°C to inactivate the enzyme.

2.11.3 Quantitative real-time PCR

Quantitative real-time PCR, referred to herein as Q PCR, is a rapid and sensitive PCR technique which enables quantification of the target gene product. It is particularly useful for large multi-gene families as the use of gene specific primers enables specific amplification of a single gene product. Q PCR was performed on 1µL first-strand cDNA by Dr Neil Shirley (ACPFG, Adelaide, Australia) according to the published method (Burton *et al.*, 2004). The reaction mixture included SYBR green

for detection of the amplification product, and amplification was performed in a RG 2000 Rotor-Gene Real Time Thermal Cycler (Corbett Research, Sydney, Australia). A melt-curve was obtained at the end of the amplification and used to detect purity of the amplification product and hence specificity of the PCR primers. The mean expression level was obtained from three replicates for each cDNA and the expression of the gene-of-interest (GOI) was normalised according to Vandesompele *et al.* (2002) using the control gene glyceraldehyde-3-phosphate dehydrogenase (*HvGAPdH*) of barley. *HvGAPdH* is a housekeeping gene which has previously been reported as a constitutive expression control in AM barley root tissue (Delp *et al.*, 2003). Q PCR primers for GOI's and the *HvGAPdH* control gene were supplied by Dr C. Huang (ACPFG, Adelaide, Australia) and the PCR products were sequenced (Section 2.13) to determine correct amplification of the target gene. The gene accession numbers, primer sets and expected amplicons are listed in Table 2.2.

Table 2.2 Gene specific primers and expected product size used for Q PCR of barley glyceraldehyde-3-phosphate dehydrogenase (*HvGAPdH*) and three P_i transporters (*HvPT1*, *HvPT2* and *HvPT8*).

Gene	GenBank accession number	Primer designation	Primer sequence	Primer T_m (°C)[1]	Amplicon size (bp)
HvGAPdH	X60343	HvGAPdH_F	GTGAGGCTGGTGCTGATTACG	51.2	198
		HvGAPdH_R	TGGTGCAGCTAGCATTTGAGAC	49.7	
HvPT1	AF543197	HvPT1_F	GCACATCTGGAGACACAGAGTCA	51.9	198
		HvPT1_R	TTGGCAATTCTTCACAAACGAATAC	47.4	
HvPT2	AF187019	HvPT2_F	GAGCTCTCCAAGGAGAACGTTG	51.6	149
		HvPT2_R	AATTACAGCAACAAAACAAGCCG	46.6	
HvPT8	AY187023	HvPT8_F	GGCAGCAACGAGGTGAAAAGTG	51.6	229
		HvPT8_R	CTGTTTGAACGTAGGCTGTGCG	51.6	

[1]Primer T_m determined in Vector NTI v10.3 (Invitrogen) based on %GC.

2.12 Agarose gel electrophoresis

RNA and DNA and the products of PCR and restriction enzyme digestion were analysed by agarose gel electrophoresis. Agarose gels (1%) including ethidium bromide (10 μg mL^{-1}) were prepared and run in 1xTAE buffer; 0.04 M Tris-acetate, 0.001 M EDTA. Samples were loaded by mixing with Orange G loading buffer; (6x) 0.15% Orange G (Sigma-Aldrich Pty. Ltd, Castle Hill, Australia) in 60% glycerol, and gels were run with a 1 kb Plus DNA ladder (Invitrogen) at 100 V for the desired time. DNA bands were visualised under a GelVue UV transilluminator (Syngene, Cambridge, UK).

2.13 Sequence analysis

The ABI Prism® BigDye™ Terminator Ready Reaction Cycle Sequence Kit (v3.1) supplied by Applied Biosystems (Scoresby, Australia) was used to sequence PCR products and confirm cloning reactions. Sequencing reactions were performed according to the manufacturer's recommendations. Purification of extension products (10 µL reaction) was achieved by addition of 75 µL freshly prepared $MgSO_4$ (0.2mM) in 70% EtOH. Tubes were vortexed, allowed to stand at RT for 15 min and centrifuged (4000 rpm, 15 min). The S/N was removed and the pellet was oven dried at 37^{o}C. Sequencing was performed by the Australian Genome Research Facility (Brisbane, Australia).

2.14 Production of transgenic barley plants

The following section details the methods used in the preparation of gene expression constructs for plant transformation and subsequent analysis of gene function.

2.14.1 General PCR method

Target DNA sequences were amplified by PCR using sequence specific primers designed with the Vector NTI gene analysis software package v10.3 (Invitrogen). PCR primers were obtained from PROLIGO (www.proligo.com); primer sequences are listed in relevant chapters. PCR reactions from gDNA or vector clones were prepared as follows using Platinum *Taq* DNA polymerase (#10966-018, Invitrogen) (µL per 25 µL reaction); template, variable; primers (10 µM), 0.5; 10x Buffer, 2.5; dNTPs (10mM), 0.5; Mg^{2+} (50mM), 0.75; Platinum *Taq* (5U μL^{-1}), 0.1; RNA/DNAse free H_2O to volume. The amount of template varied depending on the target; gDNA, 50 ng; vector DNA 0.5 ng (per 25 µL reaction). PCR amplification was performed in a DNAEngine TETRAD™2 thermocycler (Geneworks, Adelaide, Australia). Reactions were heated (94^{o}C, 2 min) to denature template and activate the enzyme prior to cycling (30 x) as follows; denature (94^{o}C, 30 s), anneal (variable ^{o}C, 30 s), extend (72^{o}C, 1 min per kb). Annealing temperature and extension time were specific to individual reactions and are provided in relevant chapters.

Where necessary the PCR reaction product was purified using a QIAquick PCR purification kit (#28104, QIAGEN) according to the manufacturer's instructions. DNA was eluted in 40 µL 10 mM Tris-HCl (pH 8.5).

2.14.2 Vectors

The plasmid vectors used for bacterial and plant transformation are described in Table 2.3 and vector maps are provided in Appendix 2. These binary vectors include a hygromycin resistance gene and are suitable for cereal transformation.

Table 2.3 Details of vectors used for bacterial and plant transformation in this work.

Vector name	Supplied by:	Selectable marker			Vector type
		Bacterial	Agrobacterium	Plant	
pWBVec8	Dr P. Schunmann	Spectinomycin	Rifampicin	Hygromycin	binary
pPZPUbi	Dr K. Oldach	Spectinomycin	Rifampicin	Hygromycin	binary

2.14.3 Restriction enzyme digestion and clone analysis

Restriction enzyme (RE) digestion was used for preparation of vector backbone and insert DNA prior to ligation and for subsequent clone analysis. Restriction enzymes were supplied by New England BioLabs (Genesearch, Arundel, Australia). Digest reactions were set up in the appropriate buffer according to the manufacturer's instructions and run for 2 hours at 37^{o}C. The reaction was terminated by heating at 65^{o}C for 20 min to inactivate the enzyme. Where necessary, buffer was removed and the reaction product was cleaned up by applying to a SUPREC™-02 size exclusion column (Scientifix, Cheltenham, Australia), centrifuging to remove S/N (8000 rpm, 4 min) and eluting DNA in 20 µL H_2O. Digestion products were checked by running out on a 1% agarose gel and staining with ethidium bromide (Section 2.12).

2.14.4 Ligation reactions

Ligations into binary vectors were performed following RE digestion using T4 DNA ligase (#M1801, Promega, Annandale, Australia) and set up according to the manufacturer's instructions. Ligations were performed overnight at 14^{o}C.

2.14.5 Bacterial transformation reactions

Preparation of competent cells and culture media are outlined in Appendix 3.

Transformation of plasmid vectors into *Escherichia coli* cells was achieved using a heat-shock procedure. Briefly, 10 µL ligation product was mixed gently with 50 µL *E. coli* DH5α competent cells, prepared according to Inoue *et al.* (1990), and placed on ice for 20 min. Following heat-shock at 42^{o}C for 50 s cells were returned to ice for 2 min and recovered in 925 µL SOC media (37^{o}C, 1.5 h). Aliquots (50 µL) were plated on LB/spectinomycin plates (50 µg mL^{-1}) and incubated overnight at 37^{o}C. Individual colonies were then transferred to liquid selection medium for amplification overnight (37^{o}C with shaking) prior to extraction of plasmid DNA (Section 2.14.6) and analysis by either RE digestion (Section 2.14.3) or sequencing (Section 2.13).

Transformation of binary vectors into *Agrobacterium tumefaciens* cells, strain AGL-1 carrying the *rifR* gene, was achieved using a freeze-thaw procedure. Competent cells (100 µL) were thawed at RT then mixed gently with ~1 µg plasmid DNA. Cells were frozen in liquid N_2 for 1 min then thawed for 5 min at 37^{o}C. Cells were recovered in 500 µL TYNG medium at 28^{o}C for 1 h prior to plating on LB/rifampicin/spectinomycin plates (50 µg mL^{-1}). Plates were incubated for 2 d at 28^{o}C and individual colonies were transferred to liquid selection medium for amplification prior to extraction of plasmid DNA (Section 2.14.6) and analysis by RE digestion (Section 2.14.3).

For maintenance of vector clones glycerol stocks were prepared from liquid cultures (750 µL *E. coli* culture in LB + 750 µL 50% glycerol), snap frozen in liquid N_2 and stored at -80^{o}C.

2.14.6 Isolation of plasmid DNA

Plasmid DNA was purified from bacterial cultures using the QIAprep Spin Miniprep Kit (#27104) supplied by QIAGEN (Doncaster, Australia) according to the manufacturer's instructions.

2.14.7 Agrobacterium-mediated transformation of barley

Agrobacterium tumefaciens-mediated transformation of *Hordeum vulgare* cv. Golden promise was carried out by Ms K. Bech-Oldach and Mr R. Singh (ACPFG, Adelaide, Australia) according to the procedure developed by Tingay *et al.* (1997) and modified by Matthews *et al.* (2001). Briefly, scutella were isolated from immature embryos of 12-16 week old barley and transformed by co-

cultivation with *A. tumefaciens* harbouring the binary expression plasmid of choice. Transformed calli were selected on callus induction medium containing hygromycin (95 μM) for 6 weeks prior to transfer to shoot regeneration medium (38 μM hygromycin) for 4-12 weeks. Regenerated shoots were excised and transferred to hormone-free callus induction medium (95 μM hygromycin) for 3-4 weeks to induce root formation. The tissue culture-derived plants were established in soil and grown to maturity. Preparation of culture media is outlined in Appendix 3.

2.15 Genotyping of transgenic plants

Genotyping of T1 and T2 transgenic seed was performed using the REDExtract-N-Amp™ Plant PCR Kit (XNAP, Sigma-Aldrich Pty. Ltd). The kit is designed for PCR amplification from leaf tissue without the necessity for DNA extraction. PCR amplification was performed using primers designed to amplify a 521 bp fragment from the hygromycin selectable marker (Table 2.4); annealing, 55^{o}C; elongation, 30 s.

Table 2.4 Primers used to PCR amplify a 521 bp fragment from the hygromycin resistance gene. These were used for genotyping transgenic plants and to generate a probe for Southern analysis.

Primer	Sequence	T_m (^{o}C)[1]
*Hyg*probe F	AGGCCATGGATGCGATCGCT	51
*Hyg*probe R	CTGCGCCCAAGCTGCATCAT	51

[1]Primer T_m determined in Vector NTI v10.3 (Invitrogen) based on %GC.

2.16 Genomic DNA extraction

Genomic DNA was extracted according to the method of Pallotta *et al.* (2000). Leaf tissue (~2 g) was snap frozen in liquid N_2 and ground in a mortar and pestle. Ground tissue was homogenised in 4.5 mL DNA buffer (pH 8.5); (g L^{-1}) Trizma base (12.1), sarkosyl (10), NaCl (5.8), Na_2EDTA (3.2), Polyvinylpolypyrolidone (20). An equal volume (4.5 mL) of Phenol/choloroform/iso-amylalcohol (PCIIAA, 25:24:1) was added and tubes were placed on an orbital rotor for 10 min and then centrifuged (4000 rpm, 10 min). The S/N was removed to a fresh tube containing an additional 4.5 mL PCIIAA, shaken for 5 min and centrifuged (4000 rpm, 5 min). The S/N was mixed with 400 μL 3 M sodium acetate (pH 4.8) followed by 4 mL isopropanol to precipitate DNA. DNA was rinsed in 70% EtOH and resuspended in 40 μg mL^{-1} RNase A in TE buffer; Tris-HCl (10 mM), EDTA (1 mM), pH 8.0. Genomic DNA was stored at 4^{o}C.

Chapter 3

Field Colonisation and Growth Responsiveness of Barley in AM Symbioses

3.1 Introduction

Cereal grains represent the most widely cultivated and consumed food staple in the world. Extensive breeding programs provide new and improved cultivars and target a diverse range of crop traits from nutrient content and quality of grain to stress response and yield improvement. Breeding programs which aim to improve P_i uptake target traits which either increase the local availability of P_i through enhanced root exudation of phosphatases and organic acids or increase access to soil P resources via altered root morphology; for example enhanced root length and branching or increased root hair length and density. Considerable genetic diversity has been reported for these traits and, at low P, demonstrated a high potential to improve P_i uptake through selection of appropriate genotypes (Gahoonia & Nielsen, 2004). Enhanced uptake through genetic engineering of particular traits has also been explored (Richardson *et al.*, 2001; Rae *et al.*, 2004).

Despite considerable interest in improving P_i uptake and P utilisation efficiency of cereal crops, few breeding programs have focussed on AM symbioses as a means to achieving this. One explanation for the lack of interest in the AM approach is the considerable variability that is observed in the response of cereals to AM colonisation. Increases of up to 50% in the yield of grain and straw have been reported for barley colonised by AM in pot experiments (Jakobsen & Jensen, 1981; Jensen, 1982), and field inoculation trials conducted by Clarke & Mosse (1981) and Powell (1981) led to increases in yield and total P_i uptake of barley colonised by introduced AM fungi over indigenous AM fungi. In contrast, field studies on AM development in barley by Black & Tinker (1979) led them to conclude that yield was negatively correlated with the degree of AM colonisation. Plenchette & Morel (1996) reported that *G. intraradices* decreased the yield of barley regardless of soil P concentrations, whilst the results of numerous pot experiments suggest that AM colonisation does not alter the growth of barley compared with NM controls (Jensen, 1983, 1984; Jakobsen *et al.*, 2005). Several authors have also reported that variability in responsiveness of cereals is related to plant cultivar. Hetrick *et al.* (1992; 1996) surveyed a range of wheat cultivars including modern varieties, landraces and ancestors. AM responses ranged from positive increases to depressions in growth and P_i uptake and there was a trend for older cultivated wheats to have a greater positive response to AM than modern cultivars. A number of studies have now demonstrated a significant

negative correlation between AM responsiveness of a cultivar and P acquisition or P utilisation efficiency, leading to the proposal that modern breeding programs which target strategies to improve P_i uptake may have led to a decline in AM responsiveness (Baon *et al.*, 1993; Yao *et al.*, 2001; Zhu *et al.*, 2001).

A second possible explanation for the lack of consideration of AM in plant breeding programs is the decline in AM fungi that is observed in high-input agricultural systems. In general, conventional cropping practices such as high P fertiliser application, tillage and long-term fallow reduce the extent of AM colonisation (Lekberg & Koide, 2005 and studies therein). As reported by Black & Tinker (1979), long-term fallow and a NM pre-crop decreased AM colonisation of field-grown barley. However, it is notable that all plots retained some level of AM infectivity. Although colonisation of commercial barley crops may be low, evidence from pot experiments suggests that even at these low levels of colonisation AM affect the growth of cereals (Hetrick *et al.*, 1992). Field surveys investigating AM colonisation of cereals provide evidence for the persistence of AM symbioses under commercial cropping conditions in a range of environments (Jensen & Jakobsen, 1980; Aliasgharzadeh *et al.*, 2001). Taken together with the recent evidence that AM colonisation alters pathways of P_i uptake regardless of plant responses it is clear that the role of AM in commercial crops cannot be ignored, and that any studies aimed at improving P_i uptake will need to consider the influence of AM fungi.

P efficiency and choice of cultivar

Due to the variability in responsiveness of cereal cultivars to AM colonisation some deliberation went into the choice of barley cultivar at the outset of the project. The overall aim of the project was to investigate the role of AM in P_i uptake of a non-responsive host. Therefore, it followed that a cultivar which was considered to be efficient in P_i uptake under low P conditions would be an appropriate choice. However, part of the experimental design was to use a transgenic approach to characterise the AM-inducible P_i transporter, HvPT8, and investigate the potential for altering P_i uptake via the AM pathway through altered expression of this transporter. The established protocol for transformation in our laboratories is based upon *Agrobacterium*-mediated transformation, the success of which is limited to cultivars which easily form callus and regenerate, such as Golden Promise (Dahleen & Manoharan, 2007). Ultimately it was decided that all glasshouse experiments would be performed on *Hordeum vulgare* L. cv. Golden Promise as it was considered important to be able to relate the results of experiments on transgenic plants to data on the physiological and

molecular characterisation of the AM pathway in non-transformed plants. Golden Promise is a two-rowed malting barley of moderate P efficiency (Glassop, 2004).

This chapter reports two experiments which aimed to: 1) determine the presence and extent of AM colonisation of barley in field soil and hence the relevance of AM investigations to field grown barley in the Australian context; and 2) quantify the growth and P response of the model species, *Hordeum vulgare* L. cv. Golden Promise, under the conditions of my experiments.

3.2 Materials and methods

3.2.1 Field colonisation of barley

Field sampling and harvest

The colonisation of two commercially-grown barley cultivars, Sloop and Keel, was investigated at Waikerie in the Riverland region of South Australia and two sites with different cultivar and treatment histories at Roseworthy in the Barossa region of South Australia (Roseworthy site 1 and Roseworthy site 2). Field sites were chosen for sampling based on accessibility. A summary of the cropping history, fertiliser application and soil characteristics at each site is provided in Table 3.1. Wheat had been cultivated at all three sites in the preceding two years and in 2004 the fertiliser applications were similar, although Waikerie received less urea. More significantly, Roseworthy site 1 received 47000 L ha^{-1} of piggery effluent in 2003. This is reflected in the high P and low pH measured for soil samples from this site (Table 3.1).

Plants were harvested in September 2004 approximately 10 weeks after planting. Four distinct locations were selected at each site and 3 barley plants were dug up at each location. Soil shaken from the roots and from the top 0-10cm of the soil pit was mixed on site and triplicate samples were taken for soil P, EC and pH analyses as described in Section 2.6. Plants were harvested in the laboratory as described in Section 2.5 and AM colonisation was determined in three root samples per location as described below. This method of sampling gave 12 measurements of each parameter per site (three replicates per location x 4 locations per site). Data was then averaged over each site.

Measurement of AM colonisation in field samples

A conservative approach was taken to the scoring of AM colonisation in field samples. Roots were stained, mounted on slides and observed under a light microscope at x100 magnification according to Section 2.9. Intersects were scored as AM, NM or Uncertain. Colonised intersects were only scored as AM if the root segment within the field of view clearly contained AM structures such as arbuscules. If there was any uncertainty or root segments only contained hyphae they were scored as Uncertain. This gave an upper and lower limit of potential AM colonisation. Only the lower limit is reported here.

Table 3.1 Crop details, fertiliser application, site history and soil characteristics for the three field sites investigated in the field colonisation study.

Site	Cultivar	Sown	Harvested	Fertiliser application (2004)[1,2]	Site history	Soil type	Olsen P[3] (mg kg^{-1})	pH[3]	EC[3] (dS m^{-1})
Roseworthy 1	Keel	24/6/04	10/9/04	DAP (90 kg ha^{-1}) deep banded; Urea (90 kg ha^{-1}) 50% w seed, remainder deep banded	2003 Krichauff Wheat 2002 Krichauff Wheat	red brown earth/clay loam	38.3 ± 6.1	6.7 ± 0.2	0.47 ± 0.07
Roseworthy 2	Sloop	29/6/04	17/9/04	DAP (100 kg ha^{-1}) 50% w seed, remainder deep banded; Urea (80 kg ha^{-1}) deep banded	2003 Krichauff Wheat 2002 Krichauff Wheat	red brown earth/clay loam	13.7 ± 0.9	8.3 ± 0.2	0.99 ± 0.17
Waikerie	Sloop	15/6/04	31/8/04	MAP (75 kg ha^{-1}) + 2% Zn; Urea (20 kg ha^{-1})	2003 Yitpi Wheat 2002 Clearfield Janz Wheat	calcareous sandy loam	13.8 ± 1.0	7.4 ± 0.1	0.47 ± 0.01

[1]In 2003 Roseworthy site 1 received 47000 L ha^{-1} of piggery effluent.

[2]DAP – Diammonium phosphate, MAP – monoammonium phosphate

[3]Soil properties determined according to Section 2.6. Values are means ± SEM of samples from four distinct locations within each site.

3.2.2 Response of barley cv. Golden Promise to AM colonisation

Experimental design

The experiment had three AM fungal treatments; non-mycorrhizal (NM) or inoculated with *G. intraradices* or *G. geosporum*, and three harvests; 2, 4 and 6 weeks. There were three replicate pots per treatment. Mallala growth medium (1.4 kg per pot) was prepared as outlined in Section 2.2, including P addition at P1 (5.7 mg kg^{-1} additional P) and 15% dry AM fungal inoculum was incorporated into the soil of AM pots only (Section 2.3.3). Seeds of barley cv. Golden Promise were surface sterilised and germinated prior to planting singly at the centre of each pot. Experimental pots were maintained in a glasshouse as outlined in Section 2.4.2. An additional 30 mg N was applied as NH_4NO_3 between the 4 week and 6 week harvests.

Harvest and calculations

Experimental pots were harvested according to Section 2.5. Plants were sampled for determination of fresh and dry weights, tissue P concentrations (Section 2.7) and AM colonisation (Section 2.9). Samples were also collected for gene expression; however, these were unfortunately destroyed during a -80^{o}C freezer failure.

The mycorrhizal growth response (MGR) (Baon *et al.*, 1993) and mycorrhizal P response (MPR) (Zhu *et al.*, 2001) were quantified in order to compare plant responses associated with AM colonisation by the two AM fungal species. MGR and MPR were calculated according to Equation 3.1 where AM and NM refer to either dry matter or total plant P, respectively.

Equation 3.1 $$MR = \frac{(AM - NM)}{NM} \times 100$$

3.3 Results

3.3.1 Field colonisation of barley

At the time of harvest barley plants from all three sites were in the stem elongation phase of growth, prior to head emergence and flowering (Figure 3.1). The results of AM colonisation are presented in Table 3.2. AM structures were observed in all samples from all field sites. Total colonisation of barley by AM fungi ranged from 9 - 47% across the three field sites. The lowest level of colonisation

was observed at Roseworthy site 1. This site had the highest Olsen P and the lowest pH. Both Waikerie and Roseworthy site 2 had low soil P (14 mg kg^{-1}) but the level of colonisation was quite different between these sites (18% and 47%, respectively).

NOTE: This figure is included on page 45 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3.1 Growth stages of cereal as defined by Large (1954) and reproduced from Reuter & Robinson (1997). Hatched arrow indicates growth stage of field crops at harvest in this experiment.

Table 3.2 Arbuscular mycorrhizal colonisation of barley crops grown commercially at 3 sites in South Australia.

Site	**Cultivar**	**% AM colonisation**[1]
Roseworthy 1	Keel	9.2 ± 3.2
Roseworthy 2	Sloop	47.0 ± 7.8
Waikerie	Sloop	18.1 ± 2.8

[1]Values are means ± SEM of sampling from four distinct locations within each site.

3.3.2 Growth and P response of barley to mycorrhizal colonisation by two AMF in a glasshouse experiment

AM Colonisation

The percentage of total mycorrhizal colonisation by both AM species increased over the 6 week growth period to a maximum of 19% in roots colonised by *G. geosporum* and 72% in those colonised by *G. intraradices* (Table 3.3). No colonisation was observed in NM control plants. Colonisation by *G. intraradices* was significantly higher than *G. geosporum* at all three harvests.

Both AM species formed *Arum*-type colonisation structures including arbuscules, and there was no difference in the proportion of colonised roots which contained arbuscules. Vesicles, lipid-rich storage structures produced in intercellular spaces of the colonised root cortex by some AM fungi, were only observed once in roots colonised by *G. geosporum* whereas vesicles were observed with increasing frequency at each harvest in roots colonised by *G. intraradices*.

Table 3.3 Colonisation of barley plants inoculated with *Glomus geosporum* or *Glomus intraradices* grown for 2, 4, or 6 weeks. Data presented as total percentage root length colonised and percentage of total colonisation as arbuscular colonisation or vesicular colonisation (See Section 2.9.2 for details).

Myc	Harvest	% Total colonisation[1]	% Arbuscular colonisation[1]	% Vesicular colonisation[1]
Glomus geosporum	Week 2	3±2[a]	50±19[a]	0[a]
	Week 4	2±0.3[a]	56±22[a]	0[a]
	Week 6	19±6[b]	38±13[a]	1±2[a]
Glomus intraradices	Week 2	57±2[c]	64±3[a]	8±2[ab]
	Week 4	61±5[cd]	62±10[a]	15±5[b]
	Week 6	72±4[d]	78±2[a]	26±3[c]

[1]Values are means ± SEM of three replicates. Values with the same letter in each column are not significantly different ($P<0.05$).

Growth and P status

There were no significant differences in growth (Figure 3.2) at the 2 week harvest for any treatments. However, at both the 4 and 6 week harvests mycorrhizal plants colonised by both AM fungi were significantly smaller than NM control plants. There was no difference in growth between plants colonised by *G. geosporum* or *G. intraradices*. The mean MGR at the 4 and 6 week harvests the MGR was -41 to -35%, respectively (Table 3.4).

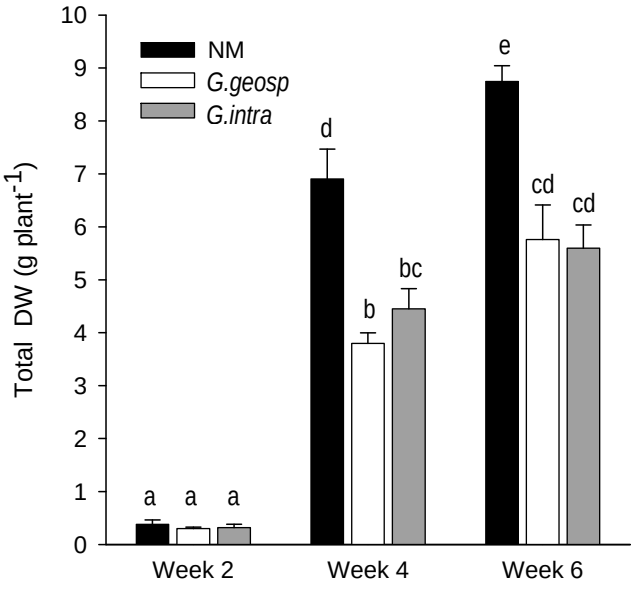


Figure 3.2 Total DW (g plant^{-1}) of barley plants grown for 2, 4, or 6 weeks. Treatments were non-mycorrhizal (NM), colonised by *Glomus geosporum* (*G.geosp*) or *Glomus intraradices* (*G.intra*). Bars are means of three replicates ± SEM; bars with the same letter are not significantly different (P<0.05).

Table 3.4 Mycorrhizal growth response (MGR) of barley at 4 or 6 weeks colonised by *Glomus geosporum* or *Glomus intraradices*.

Myc	Harvest	MGR (%)[1]
Glomus geosporum	Week 4	-45
	Week 6	-34
Glomus intraradices	Week 4	-36
	Week 6	-36

[1] Calculated according to Equation 3.1.

Shoot and root P concentrations decreased over time and there was no significant difference between AM and NM plants at any harvest (Table 3.5). As a result, total P content of barley plants mirrored the pattern observed for growth at all three harvests with AM plants taking up significantly less P than NM plants at both the 4 and 6 week harvests and no difference between AM treatments (Table 3.5). The mean MPR for both the 4 and 6 weeks harvests was -38%. The strong correlation between plant growth and P uptake is illustrated in Figure 3.3 ($y = 1.02x + 0.59$, $R^2 = 0.95$).

Table 3.5 Shoot and root P concentrations (mg kg^{-1}), total P content (mg) and MPR (%) of barley plants inoculated with *Glomus geosporum*, *Glomus intraradices*, or non-inoculated and harvested at 2, 4 or 6 weeks.

Myc	Harvest	Root P concentration (mg kg^{-1})[1]	Shoot P concentration (mg kg^{-1})[1]	Total P content (mg plant^{-1})[1]	MPR[2] (%)
Non-mycorrhizal	Week 2	823±244^{ab}	4527±490^{a}	0.8±0.1^{a}	
	Week 4	418±126^{a}	2196±98^{bc}	7.9±0.5^{c}	
	Week 6	430±40^{a}	1559±24^{bc}	9.8±0.3^{d}	
Glomus geosporum	Week 2	1122±409^{ab}	4376±258^{a}	0.8±0.2^{a}	
	Week 4	627±184^{ab}	2264±101^{bc}	5.2±0.5^{b}	-35
	Week 6	456±191^{a}	1482±14^{c}	5.8±0.1^{b}	-41
Glomus intraradices	Week 2	1465±280^{b}	4366±88^{a}	0.8±0.1^{a}	
	Week 4	576±123^{a}	2300±49^{b}	5.1±0.6^{b}	-35
	Week 6	522±143^{a}	1653±51^{bc}	5.8±0.2^{b}	-40

[1] Values are means ± SEM of five replicates. Values with the same letter in each column are not significantly different (P<0.05). [2] Calculated according to Equation 3.1.

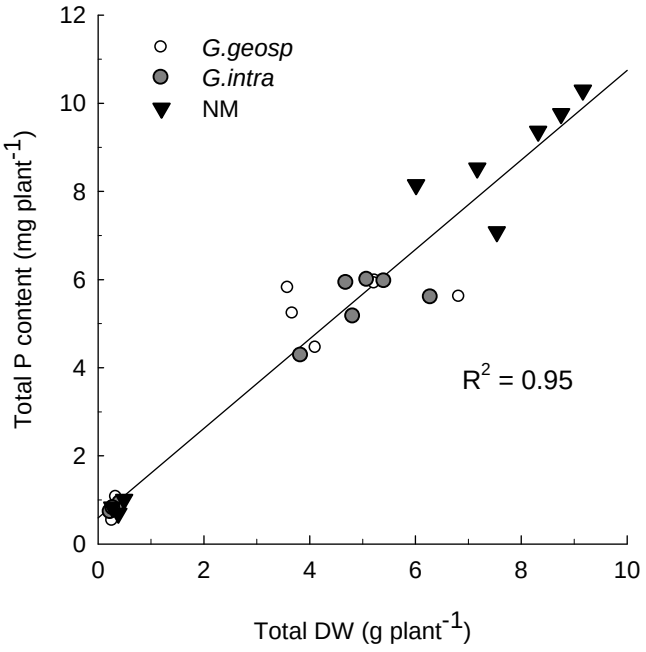


Figure 3.3 Relationship between total DW (g plant^{-1}) and total P content (mg plant^{-1}) for barley cv. Golden Promise. Treatments were non-mycorrhizal (NM), colonised by *Glomus geosporum* (*G.geosp*) or *Glomus intraradices* (*G.intra*). Data from all treatments and all three harvests (2, 4 and 6 weeks) were included in the regression.

3.4 Discussion

Colonisation of barley in the field

Barley was colonised by AM fungi at all three field sites. The lower concentration of available P at Roseworthy site 2 and Waikerie reflects the general practice of sowing malting barley in low nutrient conditions. In contrast, feed barley is usually cultivated with higher nutrient inputs (Dr D. Adcock, personal communication) as reflected by the high P level obtained for Roseworthy site 1. An Olsen P value of 38 mg kg^{-1} is significantly higher than the reported critical concentration of soil P for barley cropping in South Australia (Reuter *et al.*, 1995). Despite the relatively high level of soil P at this site AM colonisation was still observed. Previous surveys of AM colonisation under commercial cropping regimes demonstrated strong relationships between soil fertility and extent of AM colonisation in barley (Jensen & Jakobsen, 1980; Aliasgharzadeh *et al.*, 2001). Although it is not possible to assess statistical significance from this small survey, the data suggest that while available nutrient status may explain differences in colonisation at the two Roseworthy sites, the low level of colonisation at Waikerie which had the same Olsen P as Roseworthy site 2 cannot be clearly explained by soil P.

Numerous factors influence the degree of AM colonisation in field situations. Environment, soil properties, cultivation practices, previous cropping history and host genotype have all been implicated in governing AM colonisation. A more extensive survey such as that conducted by Jensen & Jakobsen (1980) would be necessary in order to draw firm conclusions defining which of these factors led to the differential colonisation observed at these field sites.

Conclusion

It has been suggested that AM fungi do not have a vital role in production agriculture (Ryan & Graham, 2002). Nevertheless, the persistence of the symbiosis under commercial conditions highlights the need to improve our understanding of AM symbioses in order to better manage and manipulate AM for improved crop nutrition and productivity. Although this survey was not extensive it provides evidence that barley crops are colonised in southern Australia and shows the relevance of AM investigations to commercial broad-acre cereal production in South Australia. It is essential to consider the role of AM fungi, whether beneficial or detrimental, in P acquisition by these crops.

Responsiveness of barley cv. Golden Promise to AM colonisation under glasshouse conditions

There were significant differences in the level of colonisation by the two AM fungi in this study. The colonisation achieved by *G. intraradices* is at the high end of reported colonisation levels in barley and is in agreement with previous reports of colonisation by this fungal species (Plenchette & Morel, 1996; Zhu *et al.*, 2003). The level of colonisation achieved by *G. geosporum* can be described as moderate for barley. Although colonisation progressed more slowly in this interaction, the presence of arbuscules indicates that initial colonisation rather than maturation of colonisation units was delayed. High variability in the degree of colonisation of barley by different AM fungi has been reported previously (Jensen, 1984) and has also been observed in wheat (Li *et al.*, 2005). However, it is important to note that in this experiment inoculum was applied on a w/w basis. This does not take into account possible differences in inoculum quality in terms of number of infective propagules per gram. Under these conditions variability in colonisation may relate to inoculum quality, the inherent colonisation strategies of the fungi or differential effects of the experimental conditions on the different species.

Under the conditions of this experiment the model cultivar, Golden Promise, demonstrated a depression in both growth and P content relative to NM controls. As discussed in Chapter 1, the negative MPR obtained in this experiment would, in the past, have been taken to indicate that the AM pathway of P_i uptake is non-functional for both *G. geosporum* and *G. intraradices* despite the high levels of colonisation formed by *G. intraradices*. However, evidence from radiotracer experiments suggests that calculation of MGR and MPR does not represent the actual contribution of the AM pathway. This data highlights the difficulties in drawing conclusions on the functionality of the AM symbiosis in non-responsive plant species based upon the conventional measures of growth, P content and % colonisation. However, some insights into the cause of the growth depression can be gained from this study.

Growth depressions in AM colonised plants are often attributed to the cost of C supply to a non-beneficial fungal symbiont. It therefore follows that the magnitude of growth depression will vary with the extent of colonisation and hence C demand of the AM fungus. In this experiment the higher colonisation and production of vesicles by *G. intraradices* suggests that C demand by this fungus would have been significantly higher than C demand by *G. geosporum*. However, there was no

difference in the MGR of plants colonised by either AM fungus. In this instance, the growth depression due to AM colonisation cannot readily be explained by C drain.

The application of mixed soil inoculum solely to AM pots results in differences in the microbial community compared to NM pots which do not receive any additional amendments. Although it is possible that the AM microbial community may cause differences in plant growth there was no evidence of pathogen activity in AM roots and previous experiments conducted in our laboratories to assess the impact of microbial community differences in AM experiments suggest that this is not a problem (Prof. SE Smith personal communication). The strong correlation between growth and total P content suggests that P was the growth-limiting factor in this experiment. This is supported by the fact that the shoot P concentration at the 4 and 6 week harvests is considered deficient for barley (Reuter & Robinson, 1997). Similar findings of growth depressions associated with low levels of colonisation have been observed in wheat (Hetrick *et al.*, 1992; Li *et al.*, unpublished 2007).

Regardless of the (unquantified) contribution of the AM pathway to plant P uptake it is evident from this experiment that AM barley took up less P than NM barley, associated with a growth depression. This suggests that P_i uptake via the direct pathway was impeded in AM colonised plants. As outlined in Chapter 1, AM colonisation often results in the down-regulation of epidermal P_i transporters; whether this can account for the decrease in uptake by the direct pathway will be investigated in following chapters. The results of this experiment highlight the shortfalls in traditional measurements of AM contribution and raise questions regarding the contribution of both AM fungal species to P_i uptake in barley. In order to gain further insight into the role of these two AM fungi it will be necessary to investigate the actual contribution of the AM pathway using ^{32}P tracer studies in compartmented pots.

Chapter 4

Measuring AM Contribution to P_i Uptake in Barley

4.1 Introduction

The experiment described in Chapter 3 demonstrated the growth-inhibiting effect of colonisation by two AM fungal species on barley at low P supply. Such growth depressions are typical of non-responsive host species. In this chapter a compartmented pot system has been used to quantify the actual contribution of these AM fungi to plant P_i uptake.

The total P content of an AM plant results from the combined operation of two pathways; the direct uptake pathway via the root epidermis and root hairs, and the AM pathway via external AM hyphae and colonised cortical cells. Compartmented pots incorporating radiolabelled hyphal compartments (HC) have been used to demonstrate significant contributions of the AM pathway to total P uptake regardless of host plant responses (see Chapter 1). Whilst demonstrating that AM fungi are capable of making significant contributions to P nutrition, even in non-responsive species, these measurements also indicate that the contribution of the direct uptake pathway can be reduced in AM colonised plants. This provides a significant link with data from gene expression studies which suggest that, in some AM interactions, AM colonisation can lead to down-regulation of expression of P_i transporters involved in P_i uptake via the direct pathway (Glassop *et al.*, 2005). This is particularly significant as transcriptional regulation has been identified as a primary control point in determining P_i transporter expression (Smith *et al.*, 2003a).

The aim of the experiment described in this chapter was therefore to: 1) quantify the contribution of the AM pathway to P_i uptake in barley; 2) quantify the expression of the P_i transporters involved in both AM and direct P_i uptake pathways and hence determine whether changes in the contribution of the two pathways are correlated with changes in gene expression. Glassop *et al.* (2005) previously reported that three of the eight P_i transporters identified in barley, *HvPT1*, *HvPT2* and *HvPT3*, were expressed in NM roots and responded to P_i concentrations in the soil. Of these, *HvPT1* and *HvPT2* were also down-regulated by AM colonisation. Therefore, the expression of *HvPT1*, *HvPT2* and the AM-inducible *HvPT8* was examined in this experiment. In order to distinguish AM effects on P_i transporter expression from the response resulting from P fertilisation two P levels were included in the experimental design.

4.2 Materials and Methods

4.2.1 Compartmented pot design and experimental setup

This experiment used a compartmented pot system comprising a small hyphal compartment (HC) within a second larger root + hyphal compartment (RHC) as described previously (Smith *et al.*, 2003b, 2004) (Figure 4.1). The HC was a small plastic container filled with 40 g NM Mallala growth medium (Section 2.2), labelled with carrier-free $H_3{}^{32}PO_4$ to provide 19.8 kBq g^{-1} and topped up with 14 g non-labelled NM growth medium as a buffer zone to prevent ^{32}P uptake by root hairs and diffusion of ^{32}P out of the HC. This compartment was covered with a 30 µm mesh which restricts access to AM hyphae only. The HC was placed 5 cm below the rim of the pot with the mesh facing inwards and the pot was filled with 1349 g growth medium. In total, experimental pots contained 1403 g Mallala growth medium of which 3.8% was contained in the HC. Preparation of Mallala growth medium is outlined in Section 2.2, including P addition. Two soil P application rates were used; P1 (5.7 mg kg^{-1} additional P) and P2 (11.4 mg kg^{-1} additional P). AM inoculum of either *G. intraradices* or *G. geosporum* was added to the RHC of mycorrhizal pots only (15% w/w), as outlined in Section 2.3.3. Inoculum used in this experiment was obtained from the same batch as used for the glasshouse experiment described in Chapter 3.

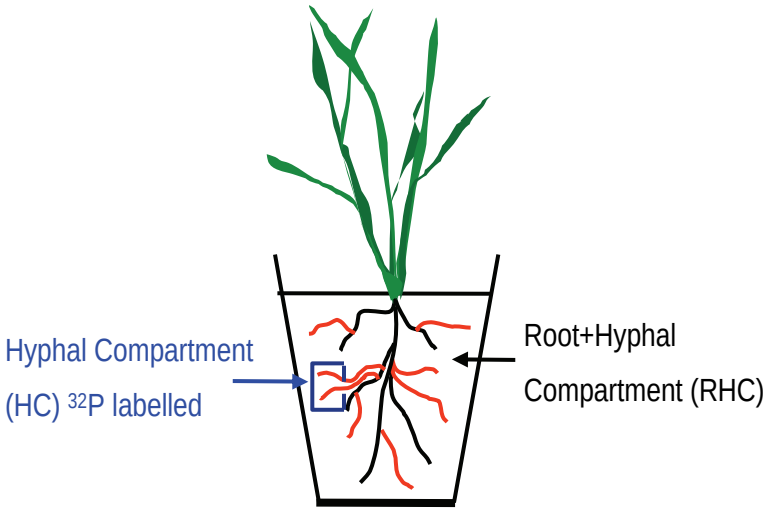


Figure 4.1 Diagrammatic representation of the compartmented pot system used in this experiment

There were five replicate pots per treatment. Three additional pots without inoculum were set up at the two P levels for measuring specific activity of ^{32}P in the HC at harvest. Seeds of barley cv. Golden Promise were surface sterilised, germinated and planted singly at the centre of each pot. Experimental pots were maintained in a glasshouse for 5 weeks as outlined in Section 2.4.2. An

additional 10 mg N as NH_4NO_3 was applied three times during the final 2 weeks of growth. The appearance of ^{32}P in the shoots was followed non-quantitatively with a hand-held monitor.

4.2.2 Harvest

Pots were harvested according to Section 2.5. Shoot and root material were sampled for fresh and dry weights, tissue P content (Section 2.7) and specific activity (Section 2.8). Roots were also sampled for AM colonisation (Section 2.9) and Q PCR analysis of the expression of three P_i transporter genes, *HvPT1*, *HvPT2* and *HvPT8* (Section 2.11). Q PCR was performed on root samples from three of the five replicates for each treatment using the gene specific primers listed in Table 2.2. Unfortunately, extracted RNA from *G. geosporum* roots grown at P1 was degraded and additional replicates were destroyed during a -80^{o}C freezer failure, consequently there is no Q PCR data for this treatment.

Soil from the RHC or HC was mixed thoroughly and sampled for plant-available P determination by both Olsen P and resin P methods (Section 2.6) and scintillation counting of these extracts (Section 2.8). Soil samples were also taken for measurement of hyphal length density (HLD) but these samples were lost and HLDs could not be determined.

4.2.3 Calculations

The contribution of the AM pathway to plant P_i uptake was calculated from the specific activity (SA) of ^{32}P in shoots and HC soil and the plant-available P in the RHC and HC according to Equation 4.1. Specific activity was calculated from the measured ^{32}P activity and P concentration in plant tissue digests or soil extracts as outlined in Section 2.8. The use of shoot SA rather than the whole plant SA avoids overestimation of hyphal P transfer by inclusion of ^{32}P retained in the intraradical hyphae. Soil SA and plant-available P were measured using both Olsen P and resin P methods and the percent contribution estimate was calculated using both values.

$$\text{Equation 4.1} \quad \%\ \text{contribution AM pathway} = \frac{\text{SA P shoot}}{\text{SA P HC}} \times \frac{\text{Soil P pot}}{\text{Soil P HC}} \times 100$$

This calculation assumes that: 1) SA of HC is a valid estimate of P availability in the RHC and HC and that these are the same; 2) hyphal length density is equal in the RHC and HC and that hyphae

access P equally in the two compartments; 3) that colonisation is rapidly established and AM fungi rapidly penetrate the HC.

The mycorrhizal growth response (MGR) and mycorrhizal P response (MPR) were calculated as described in Section 3.2.2 (Equation 3.1).

Significant interactions between mycorrhiza and P treatments were analysed by two-way ANOVA as outlined in Section 2.10 except for gene expression data. Due to the incomplete data set gene expression was analysed by one-way ANOVA, therefore the effect of AM and P cannot be separated.

4.3 Results

4.3.1 AM colonisation

The percentage AM colonisation was significantly different for barley colonised by either *G. geosporum* or *G. intraradices* (Table 4.1). Colonisation by *G. geosporum* was minimal, reaching only 2-4% regardless of P supply. Colonisation units of this fungus included arbuscules and very few vesicles. In contrast, colonisation by *G. intraradices* reached 55% of total root length at P1 and was reduced with increased P, to 43% at P2. Although there was also a decrease in the mean percentage of colonised root containing arbuscules and vesicles at P2 this was not significant. No colonisation was observed in NM control plants.

Table 4.1 Colonisation of barley plants inoculated with *Glomus geosporum* or *Glomus intraradices* grown at P1 (5.7 mg kg^{-1} additional P) or P2 (11.4 mg kg^{-1} additional P). Data are presented as total percentage root length colonised and percentage of total colonisation as arbuscular colonisation or vesicular colonisation.

Myc	P level	% Total colonisation[1]	% Arbuscular colonisation[1]	% Vesicular colonisation[1]
Glomus	P1	2±1[a]	49±19[a]	5±5[a]
geosporum	P2	4±1[a]	48±5[a]	3±2[a]
Glomus	P1	55±3[b]	64±4[a]	14±3[a]
intraradices	P2	43±6[c]	54±5[a]	12±1[a]

[1]Values are means ± SEM of five replicates. Values with the same letter in each column are not significantly different ($P<0.05$).

4.3.2 Growth and P response

The growth response of barley to AM colonisation was similar for both AM fungi (Figure 4.2). Shoot DW was significantly reduced by AM colonisation and there was no difference between plants colonised by *G. geosporum* or *G. intraradices* (Figure 4.2a). Root DW was also reduced by AM colonisation (Figure 4.2b). This was significant for all treatments except for plants colonised by *G. intraradices* at P2. Increased P supply did not significantly alter the growth of plants for any treatment at P2 compared to that at P1. The MGR was -46% for plants colonised by *G. geosporum* and plants colonised by *G. intraradices* at P1 and -38% for plants colonised by *G. intraradices* at P2 (Table 4.2).

Table 4.2 Mycorrhizal growth response (MGR) of barely colonised by *G. geosporum* or *G. intraradices* and grown at P1 (5.7 mg kg^{-1} additional P) or P2 (11.4 mg kg^{-1} additional P).

Myc	P level	MGR (%)[1]
Glomus geosporum	P1	-45
	P2	-46
Glomus intraradices	P1	-46
	P2	-38

[1]Calculated according to Equation 3.1; MGR = (AM dry wt-NM dry wt)/NM dry wt*100.

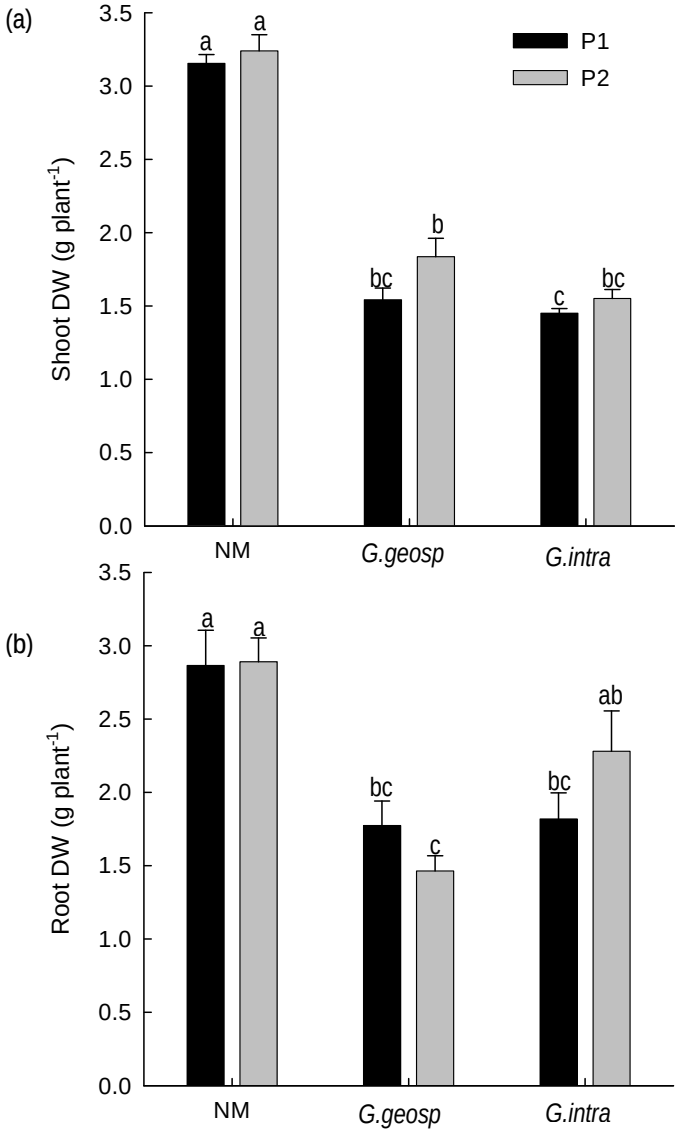


Figure 4.2 Shoot (a) and root (b) DW (g plant^{-1}) of barley plants inoculated with *Glomus geosporum*, *Glomus intraradices*, or non-inoculated (*G.geosp*, *G.intra*, NM) grown at P1 (5.7 mg kg^{-1} additional P) or P2 (11.4 mg kg^{-1} additional P). Bars are means of five replicates ± SEM; bars with the same letter are not significantly different (P<0.05).

There was no difference in shoot or root P concentrations of plants grown at P1 (Table 4.3). The shoot P concentration of plants colonised by *G. geosporum* did not change with higher P and while there was a trend for *G. intraradices* and NM plants at P2 to have higher shoot P concentrations than those at P1 this was only significant for NM plants. Consequently the total P content of NM plants at P2 was significantly greater than P1 (Table 4.3). As was observed for plant growth, both AM fungi significantly decreased the total P content of barley compared to NM control plants and there was no difference in total P content between plants grown with *G. geosporum* or *G. intraradices*. The mean MPR of mycorrhizal plants was -48%.

Table 4.3 Shoot and root P concentrations (mg kg-1), total P content (mg) and MPR (%) of barley plants inoculated with *Glomus geosporum*, *Glomus intraradices*, or non-inoculated grown at P1 (5.7 mg kg-1 additional P) or P2 (11.4 mg kg-1 additional P).

Myc	P level	Root P concentration (mg kg^{-1})[1]	Shoot P concentration (mg kg^{-1})[1]	Total P content (mg $plant^{-1}$)[1]	MPR[2] (%)
Non-mycorrhizal	P1	491±37[a]	1518±58[a]	6.2±0.2[a]	
	P2	713±57[b]	1853±65[c]	8.0±0.5[b]	
Glomus geosporum	P1	507±32[a]	1590±51[a]	3.4±0.2[c]	-46
	P2	641±47[ab]	1589±22[a]	3.8±0.2[c]	-52
Glomus intraradices	P1	546±69[ab]	1634±27[ab]	3.4±0.2[c]	-46
	P2	580±31[ab]	1789±33[bc]	4.1±0.2[c]	-49

[1]Values are means ± SEM of five replicates. Values with the same letter in each column are not significantly different (P<0.05). [2]Calculated according to Equation 3.1; MPR = (AM P-NM P)/NM P*100.

4.3.3 ^{32}P uptake and contribution of the mycorrhizal pathway

^{32}P was first detected in the shoots of plants colonised by *G. intraradices* using a hand-held monitor at 11 days. At harvest negligible levels of ^{32}P were detectable in NM plants and no ^{32}P was detectable in soil adjacent to the HC mesh, indicating the effectiveness of the buffer zone in preventing leakage of ^{32}P. Negligible levels of ^{32}P were detected in plants colonised by *G. geosporum* except for one of the five replicates at P2. High levels of ^{32}P were detectable in plants colonised by *G. intraradices* grown at both P1 and P2 as evidenced by the shoot specific activities (Figure 4.3). However, there was no significant difference between the two P treatments. The variation between replicates within each treatment was not correlated with either total colonisation or arbuscular colonisation data.

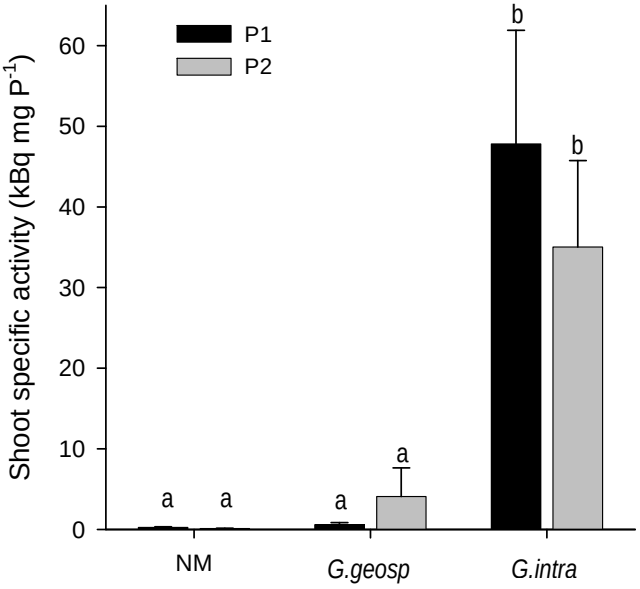


Figure 4.3 Shoot specific activity (kBq mg P^{-1}) of barley plants inoculated with *Glomus geosporum*, *Glomus intraradices*, or non-inoculated (*G.geosp*, *G.intra*, NM) grown at P1 (5.7 mg kg^{-1} additional P) or P2 (11.4 mg kg^{-1} additional P). Bars are means of five replicates ± SEM; bars with the same letter are not significantly different (P<0.05).

The specific activity of soil in the HC depended greatly on the P extraction method (Figure 4.4). Olsen P specific activities were 30-50% lower than the specific activities calculated from resin P. Olsen P is a stronger extraction method, therefore the 'exchangeable' P pool for Olsen P is greater, resulting in the lower soil specific activity. In addition, the specific activity of the HC in pots with *G. intraradices* decreased relative to the blank HC, suggesting that the HC P pool was depleted by hyphal P_i uptake. As a result the specific activity of the blank was used in calculating the contribution of the AM pathway (Equation 4.1).

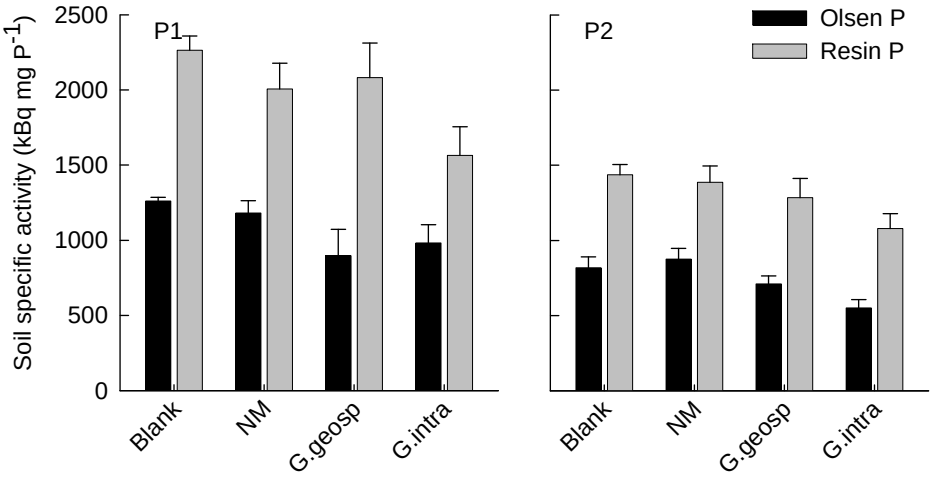


Figure 4.4 Soil specific activity (kBq mg P^{-1}) at harvest in the hyphal compartment (HC) as determined by Olsen P or resin P. Treatments were P1 (5.7 mg kg^{-1} additional P) or P2 (11.4 mg kg^{-1} additional P) without plants (blank) or with plants inoculated with *G. geosporum* (G.geosp), *G. intraradices* (G.intra) or non-mycorrhizal (NM). Bars are means of five replicates ± SEM.

As predicted from differences in the soil specific activity, the calculated contribution of the AM pathway to P uptake varied depending upon the soil extraction method. According to Equation 4.1, the lower HC specific activity for Olsen P will result in a higher estimate of the AM contribution to plant P. The calculated contribution of the AM pathway to P uptake in plants colonised by *G. intraradices* was greater than 100% using Olsen P values (clearly an impossible situation) whereas values based on resin P estimate 41-55% contribution of the AM pathway (Table 4.4).

Table 4.4 Percent contribution of the AM pathway to shoot P based on either Olsen P or resin P data (Refer to section 2.6 for details of soil extraction procedures).

Myc	P level	% contribution of AM pathway (Olsen)[1]	% contribution of AM pathway (resin)[1]
Non-mycorrhizal	P1	na	na
	P2	na	na
Glomus geosporum	P1	1±1[a]	0[a]
	P2	14±12[a]	5±4[a]
Glomus intraradices	P1	104±36[b]	41±15[b]
	P2	137±42[b]	55±17[b]

[1]Values are means ± SEM of five replicates. Values with the same letter in each column are not significantly different (P<0.05).

4.3.4 Expression of plant P_i transporter genes

The expression level of the root epidermal P_i transporters, *HvPT1* and *HvPT2*, and the AM-inducible P_i transporter, *HvPT8*, was examined in barley root tissue (Figure 4.5). *HvPT1* was variably but constitutively expressed across all treatments. In contrast, expression of *HvPT2* was significantly increased in NM roots at P2 compared to P1. Although there was a trend for down-regulation of *HvPT2* in AM roots compared to NM roots at P2, this was only significant for the *G. geosporum* P2 treatment. Transcripts of *HvPT8* were detectable at low levels in NM roots and were significantly up-regulated in roots colonised by *G. intraradices*. The variation between samples within a treatment could not be explained by colonisation data or tissue P concentrations and was not correlated with ^{32}P data. The expression of *HvPT8* was detected in all three replicates of roots colonised by *G. geosporum* and the highest level of expression occurred in the single replicate for which ^{32}P transfer was detected.

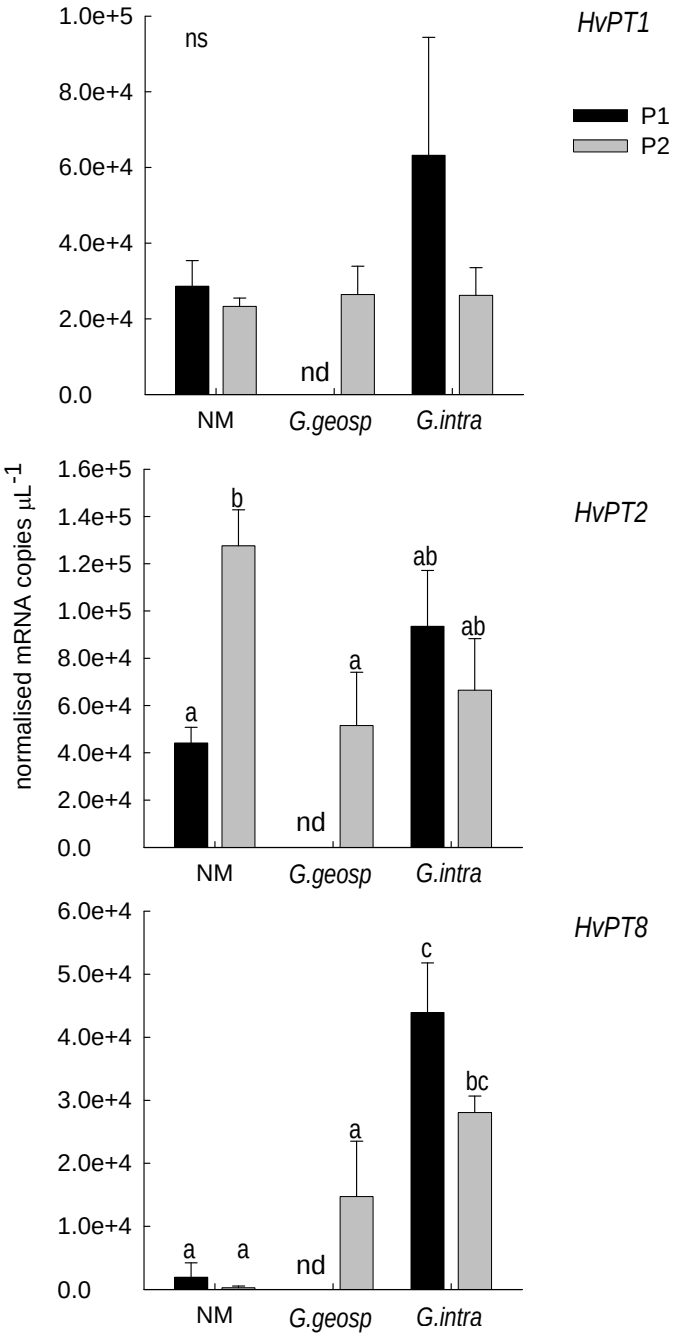


Figure 4.5 Normalised expression levels of three phosphate transporters, *HvPT1*, *HvPT2* and *HvPT8*, in roots of barley plants; non-mycorrhizal (NM) or inoculated with *Glomus geosporum* (*G.geosp*) or *Glomus intraradices* (*G.intra*). Plants were grown at P1 (5.7 mg kg^{-1} additional P) or P2 (11.4 mg kg^{-1} additional P). nd: no data available for this treatment. Bars are means of three replicates ± SEM. Values with the same letter in each column are not significantly different by one-way ANOVA (P<0.05), ns: no significant difference between treatments.

4.4 Discussion

Consistent with findings from the glasshouse experiment in Chapter 3, colonisation of barley by both *G. geosporum* and *G. intraradices* resulted in significant depressions in growth and P uptake. Once again this was not related to the extent of colonisation by either fungus. By including a radiolabelled HC in this experiment it was possible to demonstrate that hyphae of both *G. geosporum* and *G. intraradices* were capable of transferring P to the plant. This confirms and extends previous work of Zhu *et al.* (2003) who demonstrated hyphal ^{32}P transfer by *G. intraradices* to barley cultivars Clipper and Sahara, despite significant growth depressions. By measuring the soil specific activity in the HC it was possible to quantify the contribution of the AM pathway to total plant P uptake for the first time in barley, using the method of Smith *et al.* (2003b).

The quantification of AM P transfer according to Equation 4.1 is based on a number of key assumptions (see Section 4.2.3). As is evident from the over-estimation of AM contribution using Olsen P values, the accurate estimation of available P is a crucial factor in this calculation. Soil extraction methods estimate the amount of P in different soil pools and have been equated with the amount of P that is available to plants over a period of growth. However, the accuracy of these methods varies and is dependent on soil type. Anion-exchange resins simulate ion uptake characteristics of roots and tend to provide a more accurate estimate of available P than chemical extractants (McLaughlin *et al.*, 1994; van Raij, 1998). This is reflected in the more realistic estimate of AM contribution based on resin P in the current experiment. Similarly, Li *et al.* (2006) reported that Colwell P, a harsher variation of Olsen P extraction, greatly overestimated the plant-available P pool resulting in an estimate of 180% contribution of the AM pathway in wheat whereas the estimate based on resin P was 50-80%. The use of these extraction methods to estimate AM fungal contribution also assumes that fungal hyphae access the same pools of available P as plant roots. Although there is recent evidence to suggest that some AM fungi hydrolyse organic P via phosphatases (Koide & Kabir, 2000), plant roots also possess phosphatase activity and the assumption is based on evidence from plants grown in ^{32}P-labelled soil which resulted in similar specific activities in both NM and AM plants (Hayman & Mosse, 1972).

Finally Equation 4.1 assumes that colonisation of roots is rapid, that hyphal distribution is uniform in both the RHC and HC and that hyphae remove P from the RHC and HC equally. The results presented in Chapter 3 indicate that *G. intraradices* rapidly colonised barley during the first 2 weeks of growth and although HLD was not measured in this experiment, the uniform development of *G.*

intraradices hyphae in the RHC and HC has been reported previously (Smith *et al.*, 2004; Li *et al.*, 2006). However, there are no data for hyphal development of *G. geosporum* in this system. In the current experiment colonisation by *G. geosporum* was quite low; consistent with this observation *G. geosporum* did not make a large contribution to P_i uptake by barley. However, measurement of AM contribution by the two compartment method used here is dependent on AM fungal hyphae accessing the HC. The detection of ^{32}P in one of the plants colonised by *G. geosporum* indicates that this fungus is capable of P transfer to the host even at low levels of colonisation. This is supported by the presence of arbuscules in *G. geosporum* colonised roots and the expression of the AM-inducible P_i transporter *HvPT8*. The most likely explanation for the lack of ^{32}P uptake by *G. geosporum* is that hyphae of this fungus did not access the HC. In future experiments the measurement of HLDs in the HC and comparison to NM pots could confirm this assumption.

The calculation of AM contribution in barley colonised by *G. intraradices* and determined by resin P suggests that 41-55% of plant P was acquired via the AM pathway. This is in agreement with Li *et al.* (2006) who demonstrated a 50-80% contribution of *G. intraradices* to wheat using the resin P method. Taken together with the MPR of -50% these data suggest that P uptake via the direct pathway was reduced in plants colonised by *G. intraradices*. This experiment tested the hypothesis that decreased uptake via the direct pathway results from down-regulation of the root epidermal P_i transporters. There was no clear down-regulation of either *HvPT1* or *HvPT2* in roots colonised by *G. intraradices* and in fact there was a trend towards increased expression of these genes at P1 compared to NM roots at P1. This indicates that the decrease in function of the direct pathway in roots colonised by *G. intraradices* is unrelated to expression level of these genes. A similar conclusion was reached by Poulsen *et al.* (2005) for AM tomato. However, this contradicts previous findings of Glassop *et al.* (2005) showing a P-independent down-regulation of *HvPT1* and *HvPT2* in AM barley roots. At this time there is no obvious explanation for this discrepancy.

The decrease in contribution of the direct pathway in plants colonised by *G. intraradices* may be due to competition between fungal hyphae and plant roots leading to more rapid formation of a depletion zone in AM than NM plants. In addition the smaller root system of AM plants equates with a smaller soil volume explored by AM roots. Alternatively post-transcriptional or post-translational modification of P_i transporters may be involved in altering transport activity. Although regulation of P_i transporter expression is predominantly transcriptional, post-transcriptional and post-translational processes involved in the modification of regulatory components involved in the regulation of P_i

transport have begun to be elucidated (Fujii *et al.*, 2005; Miura *et al.*, 2005; Chiou *et al.*, 2006). It is likely that such mechanisms will also be involved in the AM response.

It has been suggested that growth depressions at low levels of colonisation, as observed here for *G. geosporum*, may be explained by down-regulation of direct P_i uptake in response to fungus-plant recognition, even though P flux through the AM pathway is small (Li *et al.* unpublished, 2007). Although *HvPT2* was down-regulated in roots colonised by *G. geosporum* at P2 compared to NM roots at P2, the expression level was equivalent to NM roots at P1. However, the growth of barley colonised by *G. geosporum* was significantly reduced irrespective of P treatment. Therefore, in this experiment the growth depression caused by *G. geosporum* cannot be readily explained by transcriptional regulation of epidermal P_i transporters. As discussed above, post-translational regulation of the direct pathway may be important in this response.

Despite the possible errors in calculations, overall the experiment described in this chapter demonstrated a significant (48%) contribution of the AM pathway to P uptake in barley colonised by *G. intraradices* but not *G. geosporum*. The contribution of the direct and AM pathways was not correlated with the expression of P_i transporters at the time of harvest.

Chapter 5

Spatial & Temporal Expression of P_i Transporters in AM Barley

5.1 Introduction

The experiment described in Chapter 4 assessed the response of P_i transporters involved in both the direct and AM pathways of P_i uptake to colonisation by two AM fungi. The aim of the work described in this chapter was to further investigate the spatial and temporal expression patterns of these transporters in barley colonised by *G. intraradices*.

Glassop *et al.* (2005) used an *in-situ* hybridisation approach to demonstrate the localisation of *HvPT8* transcripts to cortical cells containing arbuscules and arbusculate coils, consistent with the localisation of other AM-inducible P_i transporters in cereals and in dicotyledonous species (see Chapter 1). No expression was observed in non-colonised cortical cells of AM roots and expression in NM roots was not assessed. The aim of the first experiment described in this chapter was to confirm the spatial expression profile of *HvPT8* in both AM and NM barley. Two approaches were pursued for investigating the spatial expression of *HvPT8*. An immunolocalisation strategy was developed initially, as this technique enables detection and localisation of target protein at a sub-cellular level and would extend the findings of Glassop *et al.* (2005). However, antibody production was unsuccessful on three separate occasions; therefore this aspect of the project was suspended. The second approach, described in this chapter, utilised promoter::GFP constructs to visualise the expression of *HvPT8* in live root samples of transgenic barley.

The aim of the second experiment described in this chapter was to investigate the expression of *HvPT1*, *HvPT2* and *HvPT8* during the development of AM colonisation in wild-type barley cv. Golden Promise. Although this was assessed in the previous chapter, gene expression data in that experiment represent expression at a single time-point when plants were harvested at 5 weeks, whereas P uptake is a continuous process occurring throughout the lifecycle of the plant. Therefore, the expression of *HvPT1*, *HvPT2* and *HvPT8* was followed in a time-course experiment targeting the early stages of AM colonisation. Previous investigations of the development of AM colonisation in wheat, maize and oats reported the presence of young arbuscules 2 days after first infection whilst mature arbuscules were present at 4 days (Alexander *et al.*, 1988) and it has been reported that the entire arbuscular cycle in maize is approximately 10-12 d (Toth & Miller, 1984).

Therefore, P_i transporter expression was followed over a 17 d period in a nurse pot system designed to produce rapid and synchronous colonisation; the expression of P_i transporters was monitored by Q PCR in conjunction with assessment of development of AM colonisation.

5.2 Materials and Methods

5.2.1 Spatial expression profiling of the AM-inducible, *HvPT8*

In order to determine the expression pattern of the AM-inducible P_i transporter *HvPT8,* the gene promoter was coupled to the fluorescent reporter GFP (green fluorescent protein) and the pattern of expression was determined in transgenic barley plants by laser scanning confocal microscopy (LSCM).

Cloning of the promoter::reporter construct

The promoter region of *HvPT8* was PCR amplified from a plasmid containing the promoter and coding sequence supplied by Dr D. Glassop (CSIRO Plant Industry, Brisbane), using the primers listed in Table 5.1. These primers incorporated the appropriate restriction sites for cloning into pWBVec8. PCR was performed as outlined in Section 2.14.1 with 2.5 ng template DNA; annealing, 57^{o}C; elongation, 1 min. The amplification product was 1264 bp, composed of 1172 bp upstream of the CAAT box and 88 bp downstream, ending 48 bp before the translation start site. The amplification product was cleaned up using a QIAquick PCR purification kit (Section 2.14.1) and sequenced to ensure errors had not been introduced (Section 2.13).

Table 5.1 Primers used in the amplification of the *HvPT8* promoter. Incorporated restriction site sequences are underlined, two nucleotides were added to aid restriction enzyme (RE) digestion.

Primer	Sequence	Incorporated restriction sites	T_m (^{o}C)[1]
*HvPT8*promoter F	ga<u>TTAATTAA</u>CGCCAGTCGGTATGAATTCA	*Pac*I	61
*HvPT8*promoter R	ct<u>GGCGCGCC</u>AAGATTCAAGACGGTCCTCG	*Asc*I	76

[1] Primer T_m as reported by the manufacturer (Proligo).

The binary vector pWBVec8 (Appendix 2), incorporating the *HvPT1* promoter coupled to the first intron of the maize alcohol dehydrogenase gene (*Adh1)*, the *GFP* gene (*sgfp*S65T) and the nopaline synthase gene terminator (*Nos*) (Schunmann *et al.*, 2004), was supplied by Dr P. Schunmann (CSIRO Plant Industry, Canberra, Australia). The *HvPT1* promoter was excised by *Pac*I/*Asc*I digestion and the *HvPT8* promoter fragment was digested with these same enzymes

prior to ligation into the pWBVec8 backbone as outlined in Section 2.14.4. The ligation product was transformed into *E. coli* and individual colonies were sub-cultured overnight as outlined in Section 2.14.5. Plasmid DNA was isolated (Section 2.14.6), the vector integrity was checked by RE digestion and the full insert was sequenced. The *HvPT8*-derived expression construct is illustrated in Figure 5.1. The expression cassette was cloned in the same orientation and upstream of the selectable marker. As demonstrated by Schunmann *et al.* (2004) inclusion of the intron increases expression at least 20-fold without affecting the specificity of expression.

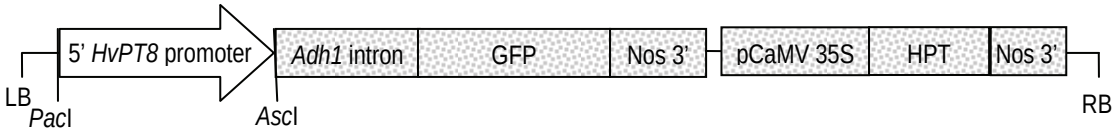


Figure 5.1 Structure of the promoter::reporter plant transformation construct. The *HvPT8* promoter was inserted at the left border, upstream of the *Adh1* intron. pWBVec8 carries the *HPT* gene driven by the CaMV35S promoter (pCaMV35S) for selection of transgenic plants on hygromycin.

The completed expression vector was transformed into *Agrobacterium* AGL-1 (Section 2.14.5) in preparation for plant transformation of *Hordeum vulgare* cv. Golden Promise as outlined in Section 2.14.7. Regenerated transformants were selected on antibiotics for the presence of the T-DNA and 14 independent lines were transferred to commercial grade potting mix and grown to maturity in a glasshouse facility.

Plant propagation and harvesting

Preliminary screening for GFP fluorescence was conducted on NM and AM primary transformants (T0) as follows. T0 plants were assessed at 8 weeks for absence of GFP expression in NM roots and leaves. Pots were gently tipped out with minimal disturbance and root samples were removed from the outer surface of the soil/root ball. Five root and leaf samples were taken per pot and immediately assessed for GFP fluorescence. In order to conduct an initial screen for strong GFP fluorescence in AM roots, two tillers from each T0 line were excised and transferred to 500 g pots containing commercial potting medium for at least 7 d to recover from excision. Tillers were then transplanted to 1.4 kg pots containing 15% w/w *G. intraradices* inoculum in Mallala growth medium and grown for at least 4 weeks. Roots were harvested according to Section 2.5 and immediately assessed for GFP fluorescence as outlined below.

Two lines (G65-16 and G65-4) which demonstrated bright GFP fluorescence were selected for further analysis. Seed from these selected T0 lines (T1 seed) was surface sterilised, germinated and planted in *G. intraradices* nurse pots as outlined in Section 2.3.4. Plants were grown for at least 3 weeks before harvesting and assessment of GFP fluorescence by confocal microscopy.

Reporter gene analysis

Expression of GFP was assessed in living tissue samples. GFP fluorescence was assessed in NM or AM roots and leaves of T0 lines using a Leica MZ FLIII fluorescence stereomicroscope with GFP Plus fluorescence filter set (GFP2, excitation 480 nm, barrier filter 510 nm) and a Leica DC 300F camera for image capture (Leica Microscopy Systems Ltd. Heerbrugg, Switzerland). For more detailed analysis in selected T1 lines, roots were counter-stained with propidium iodide (10 μg mL^{-1}) for 5 min and confocal microscopy images were collected on a Leica (sp5) spectral confocal scanning microscope (LSCM) equipped with an argon laser for excitation at 488 nm (Leica Microscopy Systems Ltd.). The GFP and propidium iodide fluorescence was collected in separate channels (500-550 nm and 600-700 nm, respectively) and then overlaid to create a composite image.

In order to co-visualise GFP fluorescence and AM fungal structures, staining of AM structures in live root tissue was attempted with acid fuchsin and a fluorescent dye conjugated to wheat germ agglutinin which binds to fungal cell walls (Genre & Bonfante, 1997). However, these dyes did not penetrate beyond the epidermal layers of live roots. Consequently, staining of AM structures was conducted following assessment of GFP fluorescence by LSCM. Root segments were cleared and stained in 5% ink and vinegar as outlined in Section 2.9.

5.2.2 Temporal expression profiling of three P_i transporters during early development of AM colonisation

Conventional methods of AM inoculation result in asynchronous colonisation so that a single root often contains colonisation units of different ages and at various developmental stages. In order to overcome this difficulty and investigate changes in P_i transporter expression during early developmental stages of AM colonisation a nurse pot colonisation system was devised according to Rosewarne *et al.* (1997). In a nurse pot system seedlings of the target plant (barley) are transplanted into pots of nurse plants (leek) supporting a pre-established AM network. This results

in rapid and synchronous development of colonisation in roots of the target plant and has proved particularly useful for molecular developmental studies (Delp *et al.*, 2003).

A time-course experiment was conducted using a nurse pot colonisation system with seven sequential plantings and a harvest at 17 d. This single-harvest strategy was devised in order to avoid pseudo-replication in a limited number of nurse pots. Three barley plants of different ages were grown in each nurse pot. There were four replicates of the 13 and 17 d plants and 8 replicates of younger plants in order to ensure enough biomass for sub-sampling. Nurse pots of leek with (AM) or without (NM) inoculum of *G. intraradices* were established as outlined in Section 2.3.4 and maintained in a glasshouse for at least 8 weeks. Uniformly sized seeds of wild-type barley cv. Golden Promise were surface sterilised and germinated prior to each planting. After 5 d germinated seeds were transplanted into nurse pots as outlined in Table 5.2. The single harvest at 17 d yielded plants that were 3, 4, 5, 7, 9, 13 and 17 days old. Nurse pots received 5 mL Long Ashton nutrient solution (Section 2.3.2) every 2 d for the duration of the experiment. Plants were harvested according to Section 2.5 and sampled for shoot and root dry weight, shoot P concentration (Section 2.7), and AM colonisation and gene expression in roots (Section 2.9, 2.11). AM colonisation was calculated as percent total root length colonised (% RLC) or percent total root length containing arbuscules or vesicles. The expression of three P_i transporter genes, *HvPT1*, *HvPT2* and *HvPT8* was determined in root samples by Q PCR according to Section 2.11.

Table 5.2 Planting and harvest timetable for time-course experiment. Crosses mark days when seeds were planted or nutrient was applied.

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Seed planted	x				x				x		x		x	x	x			
Plant age at harvest	17				13				9		7		5	4	3			Harvest
Nutrient application	x		x		x		x		x		x		x		x		x	

5.3 Results

5.3.1 Spatial expression profiling of the AM-inducible *HvPT8*

Fourteen independent transgenic lines were assessed for *HvPT8* promoter-driven expression of GFP. GFP fluorescence was not observed in shoots or roots of NM primary transformants (data not shown). Both wild-type and transgenic root tissue showed weak autofluorescence throughout the root. However, strong green fluorescence was not evident in the roots of wild-type barley colonised by *G. intraradices* (Figure 5.2 A-B). Expression of GFP was observed in the cortex of transgenic plant lines colonised with *G. intraradices* (Figure 5.2 D-F) and corresponded to regions of the root in which AM colonisation was observed by staining with ink and vinegar (Figure 5.2 C). Lines G65-16 and G65-4 were selected for further analysis by LSCM (Figure 5.3). It was not possible to obtain overlay images of AM structures and GFP fluorescence due to difficulties with penetration of fungal stains into living root tissue and the necessity to image GFP in living root tissue. Bright field observation under the LSCM enabled detection of AM structures; arbuscules and intercellular hyphae, and confirmation of co-localisation with GFP; however, no bright field images were collected. It was determined that GFP fluorescence co-localised with arbuscules in the root cortex. No fluorescence was observed around intercellular hyphae and GFP expression was not observed in non-colonised cells adjacent to cells containing arbuscule (Figure 5.3 C).

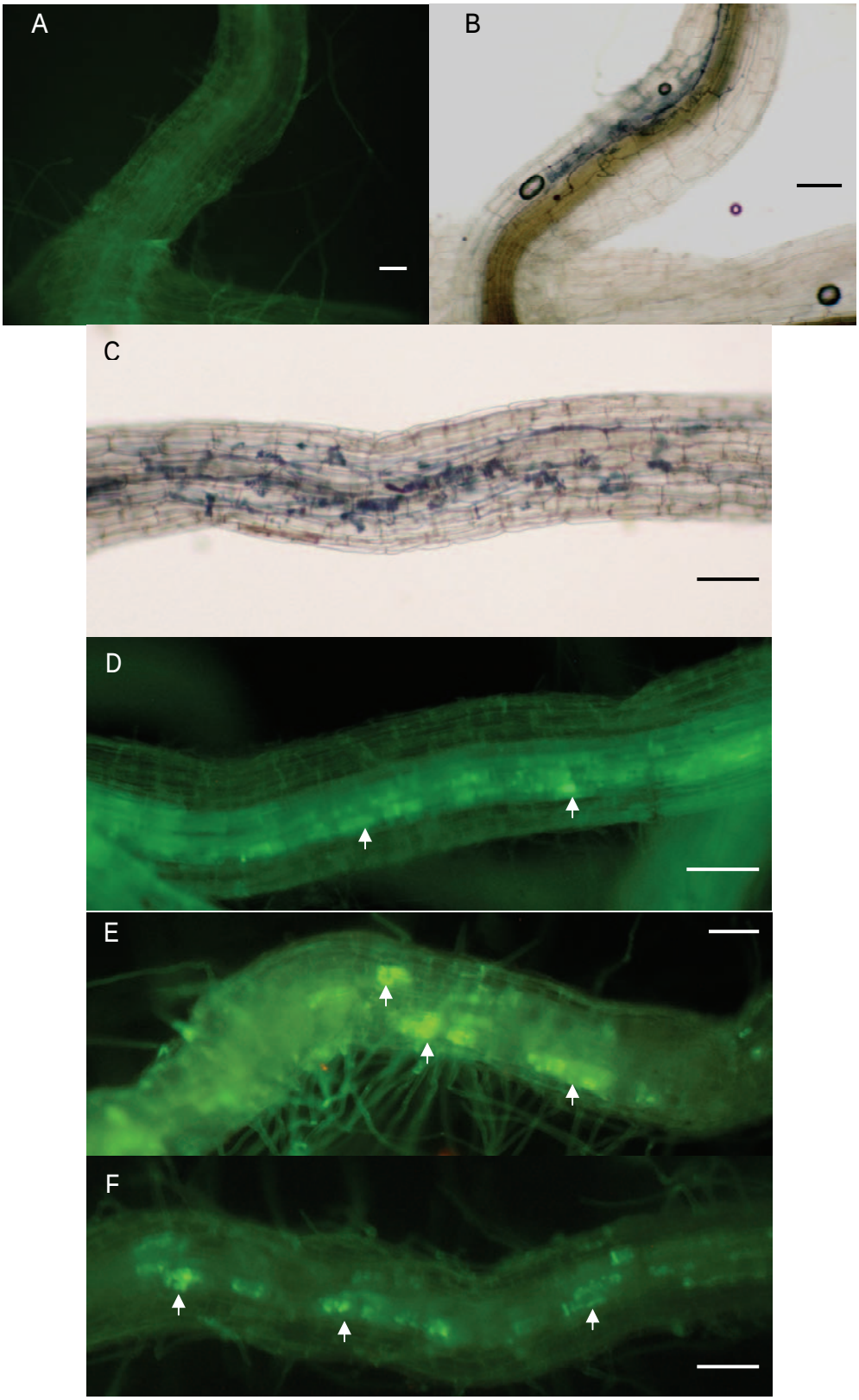


Figure 5.2 Barley roots colonised by *G. intraradices*; wild-type (A,B) or transformed with a *HvPT8* promoter::GFP construct (C-F); A) AM colonised section of root from wild-type demonstrating lack of fluorescence in the root cortex; B) Identical root section to A, stained with ink & vinegar showing location of AM colonisation unit; C-F) Representative images from transgenic roots; C) Stained with ink & vinegar to show location of arbuscules and intercellular hyphae; D-F) Arrowheads indicate expression of GFP in the root cortex. Bars equal 100 µm.

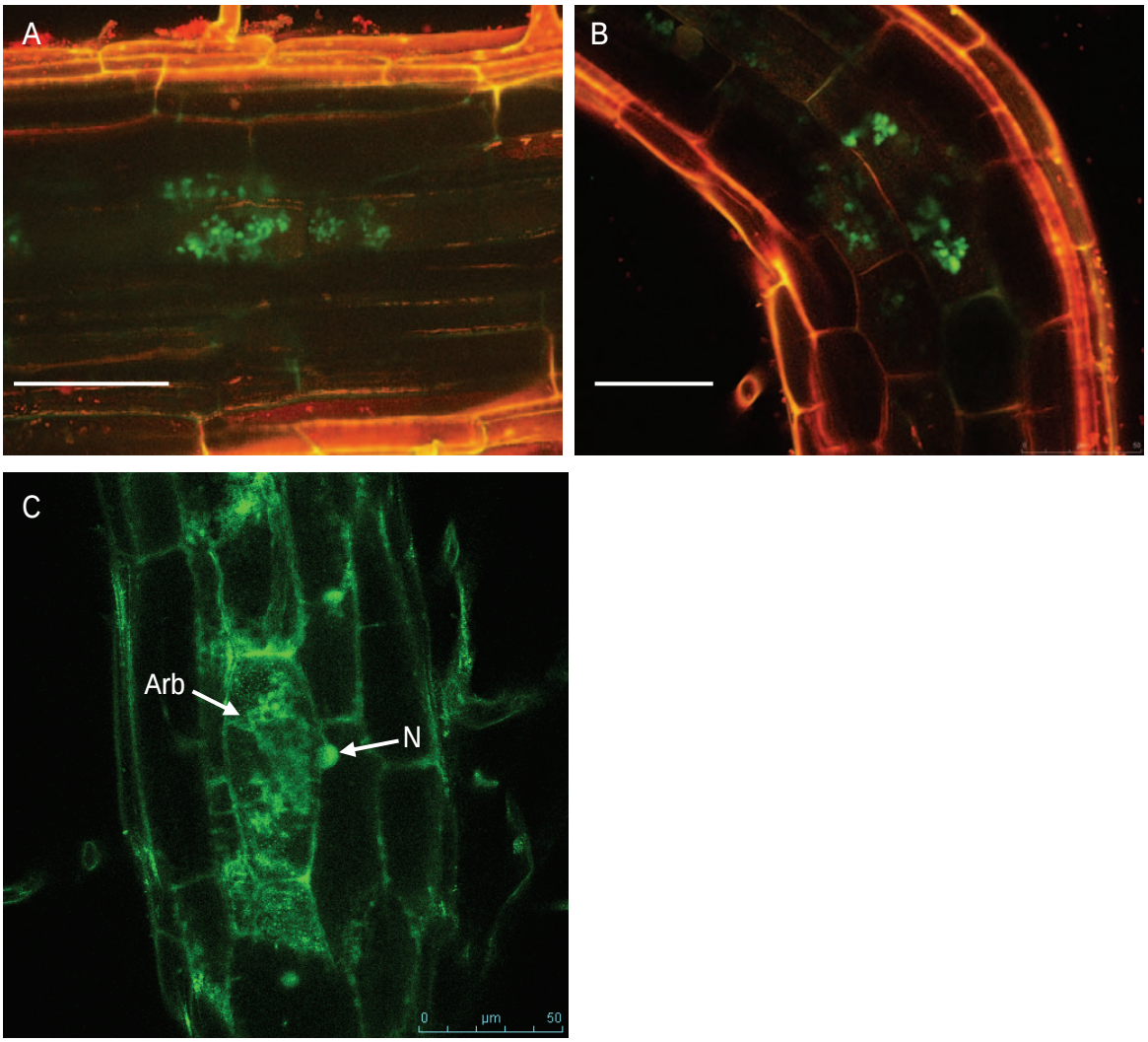


Figure 5.3 Barley roots colonised by *G. intraradices* expressing GFP under the control of the *HvPT8* promoter; A & C) Line G65-16; B) Line G65-4. Images were collected on a laser scanning confocal microscope. Roots in A & B were counter-stained with propidium iodide (red fluorescence). C) Close-up of an arbuscule-containing cortical cell (Arb) expressing GFP. There is no GFP in non-colonised adjacent cells. Nuclei (N) in peripheral position in non-colonised cells can be seen auto-fluorescing. Nuclei and cell walls auto-fluoresce green in the absence of a propidium iodide counter-stain. Bars equal 50 µm.

5.3.2 Temporal expression profiling of three P_i transporters

Colonisation of barley in nurse pots

Colonisation of barley in the nurse pot system progressed rapidly (Figure 5.4), confirming previous reports using this experimental setup (Delp *et al.*, 2003). AM fungal hyphae were detected in the roots of the youngest plants harvested 3 days after transplanting (DAT). Few arbuscules were detected 3 DAT (1.6 %RLC) but by 4 DAT arbuscular colonisation had reached 9.6 %RLC. Vesicles were first detected in plants 5 DAT and by 17 DAT total RLC had reached 70%. No colonisation was observed in NM plants.

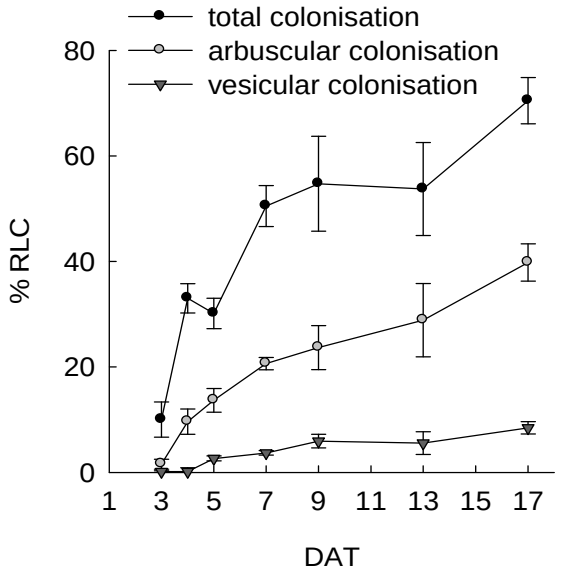


Figure 5.4 Development of AM colonisation in barley over 17 d in nurse pots inoculated with *Glomus intraradices*. DAT (days after transplanting), % RLC (percent root length colonised). Colonisation, whether total, arbuscular or vesicular, was calculated as percent of total root length. Data points are means ± SEM of 4 replicates.

Growth and P nutrition of barley in nurse pots

The growth of AM and NM plants was similar in plants up to 7 DAT but from 9 DAT onwards the growth of AM plants was reduced relative to NM plants and a significant growth depression was observed in AM plants 17 DAT (Figure 5.5). Shoot P concentration increased in AM seedlings up to 7 DAT when it reached ~2500 mg kg^{-1} (Figure 5.6). In NM plants shoot P concentration continued to increase to a maximum of 5470 mg kg^{-1} 13 DAT, then decreased. There was a significant difference in shoot P concentration of AM and NM plants at 13 DAT only. Total P content followed a similar trend to plant growth except that the total P content of NM plants was significantly greater than AM plants at both the 13 and 17 d harvests (data not shown).

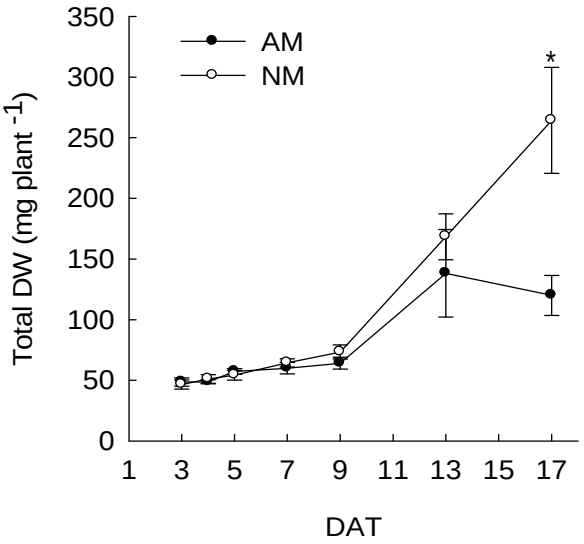


Figure 5.5 Growth of barley over 17 d in nurse pots with (AM) or without (NM) *Glomus intraradices*; DAT (days after transplanting). Data points are means ± SEM of 4 replicates. Asterisk indicates significant difference (P<0.05, Tukey test) between the two means at a particular time point.

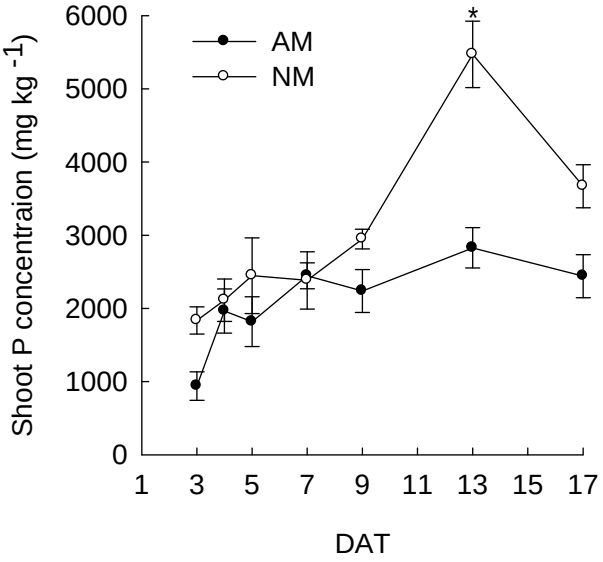


Figure 5.6 Shoot P concentration of barley grown in nurse pots with (AM) or without (NM) *Glomus intraradices*; DAT (days after transplanting). Data points are means ± SEM of 4 replicates. Asterisk indicates significant difference (P<0.05, Tukey test) between the two means at a particular time point.

Phosphate transporter expression

The expression of the root epidermal P_i transporters, *HvPT1* and *HvPT2* increased over time but there was no significant difference in expression of these transporters in AM roots compared to NM roots at any time point (Figure 5.7 A, B). There was no significant correlation between expression of the root epidermal P_i transporters and shoot P concentration in the plant. In contrast, the AM-

inducible P_i transporter, *HvPT8* was expressed in AM roots only (Figure 5.7 C). Expression of *HvPT8* was not significantly correlated with total colonisation of the root or arbuscular colonisation.

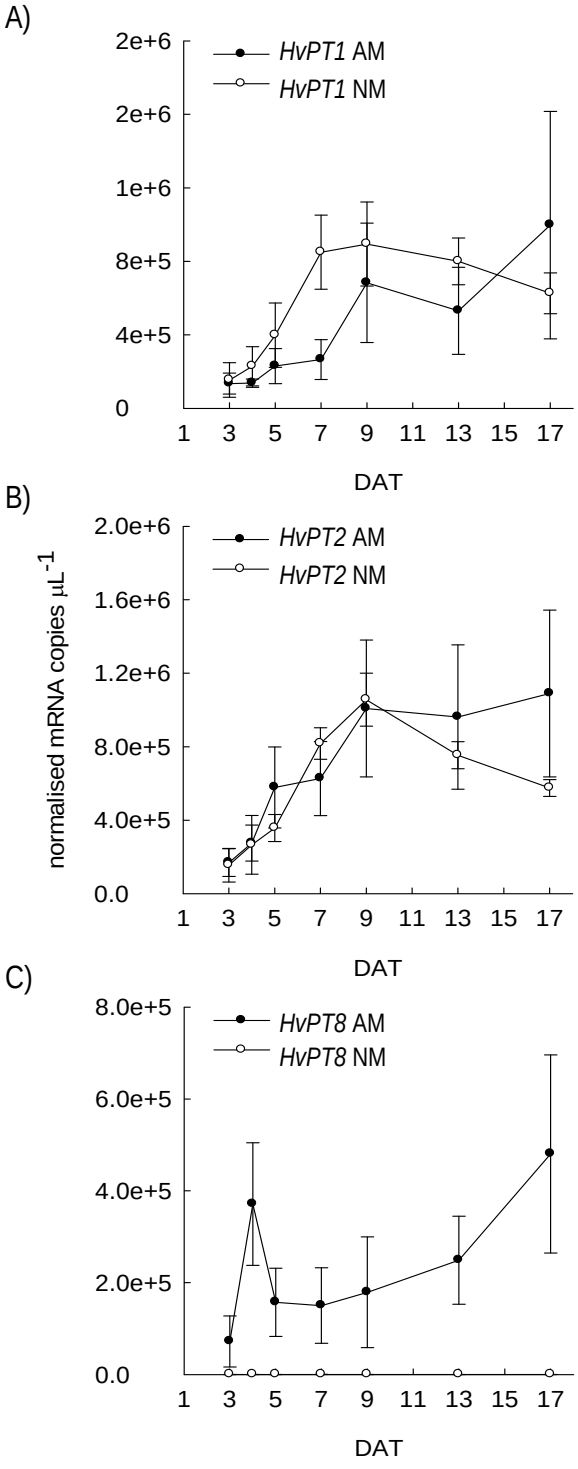


Figure 5.7 Normalised expression levels of three phosphate transporters in roots of barley plants grown for 17 d in nurse pots with (AM) or without (NM) *Glomus intraradices*; A) *HvPT1*, B) *HvPT2*, C) *HvPT8,* DAT (days after transplanting). Data points are means ± SEM of three replicates.

5.4 Discussion

The *HvPT8* promoter-driven expression of GFP in root cortical cells containing arbuscules confirms previous reports on the localisation of this transporter (Glassop *et al.*, 2005). GFP expression was observed around both mature and senescing arbuscules; however, it is not possible to conclude whether the presence of GFP around senescing arbuscules is due to continued promoter activity of *HvPT8* or 'residual' GFP expression. The half-life of the GFP variant used here, *sgfp*S65T, has not been reported but wild-type GFP is a stably expressed protein with a half-life of 26 h (Corish & Tyler-Smith, 1999). In *Medicago,* antibody localisation of MtPT4 demonstrated that the protein was primarily associated with mature arbuscules; minimal expression was observed around senescing arbuscules (Harrison *et al.*, 2002).

Contrary to findings from previous Q PCR studies (Chapter 4 and Glassop *et al.*, 2005), no GFP expression was observed in NM roots. This may be due to differences in the sensitivity of the two methods, or the low level of expression in NM roots may be masked by autofluorescence of root tissue. However, in the time-course experiment in this chapter *HvPT8* transcripts were not detected in NM roots by Q PCR suggesting that *HvPT8* may be induced in NM roots under particular conditions which are yet to be determined.

The expression of *HvPT8* was detected in roots by Q PCR three DAT, at which time arbuscular colonisation was just 1.6 %RLC. The expression of *HvPT8* increased with time but was not significantly correlated with arbuscular colonisation, as might be predicted considering the cellular localisation of this gene. In *Medicago*, Isayenkov *et al.* (2004) demonstrated that expression of *MtPT4* was more closely correlated with arbuscules than with total colonisation and these and other authors (Bucher, 2007) have suggested that the expression of AM-inducible P_i transporters will provide useful molecular markers for formation of a functional AM symbiosis. However, such assertions need to be carefully considered. In this experiment the *HvPT8* expression data alone could be taken to suggest that P transfer via the AM pathway is occurring. In contrast, the low level of colonisation suggests that it is unlikely that the AM fungus was delivering significant amounts of P to the plant via arbuscular interfaces at the first harvest. The data presented here confirm results presented in Chapter 4. In that experiment *HvPT8* expression, although variable, was reasonably high in roots with low levels of colonisation by *G. geosporum* and no ^{32}P transfer via the AM pathway was detected. Although AM-inducible P_i transporters may be localised to P_i transfer interfaces the data presented here (and previously) suggests that this is not a quantitative

correlation and that P_i transporter expression does not reflect the amount of P_i transferred via this pathway.

Contrary to previous reports (Glassop *et al.*, 2005) the down-regulation of root epidermal P_i transporters by AM colonisation was not observed in this experiment. This is consistent with the results of Chapter 4. However, the data presented here show considerable variability between replicates for a single experimental treatment. This variability is greater for AM than NM treatments suggesting that the control gene, *HvGAPdH*, is not stably expressed in AM roots. Although stable expression of *HvGAPdH* was reported by Delp *et al.* (2003), these authors were using a relative quantitative RT-PCR approach. This relies on detection of amplification product on an agarose gel which is less sensitive than the fluorescent detection system used for Q PCR. In future, data from microarray experiments will be useful to identify appropriate stably expressed control genes for use in AM experiments; however, at present a more appropriate approach would be to use random priming to generate cDNAs allowing for the use of ribosomal RNA as a Q PCR control.

Chapter 6

Characterisation of HvPT8 Kinetic Properties by Over-expression in Transgenic Barley

6.1 Introduction

As outlined in Chapter 1, functional characterisation of plant P_i transporters has been attempted by heterologous over-expression in yeast or in plant cell-suspension culture. Yeast complementation has proved problematic and in instances where complementation was successful has yielded K_m estimates ranging from 31 μM (Daram *et al.*, 1998) to 280 μM (Leggewie *et al.*, 1997) for putative high-affinity transporters. In contrast, heterologous over-expression in plant cell-suspension culture has yielded K_m estimates which are consistent with physiological measurements; 3.1 μM for Pht1:1 of *Arabidopsis* (Mitsukawa *et al.*, 1997) and 9 μM for HvPT1 of barley (Rae *et al.*, 2003). Amongst the AM-inducible P_i transporters yeast complementation has been used to demonstrate functionality of three proteins. StPT3 of potato (Rausch *et al.*, 2001), MtPT4 of *Medicago* (Harrison *et al.*, 2002) and the monocot transporter, OsPT11 of rice (Paszkowski *et al.*, 2002) all demonstrated increased uptake when expressed in yeast, relative to vector controls. The apparent K_m of StPT3 was 64 μM and, although this is reasonably high, it has been taken to suggest that StPT3 functions as a high-affinity transporter. In contrast the apparent K_m for MtPT4 was 493-668 μM. These data indicate that AM-inducible P_i transporters function in either high-affinity or low-affinity transport. Kinetic parameters were not reported for OsPT11.

The aim of the work described in this chapter was to functionally characterise and determine the kinetic properties of the AM-inducible P_i transporter HvPT8 by constitutive over-expression *in planta*. Kinetic studies on excised roots and whole plants have been used to physiologically characterise the native P_i transport systems of a range of plant species. Early physiological investigations of P_i uptake in excised barley roots identified a dual uptake system for P. Uptake was characterised by high-affinity transport at low external P_i concentrations (0 to 200-500 μM) and low-affinity transport at high external P_i (>200-500 μM) (Barber, 1972). P_i absorption at low P follows Michaelis-Menten kinetics. In intact barley plants, ^{32}P uptake at low P demonstrated an apparent K_m of 5-7 μM (Lee, 1982; Drew *et al.*, 1984) whilst Cogliatti & Santa Maria (1990) reported an apparent K_m of 26 μM for intact wheat seedlings. Measurements of the low-affinity transport system are more variable, with K_m estimates ranging from 48 μM (Furihata *et al.*, 1992) to 900 μM (Schmidt *et al.*,

1992) in *Catharanthus roseus* protoplasts or cultured cells, respectively. Although Sentenac & Grignon (1985) ascribed Michaelis-Menten kinetics to a low-affinity transport component of uptake from 10-60 μM P_i in excised maize roots, Cogliatti & Santa Maria (1990) demonstrated linear uptake for the low-affinity system above 500 μM in intact wheat plants. In the current experiment P_i uptake was investigated over a range of P concentrations from 1 μM to 1000μM P_i in order to investigate both high-affinity and low-affinity uptake.

Two methods have been reported for determining P_i influx. The first involves incubating replicate tissue samples in labelled nutrient solutions differing in P concentration and measurement of the amount of label absorbed into the tissue (Epstein *et al.*, 1963). Uptake times are usually short and it is important that the P concentration of the solution remains constant. The second technique is the solution-depletion method (Claassen & Barber, 1974). This technique involves longer incubation of tissue in labelled nutrient solution at a single initial P_i concentration. The depletion of P_i from the solution is monitored over time and used to calculate P_i influx into the tissue. The primary difference between the two methods is that the absorption method measures unidirectional influx at a steady-state P_i concentration, whereas the depletion method measures net influx in a non-steady-state system that will be adjusting for the decreasing P_i concentration. Therefore, in the experiment described in this chapter the absorption method was used to determine short-term unidirectional influx of P_i. Due to the role of the shoot in systemic P signalling and regulation of P_i uptake (Drew & Saker, 1984) initial experiments were conducted on excised roots to minimise complexities due to root-to-shoot transfer and feedback control.

6.2 Materials and methods

6.2.1 Production of transgenic plants over-expressing *HvPT8*

The binary vector pPZPUbi including the maize ubiquitin promoter (*Ubi1*) and the nopaline synthase gene terminator (*Nos*) (Appendix 2) was provided by Dr K. Oldach (ACPFG, Adelaide). The full *HvPT8* coding sequence (cds), including 33 bp upstream of the translation start and 29 bp downstream of the translation stop codon, was PCR amplified from a plasmid containing the promoter and cds supplied by Dr D. Glassop (CSIRO Plant Industry, Brisbane) using the primers listed in Table 6.1. PCR was performed as outlined in Section 2.14.1 with 3 ng template DNA; annealing, 55^{o}C; elongation, 80 s. The native *HvPT8* gene does not contain any introns. However, since introns are considered to be important for high levels of gene expression in monocots (Schunmann *et al.*, 2004), the first intron of the maize alcohol dehydrogenase gene (*Adh1*) was

incorporated in the construct. *Adh1* was PCR amplified from pWBVec8 (1.3 ng) using the primers listed in Table 6.1; annealing, 56°C; elongation, 45 s. PCR amplification products were purified as outlined in Section 2.14.1.

Table 6.1 Primers used for the design of the *HvPT8* over-expression cassette. Incorporated restriction site sequences are underlined; two nucleotides (lowercase) were added to the 5' end to aid restriction enzyme (RE) digestion.

Primer	Sequence	Incorporated restriction sites	T_m (°C)[1]	Amplicon size (bp)
*HvPT8*cds F	gtGGCGCGCCAAGAAGTGCGGACGGGCAGA	*Asc*I	81	1664
*HvPT8*cds R	taGAGCTCGATGCGCACAGCCTACGTTC	*Sac*I	68	
Adh1 F	taGGCGCGCCGCTGCACGGGTCCAGGAAAG	*Asc*I	80	544
Adh1 R	ttGTCGACGTGCAAAGGTCCGCCTTGTT	*Sal*I	71	

[1] Primer T_m as reported by the manufacturer (Proligo).

Assembly of the pPZPUbi expression cassette was performed by multipoint ligation in which individual fragments prepared with appropriate compatible cohesive ends were ligated together and inserted between the maize *Ubi1* promoter and nopaline synthase gene terminator (*Nos*) of the pPZPUbi plant transformation vector. The pPZPUbi vector backbone and the *Adh1* fragment were digested with *Sac*I and *Asc*I, respectively. The digest reactions were cleaned up as outlined in Section 2.14.3 and the eluted DNA of both fragments was digested, separately, with *Sal*I. The *HvPT8* cds fragment was digested simultaneously with *Sac*I and *Asc*I as these enzymes require the same buffer. Ligation of backbone, intron and cds was conducted in a single reaction as outlined in Section 2.14.4 and the ligation product was transformed into *E. coli* (2.14.5). Individual colonies were amplified in liquid media, plasmid DNA was isolated (2.14.6) and the vector integrity was checked by RE digestion with *Sal*I, *Asc*I/*Sac*I and *Xho*I. Plasmids demonstrating the expected fragmentation pattern were sequenced to ensure errors had not been introduced using the PZPseq primers listed in Appendix 2. The structure of the final expression cassette is illustrated in Figure 6.1. The expression cassette was cloned in the same orientation and upstream of the selectable markers.

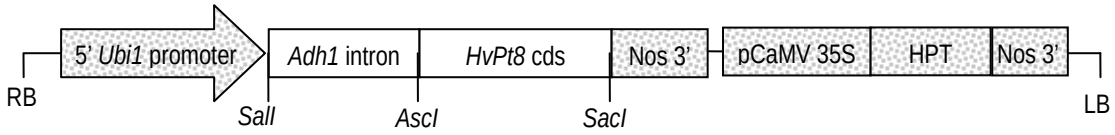


Figure 6.1 Structure of the plant transformation construct designed for constitutive over-expression of *HvPT8*. The maize *Adh1* intron and *HvPT8* cds were inserted between the maize *Ubi1* promoter and the nopaline synthase gene terminator (*Nos*) of the pPZPUbi plant transformation vector. pPZPUbi carries the *HPT* gene driven by the CaMV35S promoter (pCaMV35S) for selection of transgenic plants on hygromycin.

The completed expression vector was transformed into *Agrobacterium* AGL-1 (Section 2.14.5) and subsequently used for the transformation of *Hordeum vulgare* cv. Golden Promise as outlined in Section 2.14.7. Regenerated transformants were selected on antibiotic selection media for the presence of the T-DNA. Eight independent lines were regenerated, transferred to commercial grade potting mix and grown to maturity in a glasshouse facility.

6.2.2 Screening of primary transformants

Primary transformants (T0) were screened for expression of the transgene in root tissue by Q PCR as outlined in Section 2.11, using the Q PCR primers designed for the native *HvPT8* gene (Table 2.2). New Q PCR primers were initially designed to span the *HvPT8/Nos* junction in order to distinguish native expression from transgene expression; however, these proved incompatible with the standard Q PCR reaction conditions. As the native *HvPT8* gene is primarily expressed in AM colonised tissue, expression in non-colonised tissue can be attributed to the transgene.

6.2.3 Southern blot analysis

Primary transformants were screened by Southern blot analysis to identify plants with a single locus insertion of the transgene. Genomic DNA from the leaves of all 8 transgenic lines and 1 wild-type plant was extracted according to the protocol outlined in Section 2.16. DNA integrity was assessed on an agarose gel as outlined in Section 2.12 except that the gel was run for 1.5 h. Subsequently, ~10 µg of isolated DNA was digested with *Sac*I according to Section 2.14.3 and run on a 1% agarose gel, overnight at 33 V. The digested DNA was transferred to a Biodyne B Membrane (0.45 µm, Pall Life Sciences, AnnArbor, USA) by NaOH capillary transfer as outlined by the manufacturer. The membrane was probed with a radio-labelled 521 bp fragment from the

hygromycin resistance gene prepared as outlined in Section 2.15 with the primers listed in Table 2.4.

Labelling of the probe and membrane hybridisation were performed by Ms M. Pallotta (ACPFG, Adelaide). The random oligo-labelling method (Feinberg & Vogelstein, 1983) was used to radioactively label DNA probes with ^{32}P. Hybridisation methods were as described in Rogowsky *et al.* (1991), except that both pre-hybridisation and hybridisation were carried out in the same solution (0.9 M NaCl, 30 mM Pipes, 7.5 mM EDTA, 7.5% dextran sulphate, 0.6% BSA, 0.6% Ficoll 400, 0.6% polyvinyl-pyrollidone, 3% SDS, 250 µg denatured salmon sperm, pH 6.8). The membrane was washed under increasingly stringent conditions to remove unbound DNA, blotted dry, sealed in plastic and exposed to Fuji X-ray Medical Film (HR-T 30) at -80°C for 7 d. X-ray film was developed using an AGFA CP1000 X-ray developer.

6.2.4 Selection of homozygous lines

T0 lines carrying a single locus insertion of the transgene were grown to maturity and 12 T1 seeds per line were germinated. The T1 seedlings were genotyped as outlined in Section 2.15, all but one null segregant per line was culled and the remaining plants were grown to maturity. The resulting T2 seed was used to test for homozygosity as follows. Twelve T2 seeds per line were germinated and genotyped; the presence of the hygromycin sequence in all 12 seedlings was taken to indicate that the T1 parent line was homozygous for the insert. This process also confirmed lines which had lost the insert. These null segregants were used as transgenic controls.

6.2.5 Uptake of ^{32}P into excised roots of primary transformants

Initial uptake assays were performed on excised root segments of the 8 primary transformants. Roots of T0 plants (5 weeks old) were harvested by gently removing them from the pot and teasing out roots from the bottom and edges of the soil/root ball. Root segments, approximately 5 cm long were excised and immediately rinsed with and transferred to recovery solution consisting of 0.2 mM $CaCl_2$ and 10 µM NaH_2PO_4 (pH 5.8). It has been demonstrated previously that the rate of P_i uptake doubles in the first two hours after root excision, reaching a plateau by three hours (Gronewald *et al.*, 1979). Therefore, following excision root segments were recovered in the dark at RT on an orbital shaker (30 rpm) for at least 3 h prior to measurement of P_i uptake. In repeat experiments, roots were harvested in the afternoon and recovered overnight.

All uptake solutions were prepared in 0.2 mM $CaCl_2$, P_i was added as NaH_2PO_4 at the appropriate concentration and solutions were adjusted to pH 5.8. ^{32}P was added to uptake solutions as carrier-free $H_3{}^{32}PO_4$ to provide ~3.7 kBq mL^{-1}. Prior to measurement of uptake, root segments were pre-treated in unlabelled solution identical to uptake solutions for precisely 5 min to equilibrate. Roots were then transferred to dishes containing 20 mL ^{32}P-labelled uptake solution on an orbital shaker (30 rpm) at RT for a predetermined time. At the end of the experiment, roots were rinsed in ice cold 1000 μM P_i rinse solution (minus ^{32}P) for precisely 5 min to remove label bound in cell walls. Roots were gently blotted, weighed and transferred to plastic scintillation vials with 4 mL EcoLume™ scintillant (#882470, MP Biomedicals, Seven Hills, Australia). ^{32}P activity was measured by liquid scintillation counting on a LSC6500 scintillation counter (Beckman Instruments, Fullerton, CA). Uptake was measured on at least three replicates; lines, replicate and treatment (influx time or P_i concentration) were randomised over the course of the experiment. At intervals during the course of uptake experiments, triplicate aliquots (20 μL) of labelled uptake solution were removed for scintillation counting. These measurements were used to calculate the specific activity (SA) of uptake solutions and to monitor for depletion. P_i uptake and P_i influx were calculated according to Equation 6.1 and 6.2, respectively.

Equation 6.1 $$\text{Pi Uptake} = \frac{\text{cpm root}}{\text{SA uptake soln (cpm/nmol)}} \text{ x } \frac{1}{\text{FW root}}$$

Equation 6.2 $$\text{Pi Influx} = \frac{\text{Pi Uptake}}{\text{Flux Time}}$$

Time-courses of P_i uptake were conducted at two P_i concentrations (10 μM and 300 μM) with measurements at 0.5, 1, 2, 5, 10, 20, 40 or 60 min. Three T0 lines G64-4, G64-6 and G64-8 were selected based upon the level of transgene expression (See Section 6.3.1) and P_i uptake of these transgenic lines was compared to wild-type barley cv. Golden Promise. The pH of uptake solutions was monitored and did not change significantly during the course of experiments. ^{32}P uptake was linear over the first 10 min, therefore this time was chosen for subsequent experiments investigating the concentration dependence of P_i influx.

The concentration dependence of P_i influx for Lines G64-4, G64-6 and G64-8 and wild-type was compared using 10 min uptake at 1, 3, 10, 30, 100, 300 and 1000 μM P_i concentrations. Finally, P_i uptake during 10 min was measured in excised roots of all 8 transgenic lines and compared to wild-type at two P_i concentrations (10 μM and 100μM).

6.2.6 Growth and ^{32}P uptake of intact plants from solution culture

Two putative homozygous T2 lines, G64:7:9 and G64:8:7 and one null segregant, G64:8:9, were identified as outlined in Sections 6.2.3-6.2.4. Transgenic (T2) and wild-type seeds were germinated as outlined in Section 2.4.1. Ninety six barley seedlings (24 replicates per line) were grown in a solution culture system which consisted of a 10 L tank with aeration. The location of seedlings in the tank was completely randomised. The nutrient solution contained; (mM) KNO_3, 5.0; $Ca(NO_3)_2$, 2.0; $MgSO_4$, 2.0; Na_2SiO_3, 0.5; NH_4NO_3, 0.2; NaFe(III)EDTA, 0.05; H_3BO_3, 0.05; (μM) $ZnSO_4$, 10; $MnCl_2$, 5.0; $CuSO_4$, 0.5; Na_2MoO_3, 0.1. The KH_2PO_4 concentration varied depending on the treatment. Nutrient solution was replaced every three days. Two duplicate tanks were prepared. The high P tank received a total of 3.1 mmol P_i during the 8 d growth period whereas the low P tank received 0.7 mmol during the 9.5 d growth period. The tanks were housed in a controlled environment facility with a 16 h photoperiod and 21°C/16°C day/night temperatures.

Four replicates of each of the transgenic over-expressors and the null control line were assessed for expression of the transgene in roots by PCR from cDNA. RNA extraction and cDNA synthesis were performed according to Section 2.11 and PCR was performed as outlined in Section 2.14.1 using the Q PCR primers listed in Table 2.2 for *HvGAPdH* and *HvPT8*; annealing, 55°C; elongation, 30 s.

Whole-plant uptake assays were performed as described above for excised root experiments except that all solutions were adjusted to pH 5.6. Plants were at the three leaf stage. ^{32}P uptake was measured from 1, 3, 5, 10, 30 and 500 μM P_i solutions. Uptake assays were conducted separately on high P and low P pre-treated seedlings. Seedlings were removed from the hydroponics tray, roots were blotted and immediately transferred to unlabelled pre-treatment solution for 5 min. Seedlings were then transferred to dishes containing 100 mL ^{32}P-labelled uptake solution (~0.75 kBq mL^{-1}) on an orbital shaker (30 rpm) at RT for 10 min. Only the roots were submerged in the uptake solution. At the end of the experiment the shoot was removed and the roots were rinsed in ice cold 1000 μM P_i rinse solution (minus ^{32}P) for precisely 5 min. The ^{32}P activity in the roots was determined as described previously. In a preliminary experiment the ^{32}P activity of shoots was also assessed. This confirmed that ^{32}P transfer to the shoot during the 10 min uptake period was minimal. In subsequent experiments shoots were weighed for fresh weight and a subsample (8 randomly selected shoots per line) were used for measurement of whole-shoot P

content according to Section 2.7. Whole-plant fluxes were conducted on at least 4 replicates and treatments were randomised over the course of the experiment.

Statistical analyses

Kinetic parameters were estimated by non-linear regression using GraphPad Prism v4.03 for windows (GraphPad Software, San Diego, CA, USA). Significant differences between estimated parameters were tested using a post-hoc t-test at $P<0.05$. Fresh weight and P content data were analysed by one-way analysis of variance (ANOVA) using Genstat 8th Edition (Lawes Agricultural Trust) and significant differences between means were tested using a post-hoc Tukey test at $P<0.05$.

6.3 Results

6.3.1 Screening of transgenic barley over-expressing *HvPT8*

The level of expression of the transgene in roots of primary transformants was high but variable between transgenic lines (Figure 6.2). Based upon the level of transgene expression, three lines were selected for ^{32}P uptake assays into excised roots. These lines were G64-4, G64-6 and G64-8 demonstrating high, medium and low level transgene expression, respectively.

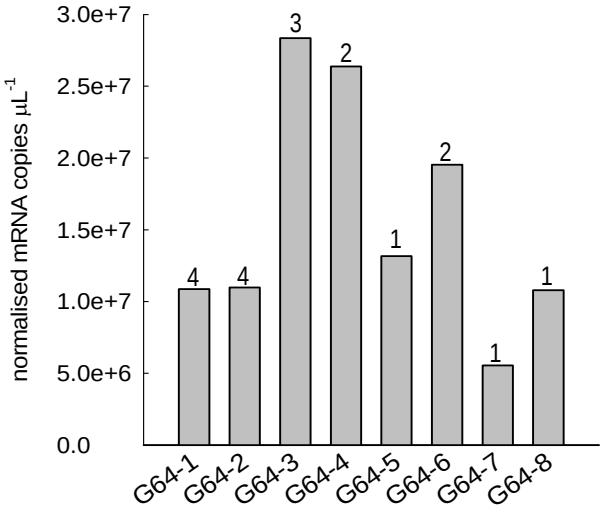


Figure 6.2 Expression level of the *HvPT8* transgene in NM roots of T0 transgenic barley. Numbers above columns indicate putative copy number based on Southern data using the *hyg* probe.

Southern analysis was used to identify primary transformants with a single locus insertion of the transgene. Three T0 lines appeared to have single locus insertions of the transgene; G64-5, G64-7

and G64-8. This result was based upon a single Southern which, unfortunately, had low exposure resulting in a weak signal (membrane not shown). Lines G64-4 and G64-6 had two (non-identical) bands, indicating two copies of the transgene. Line G64-3 had three bands whilst the Southern hybridisation patterns for lines G64-1 and G64-2 were identical (4 bands), indicating that these regenerants may have derived from the same transformation event.

6.3.2 Time-course of ^{32}P uptake

Two time-course experiments were conducted to measure P_i uptake from 10 μM and 300 μM P_i solutions over 60 min. Initially, roots for the 10 μM experiment were placed in recovery solution for 3 h after excision; however, for ease of experimentation additional replicates were conducted after overnight recovery, as were fluxes from 300 μM P_i. The rate of P_i uptake into transgenic roots was consistently greater than wild-type and was consistently highest for Line G64-8 (Figure 6.3). With a 3 h recovery, uptake from 10 μM P_i was linear over 60 min (Figure 6.3 A), whereas 10 μM P_i uptake was saturating over time in roots recovered overnight (Figure 6.3 B). Overnight recovery also increased P_i uptake significantly. The initial uptake rate from 10 μM P_i measured over the first 10 min was 4.0 nmol g^{-1} min^{-1} for Line G64-8 and 1.0 nmol g^{-1} min^{-1} for wild-type with a 3 h recovery time. This increased to 14.3 nmol g^{-1} min^{-1} in Line G64-8 and 3.4 nmol g^{-1} min^{-1} in wild-type after overnight recovery. The rate of P_i uptake from 300 μM P_i was 10.9 nmol g^{-1} min^{-1} for Line G64-8 and 5.1 nmol g^{-1} min^{-1} for wild-type (Figure 6.3 C), again after overnight recovery. Based on these data the 10 min time-point was selected for assessing the dependence of P_i influx on P_i concentration.

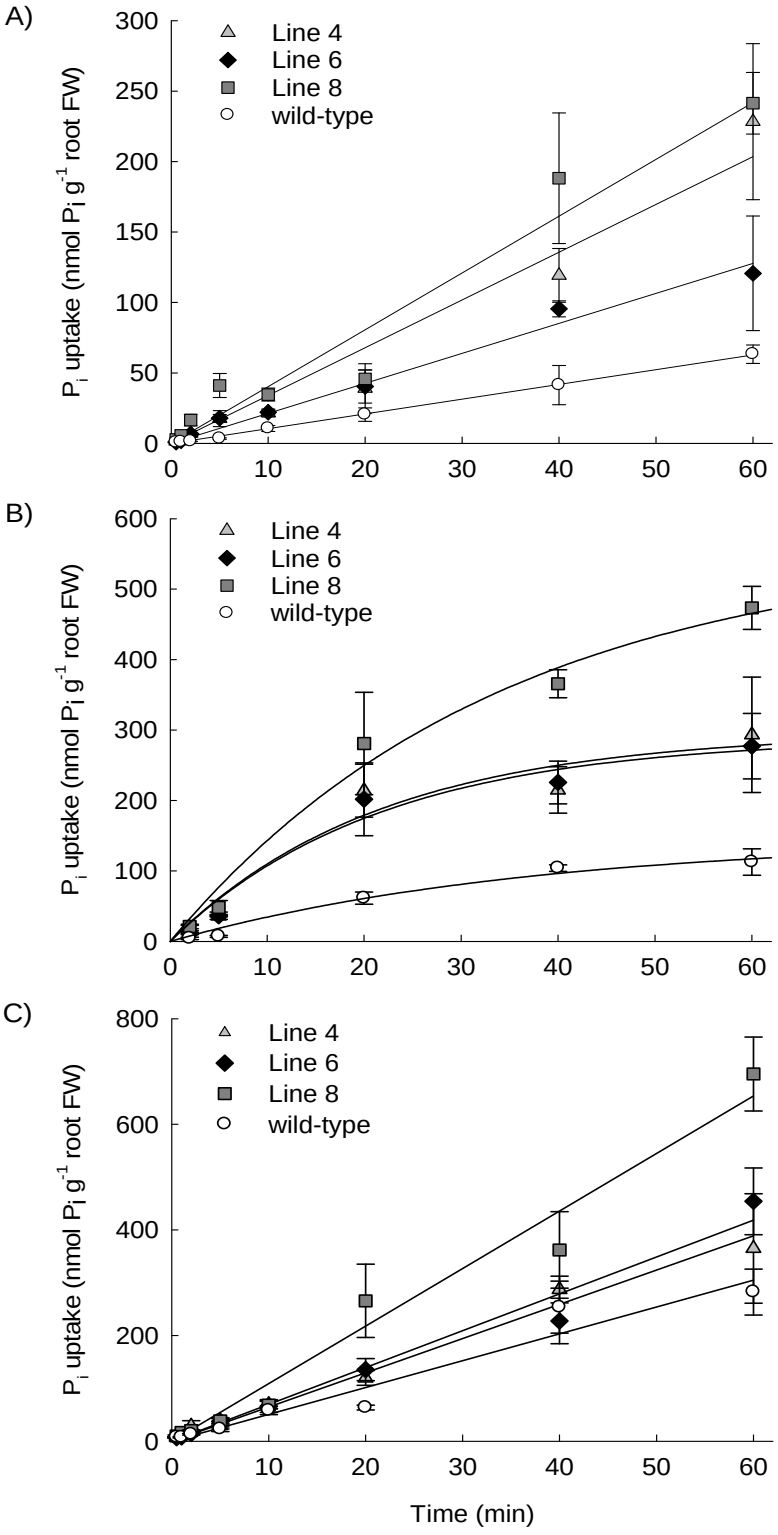


Figure 6.3 P_i uptake from 10 µM (A, B) or 300 µM (C) P_i uptake solution over 60 min. Uptake was measured in excised barley roots derived from T0 transformed lines (G64-4, G64-6 and G64-8) expressing a maize ubiquitin promoter::*HvPT8* construct or from wild-type. Roots were recovered from excision for 3 h (A) or overnight (B, C). Data are means ± SEM of three replicates for 10 µM fluxes (A, B) or means ± SEM of six replicates for 300 µM fluxes (C).

6.3.3 Concentration dependence of P_i influx

The dependence of P_i influx on external P_i concentration was assessed in two experiments. As with time-course experiments, an initial experiment was conducted with 3 h recovery of roots from excision (Figure 6.4 A) and selected concentrations were then repeated following overnight recovery (Figure 6.4 B). P_i influx in the high-affinity range appeared to approach saturation at 30 μM P_i. As was observed in time-course experiments, Line G64-8 demonstrated the highest P_i influx. With a 3 h recovery, high-affinity influx approached a V_{max} of 5.3 nmol g^{-1} min^{-1} in Line G64-8 compared to 4.2 nmol g^{-1} min^{-1} in wild-type plants. Overnight recovery increased P_i influx by 4- to 7-fold in transgenic lines and 2-fold in wild-type plants.

Comparison of P_i influx in root segments of all transgenic lines to wild-type demonstrated increased influx for seven of the eight transformed lines measured (Figure 6.5). P_i influx in Line G64-3 was not different to wild-type; however, pots of Line G64-3 were water-logged and the soil was anaerobic. Therefore, data for this line are not shown. P_i influx in the remaining seven lines over-expressing *HvPT8* increased 1.5- to 8.3- fold relative to wild-type.

Kinetic parameters were calculated for Lines G64-4, G64-6, G64-8 and wild-type by non-linear regression, which is both more accurate and more precise than methods based on linear transformations (Ranaldi *et al.*, 1999). There was no significant difference between kinetic parameters derived from experiments in which a 3 h recovery time was used. Overnight recovery from root excision increased V_{max} which was significantly higher in transgenic lines than wild-type (Table 6.2).

Table 6.2 Estimates of kinetic parameters derived from non-linear regression of influx data for excised root segments recovered for 3 h or overnight.

Genotype	3 h		Overnight	
	K_m (ns) (μM)[1]	V_{max} (ns) (nmol g^{-1} FW min^{-1})[1]	K_m (ns) (μM)[1]	V_{max} (nmol g^{-1} FW min^{-1})[1]
Wild-type	30.5±17.1	5.5±0.7	38.7±18.2	11.2±1.3[a]
Line G64-4	36.3±14.0	7.3±0.7	23.8±10.2	22.1±2.1[b]
Line G64-6	28.4±12.0	6.7±0.7	29.1±14.7	26.4±3.2[b]
Line G64-8	8.2±3.1	6.3±0.5	41.9±21.7	51.2±6.9[c]

[1]Data are estimates ± std dev as reported in GraphPad Prism output. Values followed by the same letter are not significantly different at $P<0.05$. ns – no significant difference between treatments.

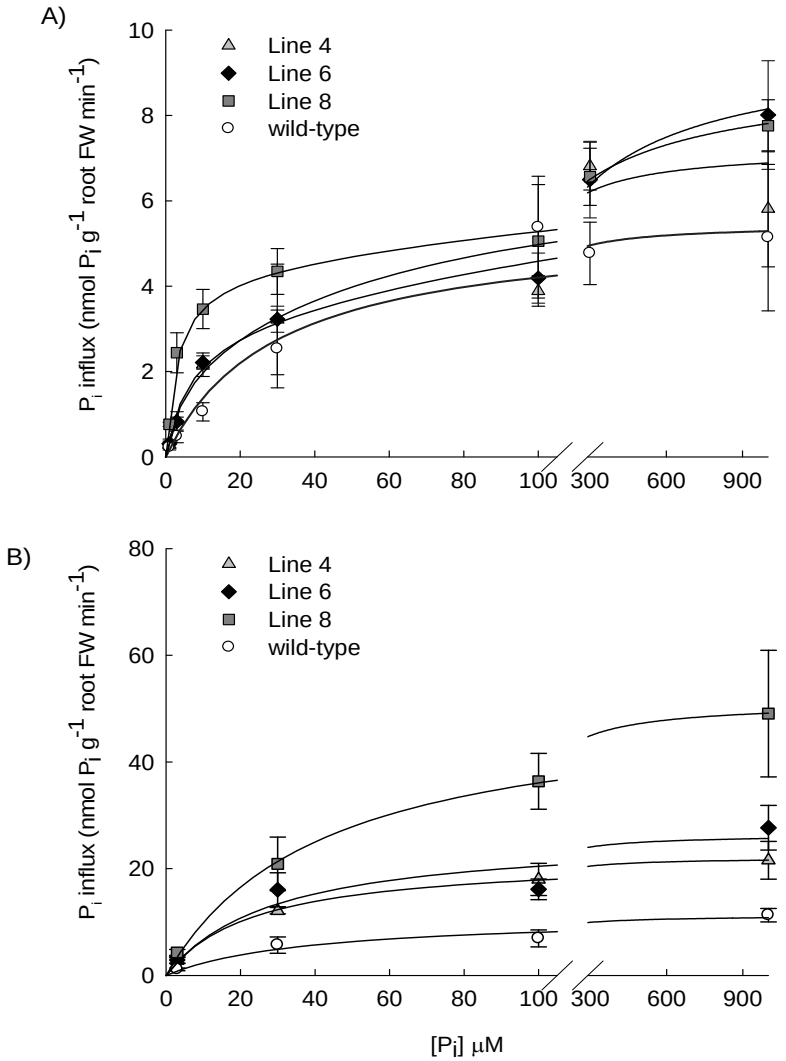


Figure 6.4 P_i influx as a function of increasing solution P_i concentration in excised roots of barley derived from wild-type or T0 transformed lines (G64-4, G64-6 and G64-8) expressing a maize ubiquitin promoter::*HvPT8* construct. Roots were recovered from excision for 3 h (A) or overnight (B). P_i influx was calculated from 10 min of uptake at the desired concentration. Data are means ± SEM of three replicates.

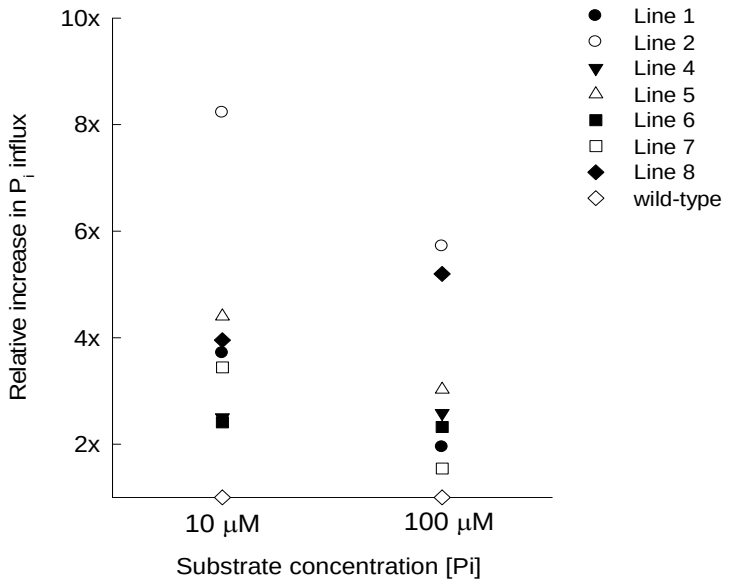


Figure 6.5 Relative increase in P_i influx from 10 μM or 100 μM P_i uptake solution by excised barley roots derived from seven transgenic lines expressing a maize ubiquitin promoter::*HvPT8* construct compared to wild-type roots. Data derived from 5 replicate measurements per line.

6.3.4 ^{32}P uptake assays in intact plants

Two putative homozygous single-insert lines G64:8:7 and G64:7:9 and one null segregant, G64:8:9 were identified as outlined in Section 6.2.4. The expression of the transgene in non-mycorrhizal root tissue of T2 plants was assessed non-quantitatively by PCR from cDNA and the expression of the control gene, *HvGAPdH* was used to confirm cDNA integrity. *HvPT8* was expressed in lines G64:8:7 and G64:7:9 but not in the null segregant (Figure 6.6). Seedlings of these three lines and wild-type barley were grown in solution culture at high or low P as outlined in Section 6.2.6 and subsequently used for measuring ^{32}P uptake in intact plants.

Barley seedlings grown at high P had an average shoot P concentration of 9576 mg kg^{-1} whereas the shoot P concentration was 2811 mg kg^{-1} in seedlings grown at low P (data not shown). P_i influx increased 1.5-fold in plants grown at low P compared to high P (Figure 6.7). In seedlings grown at high P there was a significant difference in K_m and V_{max} of line G64:7:9 compared to both wild-type and the transgenic control line but not for Line G64:8:7 (Table 6.3). There was no difference in K_m and V_{max} of seedlings grown at low P.

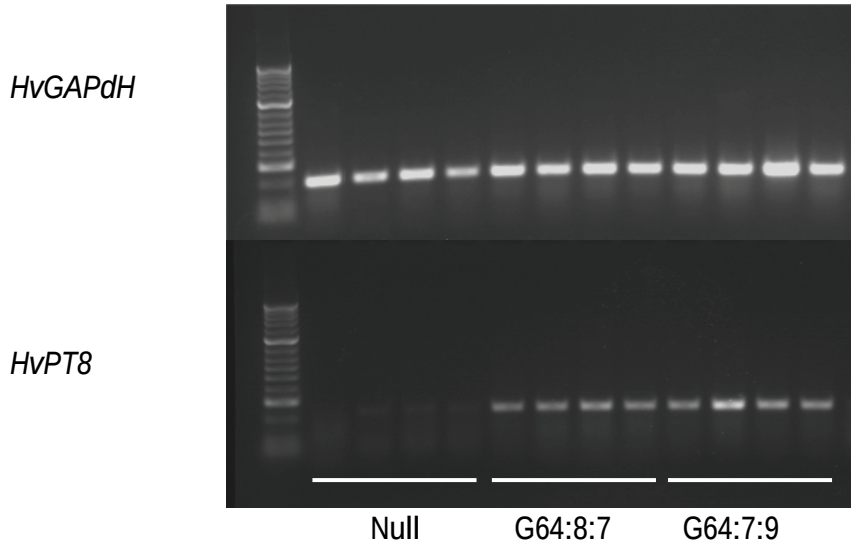


Figure 6.6 PCR analysis of *HvGAPdH* and *HvPT8* in cDNA prepared from root tissue of transgenic barley. Null - transgenic control G64:8:9; transgenic line G64:8:7 and G64:7:9 expressing a maize ubiquitin promoter::*HvPT8* construct.

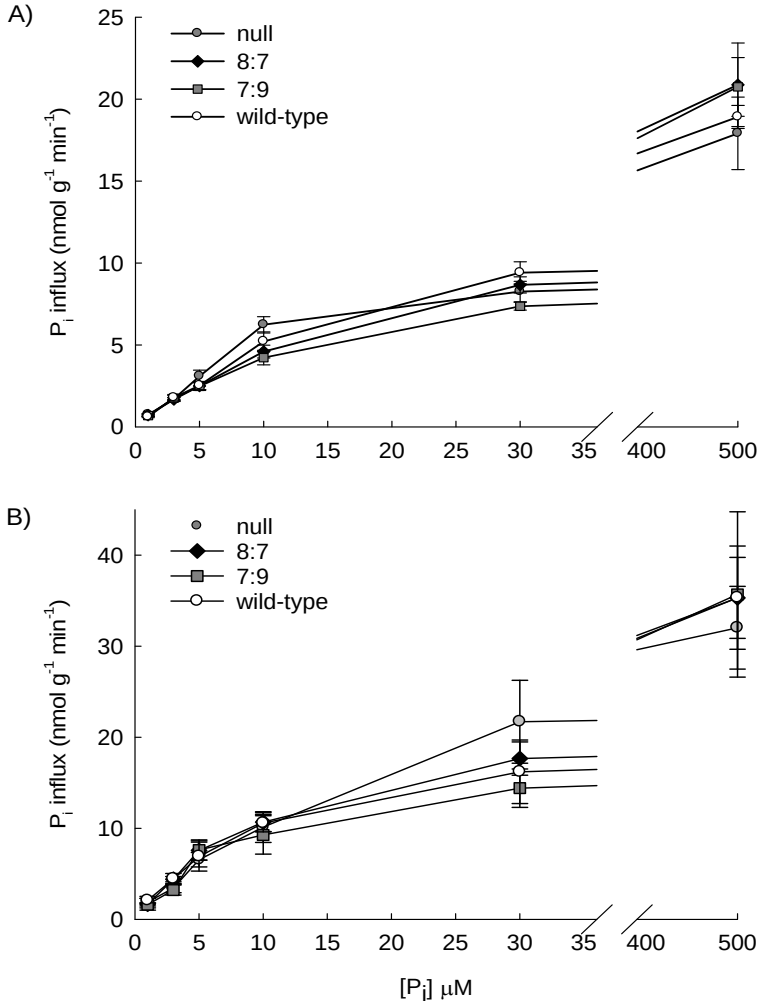


Figure 6.7 P_i influx as a function of increasing solution P_i concentration in intact barley seedlings grown in high P (A) or low P (B) nutrient solution for 8 d or 9.5 d, respectively. Seedlings were wild-type, transgenic control G64:8:9 (null) and transgenic lines G64:8:7 and G64:7:9 expressing a maize ubiquitin promoter::*HvPT8* construct. P_i influx was calculated from 10 min of uptake at the desired concentration. Data are means ± SEM of four replicates.

Table 6.3 Estimates of kinetic parameters derived from non-linear regression of influx data for whole barley seedlings grown at high P or low P.

Genotype	High P		Low P	
	K_m (μM)[1]	V_{max} (nmol g^{-1} FW min^{-1})[1]	K_m (ns) (μM)[1]	V_{max} (ns) (nmol g^{-1} FW min^{-1})[1]
Wild-type	32.1±2.8^{a}	20.1±0.5^{ab}	29.7±7.7	36.9±3.0
Null	28.6±5.3^{a}	18.6±1.1^{a}	20.1±5.6	33.7±2.8
Line G64:8:7	44.5±7.8^{ab}	22.6±1.2^{bc}	26.4±4.9	36.7±2.2
Line G64:7:9	53.9±7.7^{b}	22.9±1.0^{c}	37.3±14.2	37.9±4.5

[1]Data are estimates ± std dev as reported in GraphPad Prism output. Values followed by the same letter are not significantly different at $P<0.05$. ns – no significant difference between treatments.

6.3.5 Growth and P content of transgenic plants in solution culture

The growth of barley seedlings during 8 d at high P and 9.5 d at low P is illustrated in Figure 6.8. Growth at high and low P cannot be compared due to the difference in growth period; however, the root:shoot ratio increased significantly in seedlings grown at low P. There was no significant difference in shoot or root growth between genotypes grown at high P. Significant differences in growth at low P were observed between Line G64:8:7 and wild-type only; neither transgenic line was significantly different to the transgenic control (null).

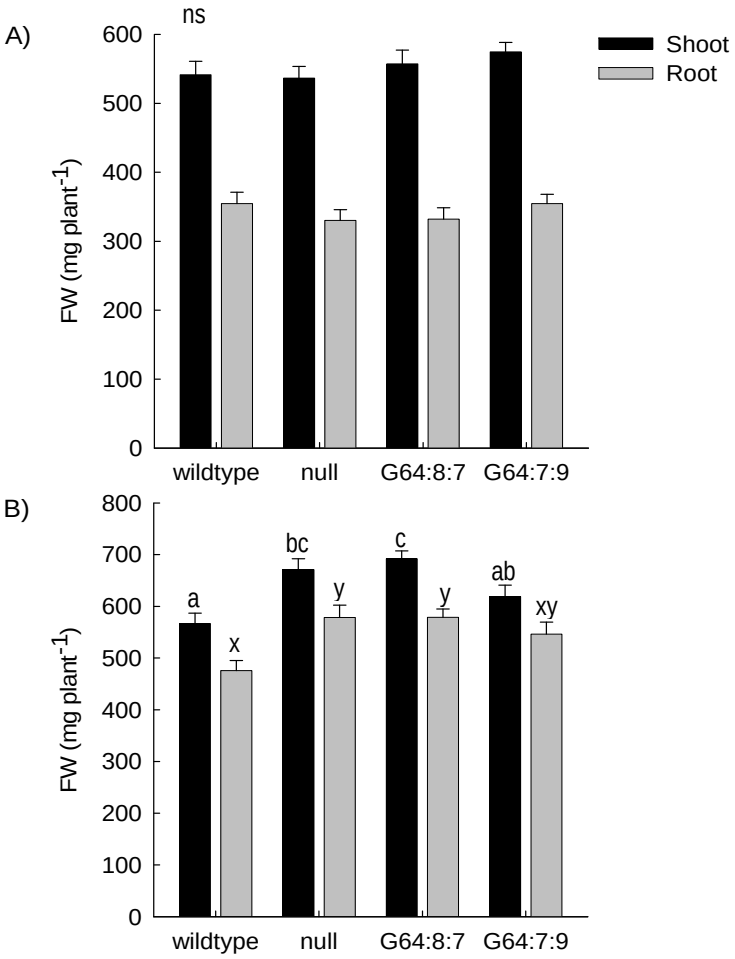


Figure 6.8 Shoot and root fresh weights of barley seedlings grown in high P (A) or low P (B) nutrient solution for 8 d or 9.5 d, respectively. Seedlings were wild-type, transgenic control G64:8:9 (null) and transgenic lines G64:8:7 and G64:7:9 expressing a maize ubiquitin promoter::*HvPT8* construct. Bars are means ± SEM of twenty four replicates, bars with the same letter are not significantly different (P<0.05). Shoot and root data were analysed separately and letters only apply within a tissue. ns - no significant difference between treatments.

There was no significant difference in shoot P content of seedlings grown at high P (Figure 6.9). In seedlings grown at low P, the shoot P content of both transgenic lines G64:8:7 and G64:7:9 was significantly higher than wild-type but not different to the transgenic control line.

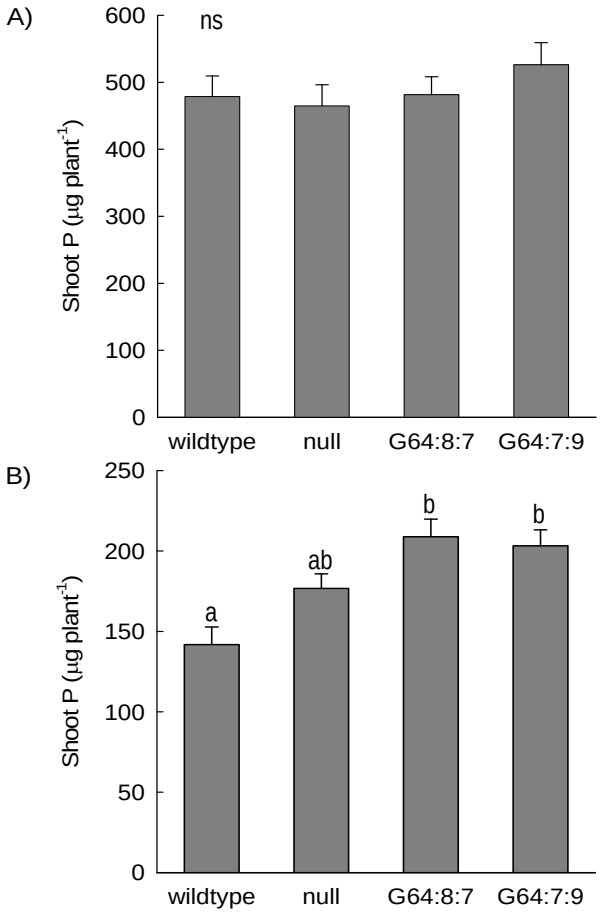


Figure 6.9 Shoot P content (µg plant^{-1}) of barley seedlings grown in high P (A) or low P (B) nutrient solution for 8 d or 9.5 d, respectively. Seedlings were wild-type, transgenic control G64:8:9 (null) and transgenic lines G64:8:7 and G64:7:9 expressing a maize ubiquitin promoter::*HvPT8* construct. Bars are means ± SEM of eight replicates, bars with the same letter are not significantly different (P<0.05). ns - no significant difference between treatments.

6.4 Discussion

Transformation of barley with the maize ubiquitin promoter::*HvPT8* construct successfully produced plants which mis-expressed the transgene at high levels in non-mycorrhizal root tissue. Over-expression of *HvPT8* increased P_i uptake into excised roots of transgenic plants compared to wild-type indicating that *HvPT8* encodes a functional P_i transporter. Although there was no significant difference in the estimated K_m for excised roots obtained from either transgenic or wild-type plants, the increase in P_i influx into excised roots of transgenic plants at low P indicates that HvPT8 functions in the high-affinity P_i uptake range and is likely to have a K_m equal to that of the native high-affinity uptake system. The estimated K_m (~25 µM) is higher than predicted previously in cell-suspension culture for HvPT1 of barley and Pht1:1 of *Arabidopsis* (K_m = 9 µM and 3 µM, respectively) but is within the range of previous estimates for whole-plant uptake in wheat

(26 μM: Cogliatti & Santa Maria, 1990) and potato (21 μM: Cogliatti & Clarkson, 1983). This data provides the first evidence of the kinetic properties of an AM-inducible P_i transporter from a monocot, indicating that HvPT8 is a high-affinity transporter similar to the AM-inducible P_i transporter StPT3 of potato.

The observed increase in P_i influx after overnight pre-treatment is consistent with the enhanced P_i uptake that is typically observed after P starvation (Cogliatti & Clarkson, 1983; Drew *et al.*, 1984). The doubling of P_i influx in excised roots of wild-type plants can be explained by increased transcription of P_i transporters leading to increased abundance in the membrane. However, the 4- to 7-fold increase in P_i influx in excised roots of transgenic lines suggests that P starvation increased the activity of these roots over-expressing HvPT8 to a greater degree than wild-type. A possible explanation for this discrepancy is that P_i starvation alleviated allosteric regulation of the HvPT8 transgene, resulting in a greater increase than observed for wild-type roots. However, it has been suggested that allosteric regulation of the P_i transporters involved in uptake of P from soil is unlikely and is inconsistent with the observation that plants maintain P_i homeostasis in the cytoplasm (Lee *et al.*, 1990; Dong *et al.*, 1999) (see below). Whether AM-inducible P_i transporters are differentially regulated with respect to the P_i transporters expressed in the epidermis remains to be determined.

Estimates of kinetic parameters from intact plant ^{32}P uptake assays were similar to those from excised roots. In plants grown at high P, V_{max} was higher in transgenic lines than wild-type and transgenic control, although this was only statistically significant for Line G64:7:9. However, there was no difference in growth or P content of transgenic lines compared to control lines grown at high P. This discrepancy is likely due to regulatory mechanisms which are important in maintaining the internal P_i concentration below toxic levels. Short term measurements of ^{32}P uptake estimate uni-directional P_i influx whereas the long term accumulation of P in the plant results from the combined processes of influx and efflux. The concentration of P_i in the cytoplasm is tightly controlled by transport and storage of P_i in the vacuole (Lee *et al.*, 1990; Mimura *et al.*, 1990) and at high external P by P_i efflux. The proportion of P_i efflux increases with increasing external P_i concentration (Cogliatti & Santa Maria, 1990) and it has been proposed that under non-limiting conditions P_i homeostasis is primarily controlled by P_i efflux (Elliott *et al.*, 1984; Raghothama, 1999). In order to test the hypothesis that P_i efflux is enhanced in transgenic plants over-expressing *HvPT8,* the dual labelling method of Elliot *et al.* (1984) could be applied to simultaneously measure ^{32}P influx and ^{33}P efflux from both wild-type and transgenic plants.

As reported previously for barley (Drew *et al.*, 1984) low P pre-treatment did not alter the K_m but increased V_{max} relative to plants pre-treated at high P. This is consistent with increased synthesis of P_i transporters in response to P_i starvation. However, there was no difference between transgenic and control lines, indicating that increased transcription of native P_i transporters at low P is sufficient to increase P_i influx to a comparable level to that found in transgenic plants over-expressing HvPT8. Although growth and P content of plants grown at low P was higher in transgenic lines compared to wild-type plants, there was no significant difference to the transgenic control plants. This is in agreement with previous reports by Rae *et al.* (2004). In that experiment constitutive over-expression of *HvPT1* in barley did not increase P_i uptake or growth of plants in solution culture or from soil. Taken together these data suggest that transporter abundance may not be the limiting factor governing P_i uptake from solution. As mentioned in Chapter 4 and discussed further in Chapter 8, regulatory components which are involved in plant P_i starvation responses have begun to be elucidated (Fujii *et al.*, 2005; Miura *et al.*, 2005). The role that these regulatory components play in governing plant P_i uptake remains to be determined.

Chapter 7

Response of Barley Over-expressing HvPT8 to AM colonisation

7.1 Introduction

The experiments described in the previous chapter produced transgenic plants constitutively over-expressing the AM-inducible HvPT8 transporter. Increased P_i uptake was observed in excised roots of transgenic plants but had no effect on the overall P content of intact plants grown in solution culture. The aim of the experiment described in this chapter was to investigate the response of these transgenic plants to AM colonisation. A compartmented pot system as described in Chapter 4 was used to investigate the contribution of the AM pathway to plant P uptake.

7.2 Materials & methods

7.2.1 Experimental design

The experimental design consisted of two P application rates, two AM fungal treatments (NM or inoculated with *G. intraradices*) and four plant lines (wild-type, Line G64:5, Line G64:8, transgenic control). There were 5 replicates of all treatments.

Compartmented pots were prepared as outlined in Chapter 4 except that the nutrients added to the 1:9 Mallala soil sand mix were adjusted to provide sufficient nutrients for the duration of the experiment. Mineral nutrients were mixed thoroughly into the 1:9 soil sand mix at the following rates (mg kg^{-1} dry soil); NH_4NO_3, 320.2; KNO_3, 283.1; $Ca(NO_3)_2$, 204.6; K_2SO_4, 104.6; $MgCl_2$, 19.0; $FeSO_4$, 4.9; $CuSO_4$, 2.4; $ZnSO_4$, 1.9; $MnSO_4$, 1.8; Na_2MoO_4, 0.3; H_3BO_4, 0.2 (Murphy *et al.*, 1997). The P application rate was also increased relative to previous experiments. Phosphorus was added as $CaHPO_4$ to provide an additional 25 or 50 mg P kg^{-1}, P1 and P2, respectively. AM inoculum of *G. intraradices* was applied at 15% w/w to the RHC of AM pots only. HCs were labelled with carrier-free $H_3{}^{32}PO_4$ to provide 10.3 kBq g^{-1} soil. In total, experimental pots contained 1403 g Mallala growth medium of which 3.8% was contained in the HC.

The experiment was conducted on T1 transgenic seed of two putative single insert lines, Line G64:8 and G64:5, identified by Southern hybridisation as outlined in Chapter 6, null segregants of

these lines (see below) and wild-type barley cv. Golden Promise. Uniformly sized seeds were surface sterilised and germinated (Section 2.4.1). Germinated seeds were transferred to a small-scale solution culture set up for 10 d to enable genotyping prior to planting. The nutrient solution consisted of: (mM) KNO_3, 2.5; $Ca(NO_3)_2$, 1.0; $MgSO_4$, 1.0; Na_2SiO_3, 0.25; NH_4NO_3, 0.1; NaFe(III)EDTA, 0.025; H_3BO_3, 0.05; (µM) $ZnSO_4$, 10.0; $MnCl_2$, 5.0; $CuSO_4$, 0.5; Na_2MoO_3, 0.1. There was no P addition. Seedlings were maintained in a controlled environment facility with a 10 h photoperiod at 21°C. Transgenic T1 seedlings were genotyped as outlined in Section 2.15 in order to identify null segregants which had lost the insert. Eleven null segregants were identified from 36 seedlings of Line G64:5 and 9 null segregants were identified from 60 seedlings of Line G64:8. Statistical analysis of the segregation ratio was tested using a chi^2 test. Both lines were significant (P<0.05) for a 1:3 segregation ratio (characteristic of a single insert Mendelian segregation ratio) but not for 1:15 (characteristic of a double insert Mendelian segregation ratio) supporting the assignment of these lines as single-copy. In the following analyses data for the null segregants of the two lines have been pooled to provide transgenic controls. After genotyping seedlings were transplanted singly into compartmented pots.

Plants were grown in a controlled environment facility with a 16 h photoperiod, photon irradiance of 360 - 460 µmol m^{-2} s^{-1} and 21°C/16°C day/night temperatures. Pots were watered to 10% w/w with RO water every 2 d. The appearance of ^{32}P in the shoots was followed non-quantitatively with a hand-held monitor; ^{32}P counts were first detectable after 20 d.

7.2.2 Harvest

Pots were harvested 32 d after planting, according to Section 2.5. Shoot and root material were sampled for fresh and dry weights, tissue P concentration and specific activity (Section 2.7, 2.8). Total AM colonisation (% RLC) of roots was assessed using the grid intersect method (Giovannetti & Mosse, 1980). Stained roots were spread on a dish with a 1 cm x 1 cm grid and intersects with AM colonisation units were scored at x 40 magnification. A random sub-sample of roots from each treatment was also observed at x 100 magnification with a stereoscopic microscope in order to investigate structural differences between colonisation units. Roots were also sampled for Q PCR analysis of the expression of the three P_i transporter genes, *HvPT1*, *HvPT2* and *HvPT8* (Section 2.11). Q PCR was performed on root samples from three of the five experimental replicates from each treatment using the primers listed in Table 2.2. As discussed in Chapter 6, the *HvPT8* Q PCR primers detect the expression of both native *HvPT8* and the transgene. Genotyping of all transgenic

plants was confirmed post-harvest by PCR amplification of the hygromycin resistance gene from cDNA using the primers and conditions listed in Section 2.15 (data not shown).

Soil from the RHC or HC was mixed thoroughly and sampled for determination of plant-available P by both Olsen P and resin P methods and of specific activity (Section 2.6, 2.8). Although ^{32}P counts were detectable in the shoots of AM plants at harvest using a hand-held monitor, the activity was low and there were considerable delays with access to the scintillation counter. As a result the ^{32}P activity of shoot and root tissue digests had decayed to background level by the time of counting. Therefore, the contribution of the AM pathway could not be calculated in this experiment.

7.3 Results

7.3.1 Expression of plant P_i transporters

The expression levels of the AM-inducible P_i transporter, *HvPT8*, and the root epidermal P_i transporters, *HvPT1* and *HvPT2*, were examined in root tissue. A low level of background expression of *HvPT8* was detected in NM roots of both wild-type and null control lines (Figure 7.1). *HvPT8* expression increased in control AM roots compared to NM roots and was generally higher at P1 than P2. The Q PCR primers designed for amplification of *HvPT8* detect both native and transgene expression. Therefore, the expression level of *HvPT8* in transgenic AM plants represents the combined expression of these gene copies, whereas the expression in transgenic NM roots can be primarily attributed to the transgene. The expression of the transgene was evident in both transgenic lines. *HvPT8* expression was significantly greater in both NM and AM transgenic plants with respect to both AM wild-type and AM null controls (Figure 7.2). The increase in *HvPT8* expression in transgenic AM roots was 14-fold and 61-fold at P1 relative to AM wild-type and null controls, respectively and there was an approximately 1300-fold increase in *HvPT8* expression in NM transgenic roots relative to NM controls, at P1. At P2 the relative increase was 325-fold and 592-fold with respect to AM or NM controls, respectively.

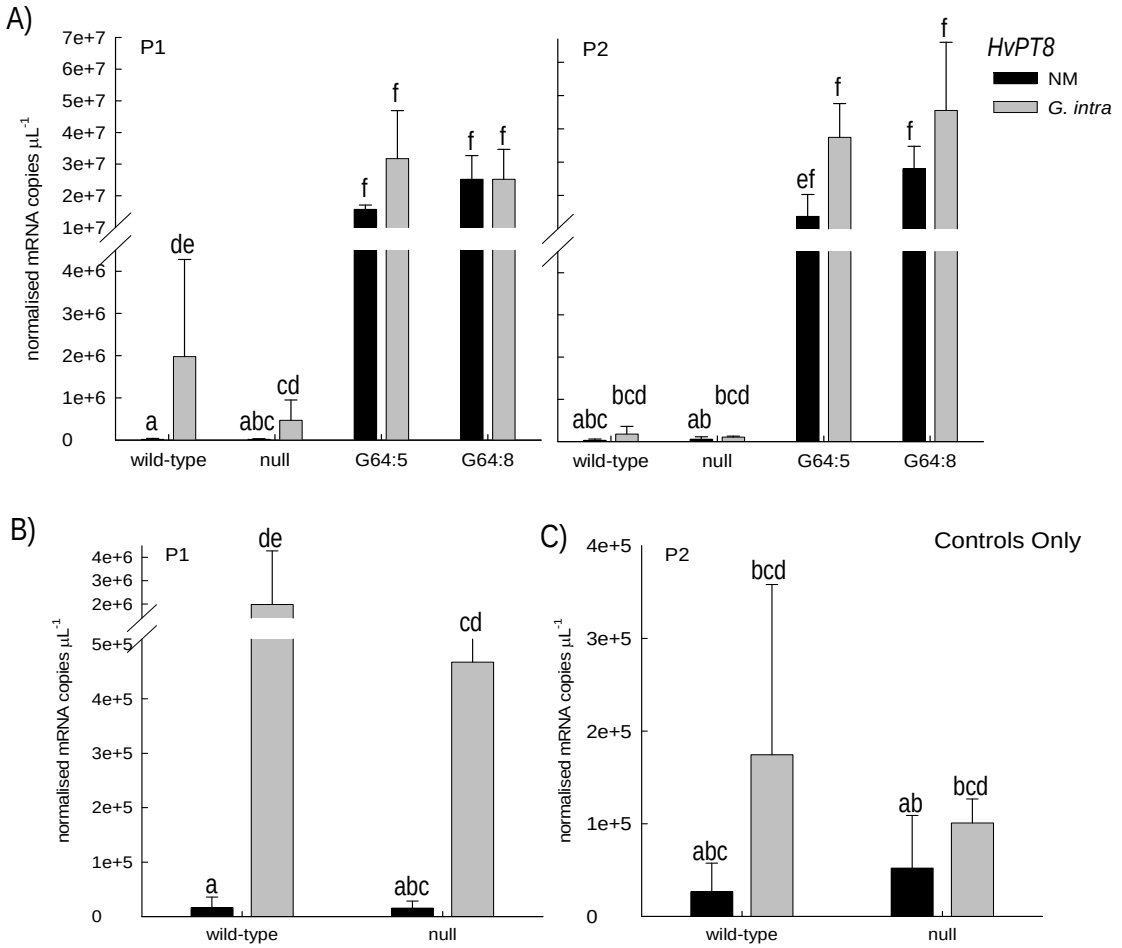


Figure 7.1 Normalised expression levels of *HvPT8* in roots of transgenic T1 barley plants (G64:5 and G64:8) and their controls (wild-type or null segregant) inoculated with *G. intraradices* or non-mycorrhizal (*G. intra*, NM) grown at P1 (25 mg kg^{-1} additional P) or P2 (50 mg kg^{-1} additional P). A) Normalised expression level of *HvPT8* across all treatments; B and C present the same data as for A but in the control treatments only; B) P1; C) P2. Bars are means of three replicates ± SEM; bars with the same letter are not significantly different (P<0.05).

Both *HvPT1* and *HvPT2* were variably but constitutively expressed at each P level (Figure 7.2). There was a trend towards decreased expression at P2 compared to P1, although this was not significant. The expression of *HvPT1* and *HvPT2* was not significantly altered by over-expression of *HvPT8*.

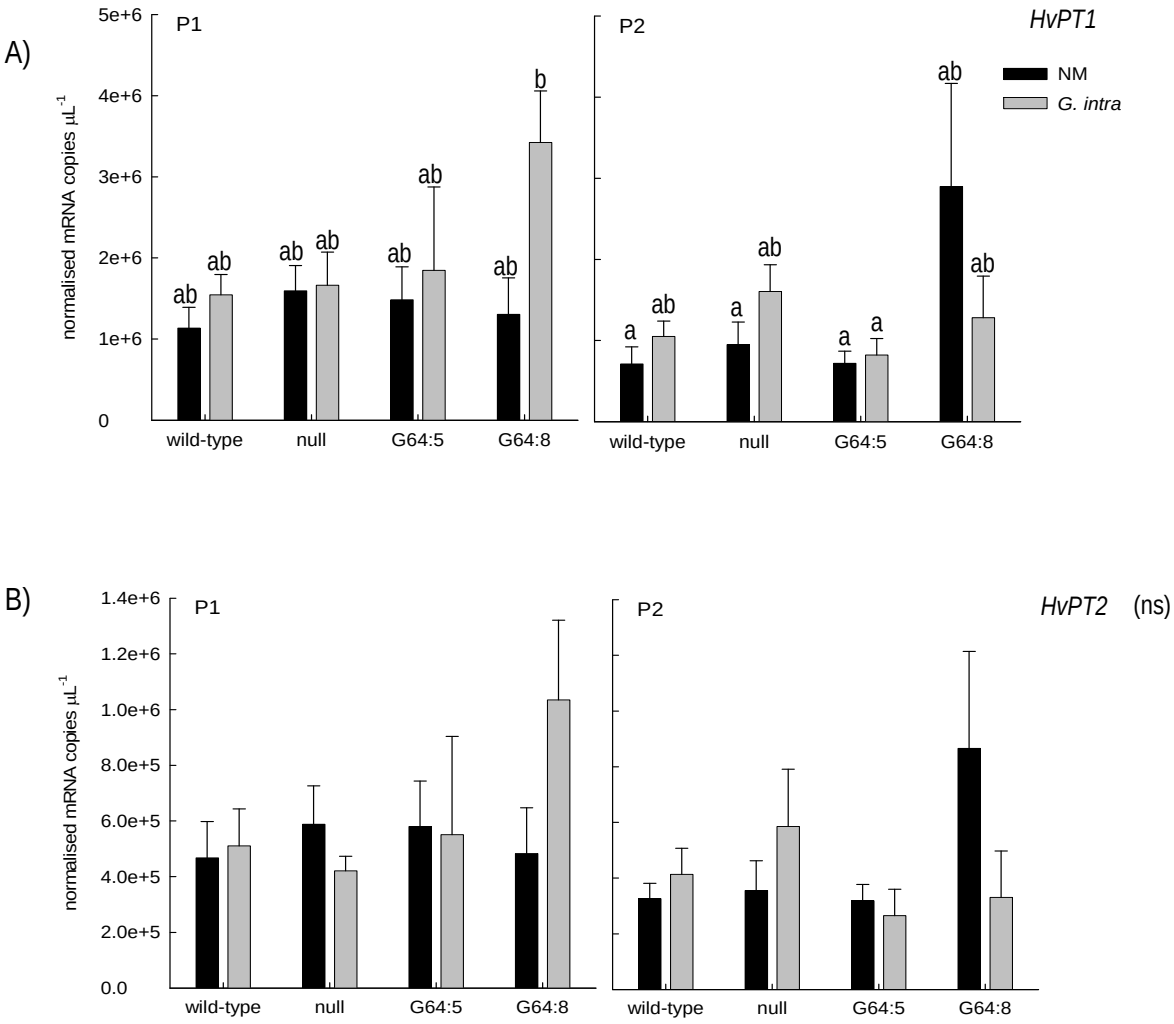


Figure 7.2 Normalised expression levels of the root epidermal P_i transporters, *HvPT1* and *HvPT2* in roots of transgenic T1 barley plants (G64:5 and G64:8) and their controls (wild-type or null segregant) inoculated with *G. intraradices* or non-mycorrhizal (*G. intra*, NM) grown at P1 (25 mg kg^{-1} additional P) or P2 (50 mg kg^{-1} additional P). Bars are means of three replicates ± SEM; bars with the same letter are not significantly different (P<0.05). ns - no significant difference between treatments.

7.3.2 AM colonisation

No colonisation was observed in non-inoculated plants. There was no significant difference in the total colonisation of transgenic lines compared to wild-type or transgenic controls at either soil P level (Table 7.1).The mean colonisation at P1 was 25 %RLC whereas the mean colonisation at P2 was 13 %RLC. There were no apparent differences in the structure of colonisation units (data not shown).

Table 7.1 AM colonisation of transgenic T1 barley plants (G64:5 and G64:8) and their controls (wild-type or null segregant) inoculated with *Glomus intraradices* grown at P1 (25 mg kg^{-1} additional P) or P2 (50 mg kg^{-1} additional P). %RLC (percent root length colonised).

Genotype	P level	% RLC[1]
Wild-type	P1	25.6±1.6[a]
	P2	10.6±1.5[b]
Null	P1	17.5±7.7[ab]
	P2	15.6±4.6[ab]
G64:5	P1	24.9±4.7[a]
	P2	12.1±2.1[ab]
G64:8	P1	24.7±4.2[a]
	P2	12.8±1.8[ab]

[1]Values are means ± SEM of five replicates. Values with the same letter in each column are not significantly different ($P<0.05$).

7.3.3 Growth and P response

The growth of NM transgenic lines and wild-type or null controls was not significantly different at either P level (Figure 7.3). Although it appears that there was a depression in shoot growth of wild-type barley colonised by *G. intraradices* at P1 compared to NM wild-type, this was not significant and the trend was reversed at P2. Shoot growth of NM null controls was similar to AM colonised plants at both P levels whereas shoot growth of transgenic plants colonised by *G. intraradices* tended to be greater than NM plants. However, this was only significant for Line G64:5 at P2. There was no significant difference in root DW for any treatment.

There were no significant differences in shoot and root P concentrations at either P level. The mean shoot P concentration was 2708 mg kg^{-1} and the mean root P concentration was 1520 mg kg^{-1} (Table 7.2). The total P content followed a similar trend to shoot DW (Figure 7.4). However, there were no significant differences in total P content for any treatment at either P1 or P2.

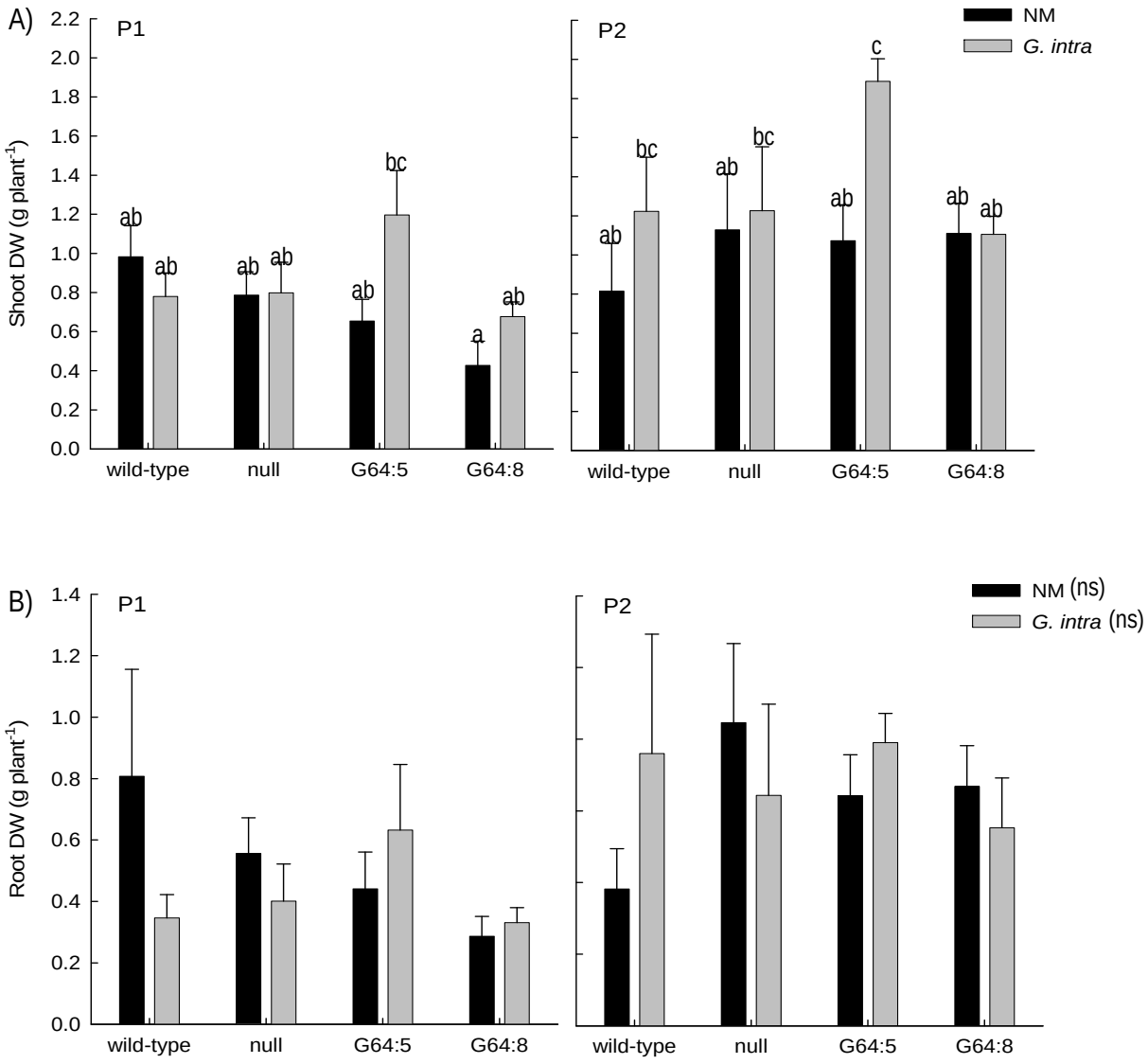


Figure 7.3 Shoot (A) and root (B) dry weight of transgenic T1 barley plants (G64:5 and G64:8) and their controls (wild-type or null segregant) inoculated with *G. intraradices* or non-mycorrhizal (*G. intra*, NM) grown at P1 (25 mg kg^{-1} additional P) or P2 (50 mg kg^{-1} additional P) for 32 d. Bars are means of five replicates ± SEM; bars with the same letter are not significantly different (P<0.05). ns - no significant difference between treatments.

Table 7.2 Shoot and root P concentrations (mg kg^{-1}) of transgenic T1 barley plants (G64:5 and G64:8) and their controls (wild-type or null segregant) inoculated with *G. intraradices* or non-mycorrhizal, grown at P1 (25 mg kg^{-1} additional P) or P2 (50 mg kg^{-1} additional P).

Genotype	P Level	Shoot P concentration (mg kg^{-1})[1]		Root P concentration (mg kg^{-1})[1]	
		Non-mycorrhizal	*G. intraradices*	Non-mycorrhizal (ns)	*G. intraradices* (ns)
Wild-type	P1	2571±253^{ab}	2554±137^{ab}	1334±86	1630±112
	P2	2751±236^{ab}	2541±223^{ab}	1510±116	1514±91
Null	P1	2751±285^{ab}	2434±272^{ab}	1449±168	1578±210
	P2	2917±432^{ab}	2760±167^{ab}	1467±113	1602±226
Line 5	P1	2949±101^{ab}	2179±259^{a}	1488±140	1427±356
	P2	3297±229^{b}	2468±151^{ab}	1763±175	1515±92
Line 8	P1	2970±131^{ab}	2548±165^{ab}	1708±125	1465±72
	P2	2769±194^{ab}	2864±170^{ab}	1324±86	1542±112

[1]Values are means ± SEM of five replicates. Values with the same letter in each column are not significantly different (P<0.05). ns – no significant difference between treatments.

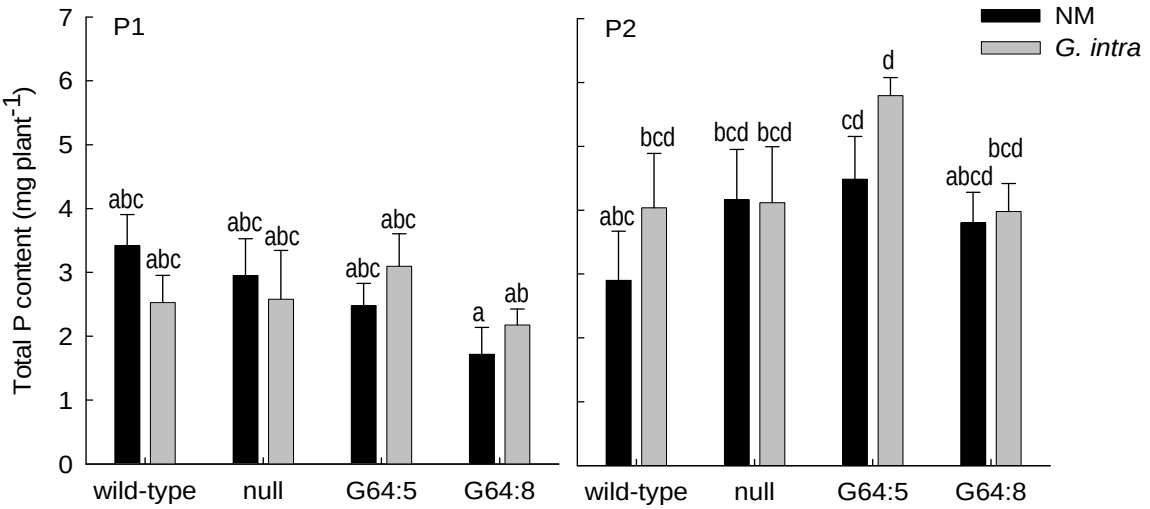


Figure 7.4 Total P content (mg) of transgenic T1 barley plants (G64:5 and G64:8) and their controls (wild-type or null segregant) inoculated with *G. intraradices* or non-mycorrhizal (*G. intra*, NM) grown at P1 (25 mg kg^{-1} additional P) or P2 (50 mg kg^{-1} additional P) for 32 d. Bars are means of five replicates ± SEM; bars with the same letter are not significantly different (P<0.05).

7.3.4 Discussion

AM colonisation of barley by *G. intraradices* decreased with increased P application and was reduced relative to previous experiments in which a lower P application rate was used (Chapters 3-5). This reduction in colonisation with increasing P supply is typical of many AM hosts (Baon *et al.*, 1994; Fay *et al.*, 1996; Khaliq & Sanders, 1997, 2000). Contrary to previous experiments reported herein, a significant growth depression was not observed in wild-type barley colonised by *G. intraradices*. Variability in the magnitude of growth responses is a common occurrence amongst non-responsive AM hosts (Graham & Abbott, 2000; Li *et al.*, 2005). In this instance both the improved nutrient status and/or the decreased colonisation (relative to previous experiments) may be responsible.

AM colonisation did not down-regulate the expression of *HvPT1* or *HvPT2* (see Chapter 5 discussion). Both genes were variably but constitutively expressed across all treatments, although there was a trend towards reduced expression at P2. In contrast to the experiment described in Chapter 4, *HvPT2* was not up-regulated with increased P supply; however, as both levels of P applied in the current experiment were higher than previous experiments, these results cannot be directly compared. Over-expression of *HvPT8* in two transgenic barley lines increased expression significantly relative to native *HvPT8* expression in AM colonised control roots. Consistent with the results of the solution culture experiment in Chapter 6, over-expression of the HvPT8 transporter did not increase the growth of NM plants in soil. This is also consistent with the results of Rae *et al.* (2004) for plants over-expressing *HvPT1* grown in soil (see Chapter 6 discussion).

To date there are no data for the response of AM colonisation to over-expression of an AM-inducible P_i transporter. As discussed in Chapter 1, knockdown of AM-inducible P_i transporters in *Lotus* (Maeda *et al.*, 2006) and *Medicago* (Javot *et al.*, 2007a) resulted in decreased AM colonisation and reduced growth of these highly responsive plants. Clearly, nutrient exchange via AM-inducible P_i transporters plays a pivotal role in the development and function of AM symbioses. The experiment described in this chapter tested the hypothesis that over-expression of an AM-inducible P_i transporter would enhance AM colonisation and/or P_i uptake via the AM pathway. Mutants which exhibit enhanced AM colonisation (Myc++) have been reported. In the super-nodulating legume mutants this enhanced colonisation has been attributed to increased arbuscule formation (Morandi *et al.*, 2000; Solaiman *et al.*, 2000) whereas the maize *pram1* (*precocious arbuscular mycorrhiza 1*) mutant is characterised by accelerated development of AM colonisation

(Paszkowski *et al.*, 2006). Over-expression of *HvPT8* did not alter the colonisation of barley by *G. intraradices* at the single 4 week harvest in this experiment. Both total colonisation and morphology of colonisation units were unaltered in transgenic plants, suggesting that the AM phenotype was unaffected. However, the data collected in this experiment would not identify accelerated development of colonisation if this occurred. Interestingly, data from Myc++ mutants suggest that increased AM colonisation does not enhance the AM response of the plant. The growth of Myc++ AM mutants was unaffected in *Lotus* (Solaiman *et al.*, 2000) and maize (Paszkowski *et al.*, 2006), whereas the growth of Myc++ mutants of pea (*Pisum sativum*) and *Medicago* was reduced (Morandi *et al.*, 2000). However, all of these species are AM responsive; in future work it will be useful to identify Myc++ mutants of non-responsive species to investigate whether growth increases are observed or growth depressions become less negative.

In the current work, the growth of transgenic plants colonised by *G. intraradices* tended to be greater than NM transgenic plants. However, this was not significant and no differences in P concentration or total P content were evident, indicating that over-expression of *HvPT8* did not increase P uptake in AM plants and corroborating the results obtained for NM plants in soil. Unfortunately, due to the lack of ^{32}P data it is not possible to determine whether the absolute contributions of the AM and direct pathways were altered. In future experiments it would be useful to use a lower soil P level at which a growth depression of wild-type AM plants is observed (as in Chapter 4) in order to directly detect effects of over-expression on the AM pathway. The reduced ^{32}P transfer in this experiment may be due to the decreased colonisation (relative to Chapter 4) and a delay in "switching-on" the AM pathway. Comparison of HLDs in the RHC and HC would be useful to confirm whether AM fungal hyphae had reached the HC. However, ^{32}P counts were detected at 20 d using a hand-held monitor in both transgenic and wild-type AM plants. This indicates that fungal hyphae were present in the HC at this time and that some P transfer via the AM pathway had occurred. This is somewhat slower than in the experiment described in Chapter 4, where ^{32}P counts were first detected in some plants 11 d after planting, and supports the suggestion that the AM pathway was functional but delayed in this experiment.

Overall the experiment described in this chapter strengthens previous assertions that over-expression of P_i transporters does not increase plant P_i uptake. The results indicate that P transfer via the AM pathway was not enhanced in plants over-expressing *HvPT8*; however, a direct measurement of the AM pathway is necessary to confirm this assumption. In addition, it would be

useful to investigate whether the *HvPT8* transgene or protein is actually expressed on the periarbuscular membrane, either by in-situ hybridisation or antibody localisation, respectively.

Chapter 8
General Discussion & Future Work

8.1 Review of thesis aims

The general aim of the work described in this thesis was to investigate the role of plant P_i transporters in governing the contribution of the AM pathway to P_i uptake in a non-responsive plant. The specific aims were to: 1) Determine the AM contribution to P uptake in barley; 2) Investigate the correlation between contributions of the AM and direct pathways and expression of plant P_i transporters; 3) Functionally characterise the AM-inducible P_i transporter, HvPT8; and 4) Investigate the potential for altering P_i uptake via the AM pathway through altered expression of this transporter.

8.2 Contribution of the direct and AM P_i uptake pathways in a non-responsive plant

The growth and P content of barley cv. Golden Promise in symbiosis with *G. intraradices* or *G. geosporum* was significantly reduced at low P compared to NM controls (~MGR -40%, ~MPR -40%). This growth depression was observed consistently (Chapters 3, 4 and 5). The magnitude of the growth depression was not related to AM fungal species or extent of colonisation; equivalent growth depressions were observed with *G. geosporum* (~3% RLC) and *G. intraradices* (~49% RLC) (Chapter 4). A similar phenomenon has been reported previously in wheat (Hetrick *et al.*, 1992; Li *et al.*, unpublished 2007). In the experiment reported by Hetrick *et al.* (1992), the growth depression caused by *G. versiforme* or *G. mosseae* was equal (MGR -50%) but these fungi colonised 61% and 5% of the root length, respectively. Such disparate colonisation suggests that the C demand of these AM fungi is likely to be quite different; in the current work this suggestion is further supported by the production of vesicles (lipid-rich storage structures) in roots colonised by *G. intraradices* but not *G. geosporum*. These data suggest that the conventional explanation of growth depressions resulting from C drain to the fungal symbiont does not hold in all cases. The strong correlation between tissue P content and growth, presented in Chapter 3, suggests that P was the limiting factor in plant growth in that experiment. This suggestion is supported by the observation that colonisation by *G. intraradices* did not result in a growth depression when plants were grown at a

higher soil P level in the experiment described in Chapter 7, although reduced AM colonisation may also be implicated in that response.

As demonstrated by previous authors (Pearson & Jakobsen, 1993; Smith *et al.*, 2003b, 2004; Poulsen *et al.*, 2005), the contribution of the AM pathway was unrelated to the growth and P response of the plant. Despite possible problems with calculations (discussed in Chapter 4), *G. intraradices* made a significant contribution (~48%) to plant P_i uptake and although the contribution of *G. geosporum* could not be quantified, this fungus was capable of P transfer (Chapter 4). These data raise significant issues regarding the contribution of the direct, epidermal P_i uptake pathway in AM barley. The contribution of the direct pathway was reduced in AM plants and it can be inferred from the measurement of AM contribution that direct uptake was reduced to a greater extent in barley when colonised by *G. intraradices* than when colonised by *G. geosporum*. This is particularly significant because root growth was similar in these AM plants and, assuming no change in length:weight ratio, this indicates an equivalent surface area was presented to the soil.

It is possible that the responses of barley to *G. geosporum* and *G. intraradices* result from two separate phenomena; *G. geosporum* caused a growth depression at low colonisation and did not make a significant contribution to plant P, whereas *G. intraradices* colonised at least 50% of the root length and the AM pathway accounted for 48% of plant P_i uptake. However, the magnitude of growth depression was equal for both AM fungi and, as demonstrated in Chapter 3, neither AM fungus reduced the growth at the 2 week harvest despite colonisation by *G. intraradices* having reached 57% compared to 3% for *G. geosporum*. The work described here (Chapters 4, 5 and 7) investigated the hypothesis that decreased P_i uptake via the epidermal pathway is related to AM-induced changes in the expression of plant P_i transporters.

This study provided the first data integrating measurements of AM contribution to plant P_i uptake with molecular characterisation and quantification of P_i transporter expression in a non-responsive AM plant (Chapters 4, 5 and 7). In contrast to previous reports investigating gene expression in AM barley (Glassop *et al.*, 2005), down-regulation of the root epidermal P_i transporters, *HvPT1* and *HvPT2*, was not observed in AM roots colonised by either *G. geosporum* or *G. intraradices* (Chapters 4, 5 and 7). In the present work the expression of these genes was investigated over a range of soil P concentrations (5 mg kg^{-1}, Chapter 4; 50 mg kg^{-1}, Chapter 7), at varying levels of AM colonisation and during a developmental time-course of AM colonisation (Chapter 5). The decrease in contribution of the direct pathway was not correlated with decreased expression of the root

epidermal P_i transporters. Poulsen *et al.* (2005) reached a similar conclusion using tomato, although in that case AM colonisation resulted in increases in plant growth and P uptake. Interestingly, in the interaction between tomato and *G. intraradices* BEG 87, the AM pathway accounted for only 20% of plant P_i uptake, indicating that the MPR of 116% was due to an increase in P_i uptake via the direct pathway. However, there was no clear correlation between the contribution of the direct uptake pathway and changes in expression of the epidermal P_i transporters. A similar observation of enhanced P_i uptake by AM roots has been reported for non-responsive cucumber (*Cucumis sativus*) colonised by *S. calospora* (Pearson & Jakobsen, 1993). These data emphasise that there can be considerable changes in the relative contributions of direct and AM P_i uptake pathways, whether up or down. The mechanisms for these changes remain to be elucidated.

Two issues with respect to these data relate to the methods used. The analysis of gene expression data requires sensitive and precise quantification of specific mRNA sequences. In the experiments reported in the current work a single control gene was used for standardisation of Q PCR data, whereas it has been suggested that multiple internal controls provide the most accurate and un-biased quantification of gene expression (Vandesompele *et al.*, 2002; Nicot *et al.*, 2005). In future work, the identification of multiple genes which do not change their expression in response to AM and P status of the plant will enable more accurate detection of gene expression changes. Microarray data produced from AM barley or other monocots as presented by Güimil *et al.* (2005) for rice, may prove useful in identifying these genes (see Chapter 5 discussion). A second confounding factor in gene expression studies of AM symbioses is the use of root samples obtained from the whole root system for analysis of changes in gene expression. AM colonisation is nonsynchronous; the plant root system is patchily colonised and colonisation units vary in age and stage of development. Sampling whole roots may mask cell-type-specific changes in transcript accumulation or changes in the localisation of gene expression. A number of studies have reported that expression of *StPT1* and *StPT2* of potato is not altered in AM roots (Karandashov *et al.*, 2004; Nagy *et al.*, 2005). However, using a split-root system Rausch *et al.* (2001) demonstrated localised down-regulation of these genes in the colonised half of the root system only. These observations, together with those of Gordon-Weeks *et al.* (2003) demonstrating differential expression of StPT2 during root development, highlight the need for targeted sampling in gene expression studies. New technologies, such as laser microdissection (Day *et al.*, 2007) or fluorescence-activated cell sorting of protoplasts expressing an AM activated fluorescent marker (Birnbaum *et al.*, 2005), which enable analysis of cell-type-specific gene expression, may prove useful in furthering our understanding of

the role that P_i transporter expression plays in governing P_i fluxes via the direct and AM pathways. At the very least, sampling methods should be detailed in scientific papers in the hope of clarifying inconsistencies in reported results.

Nevertheless, the data presented here suggests that P_i uptake via the direct pathway was suppressed in AM plants. As discussed in Chapter 4, decreased contribution of the direct pathway resulting from AM colonisation has also been explained by an increased rate of depletion of soil P adjacent to AM roots, compared with NM roots (Poulsen *et al.*, 2005). This is consistent with observations from studies comparing ^{32}P uptake by roots of AM and NM tomato (Cress *et al.*, 1979) and clover (Schweiger *et al.*, 1999) which demonstrated that AM roots had a significantly lower K_m than NM roots. However, this explanation does not satisfactorily explain the equivalent growth depression of barley when colonised by *G. geosporum,* which could be expected to have a small degree of external hyphal development and hence present minimal competition with roots, or when colonised by *G. intraradices* (Chapters 3 and 4).

Although transcriptional regulation has been identified as an important primary control point for plant P_i transport, recent advances suggest that both post-transcriptional and post-translational modification of regulatory components are also important in determining plant P_i uptake (Fujii *et al.*, 2005; Miura *et al.*, 2005; Chiou *et al.*, 2006). It is plausible that AM regulation of the direct uptake pathway occurs through modification of regulatory components rather than direct regulation of P_i transporter expression. The role of post-transcriptional and post-translational processes in determining P_i fluxes via both the direct and AM uptake pathways in an AM plant remain to be determined. This will be a critical area for future research. If AM fungi have differential ability to directly or indirectly regulate plant P_i uptake pathways this may be pivotal to understanding the observed diversity in plant responses to AM colonisation.

8.3 Expression profiling of *HvPT8* in AM barley

Analysis of *HvPT8* promoter activity using a promoter::GFP gene fusion identified expression in cortical cells containing arbuscules (Chapter 5), confirming the previous report by Glassop *et al.* (2005) in which *HvPT8* transcript was localised to arbuscules and hyphal coils using *in-situ* hybridisation. However, in contrast to the GFP localisation results, the developmental time-course (Chapter 5) indicated that expression of *HvPT8* was induced at reasonably high levels during the early stages of colonisation when few arbuscules were present, suggesting that *HvPT8* expression

may be induced prior to arbuscule formation. In future work it would be useful to combine the visual reporter system with a detailed developmental time-series to determine at what stage of AM development *HvPT8* expression is induced. Identification of the inducible signal, whether plant or fungal, should also be a target of future work.

GFP expression was not detected in NM roots and the localisation of *HvPT8* transcripts which were detected in NM roots by Q PCR remains to be determined (see Chapter 5). This discrepancy between *HvPT8* promoter activity detected using the fluorescent reporter and Q PCR data may be due to differences in the sensitivity of these two methods. Although a direct comparison of the sensitivity of Q PCR with reporter gene analyses, *in-situ* hybridisation or indeed Northern hybridisation has not been reported, it is likely that Q PCR will be the more sensitive method for detection of low abundance gene expression. Indeed, differences in sensitivity may explain why shoot expression of potato *StPT3* was not detected by Northern analysis in the original paper (Rausch *et al.*, 2001), but was later detected in both shoot and NM roots by RT PCR (Karandashov *et al.*, 2004). However, it is also possible that the *HvPT8* promoter fragment used in the reporter construct does not include all of the regulatory domains which direct expression of the full-length *HvPT8* native promoter.

Localisation of AM-inducible P_i transporters to intracellular symbiotic interfaces, as was observed here for barley *HvPT8* (Chapter 5), has been reported for a range of plant species (see Chapter 1). However, a recent report indicates that expression of the AM-inducible P_i transporters is not always restricted to intracellular interfaces. Using laser microdissection, coupled with gene expression analyses of AM tomato roots, Balestrini *et al.* (2007) demonstrated expression of the AM-upregulated *LePT3* and AM-specific *LePT4* transporters exclusively in arbuscule-containing cortical cells. The AM-up-regulated *LePT5* was also expressed in non-colonised cells of AM roots and was assumed to be associated with intercellular hyphae. This is particularly significant as intracellular interfaces, particularly arbuscules, have often been considered the primary site for P_i exchange in AM symbioses (see Chapter 1). Expression of *LePT5* in non-colonised cortical cells suggests that the plant is involved in P_i capture from intercellular hyphae. Whether P_i release by intercellular hyphae occurs by leakage or an efflux mechanism similar to that invoked in arbuscular P_i release will require further investigation. I hypothesise that *LePT5* will be a high-affinity transporter, possibly with a higher K_m than P_i transporters involved in P_i uptake from the intracellular interfaces, reflecting a role in P_i scavenging. Ultimately these data highlight the necessity for careful scrutiny of

experimental results with respect to both the sensitivity of the methods used and the inferences that are made.

8.4 Functional characterisation of HvPT8

Over-expression of *HvPT8* in barley increased P_i uptake from solution by excised roots at low P, demonstrating that HvPT8 functions as a high-affinity transporter (K_m ~8 μM for Line G64:8) (Chapter 6). This is the first *in planta* characterisation of a plant P_i transporter and provides the first kinetic data for an AM-inducible P_i transporter from a monocot. As discussed in Chapter 1, the P concentration in the interfacial apoplast is unknown; however, the localisation of an AM fungal P_i transporter, *GmosPT*, and H^+-ATPase, *GmHA5*, to the fungal arbuscular membrane (Balestrini *et al.*, 2007) suggests that the plant will require a P_i uptake system capable of competing with the fungal symbiont for P_i acquisition from symbiotic interfaces. This is consistent with the characterisation of HvPT8 as a high-affinity transporter.

The occurrence of high-affinity AM-inducible P_i transporters in potato (StPT3) (Rausch *et al.*, 2001) and barley (HvPT8) and a low-affinity AM-inducible P_i transporter in *Medicago* (MtPT4) (Harrison *et al.*, 2002) raises significant questions regarding symbiotic function in these plant species. The low-affinity capacity of the MtPT4 transporter implies that in *Medicago* the P concentration in the interfacial apoplast is relatively high, and that a P_i scavenging system is not required. Whether this reflects an inherent difference in symbiotic function in this highly responsive AM host compared to less responsive species remains to be determined. It is not yet known whether *Medicago* or barley possess additional AM-inducible P_i transporters, as has been reported for five members of the Solanaceae (Nagy *et al.*, 2005; Chen *et al.*, 2007) and for rice (Güimil *et al.*, 2005; Glassop *et al.*, 2007). However, with the sequencing of the *Medicago* genome nearing completion (http://www.medicago.org/genome/index.php) it should soon be possible to identify the complete PhT1 family as has been done for *Arabidopsis* (Mudge *et al.*, 2002) and rice (Paszkowski *et al.*, 2002). It will be interesting to determine whether *Medicago* possesses multiple AM-inducible P_i transporters and whether these differ in kinetic properties. Investigation of the kinetic properties of the additional AM-inducible P_i transporters of potato, StPT4 and StPT5, should also be a target of future endeavour.

The identification of additional AM-inducible P_i transporters in barley was not a key aim of the current work. However, in order to further characterise the role of HvPT8 in AM symbiotic function,

knockout transformants were prepared by transformation with an RNA interference (RNAi) construct (see Appendix 4). These plants could also be expected to provide insight into whether barley has additional transporters which are capable of compensating for loss of HvPT8 function. These plants were not analysed due to time constraints and therefore they were not presented in the experimental section of this work. However, since the project began three reports on knockdown of AM-inducible P_i transporters have been published (Nagy *et al.*, 2005; Maeda *et al.*, 2006; Javot *et al.*, 2007a). As discussed in Chapter 1, data from a *lept4-1* tomato mutant suggests that LePT3 and LePT5 are able to compensate for loss-of-function of the LePT4 transporter (Nagy *et al.*, 2005), whereas *ljpt3-1* and *mtpt4-1* mutants demonstrated that these transporters are essential for symbiotic P_i transfer and AM development in *Lotus* (Maeda *et al.*, 2006) and *Medicago* (Javot *et al.*, 2007a), respectively. It will be interesting to see whether double and triple knock-out mutants of the tomato AM-inducible P_i transporters display the same reduced mycorrhization phenotype as members of the Fabaceae. Future characterisation of the barley RNAi transformants produced during the course of this work would provide valuable insight into the role of AM-inducible P_i transporters in barley and a valuable comparison to these published data obtained for dicotyledonous plants. I hypothesise that if HvPT8 is the sole P_i transporter involved in P_i uptake via the AM pathway in barley, then RNAi knockout of this gene will inhibit P_i transfer via this pathway and may result in a reduced mycorrhization phenotype as observed for *Medicago mtpt4-1* mutants (Javot *et al.*, 2007a). Although, since the full complement of barley PhT1 transporters has not been identified, it would not be possible to rule out cross-reaction of the RNAi construct with as yet unidentified P_i transporters with high homology to HvPT8.

8.5 Manipulation of P_i uptake in an AM plant

Constitutive over-expression of *HvPT8* in barley did not increase P_i uptake in NM plants grown in solution culture or in soil (Chapters 6 and 7). This is consistent with previous findings by Rae *et al.* (2004). As suggested in Chapter 6, the failure of plants grown at high P to accumulate more P than control plants despite the measured increase in P_i influx in transgenic plants is consistent with enhanced P_i efflux from transgenic plants, a process that is crucial for the maintenance of P_i homeostasis (Cogliatti & Santa Maria, 1990). Although P_i influx increased at low P, this was observed in both transgenic and control lines indicating that up-regulated expression of native P_i transporters is sufficient to maximise P_i uptake at low P. These data support previous suggestions (Rae *et al.*, 2004) that P_i transporter expression is not the limiting factor in P_i uptake under low P conditions, either from solution culture or soil.

Constitutive over-expression of *HvPT8* in AM barley did not affect AM colonisation or total P content compared to control AM plants (Chapter 7). As ^{32}P transfer was not detected in AM plants in this experiment the quantitative effect of increased *HvPT8* expression on P_i transfer via the AM pathway could not be assessed. However, these data provide further insight into the regulation of the direct P_i uptake pathway in AM plants. Based on data presented in Chapter 6, it is evident that the *HvPT8* transgene was expressed in the epidermis and was functional in terms of P_i uptake. If AM fungi transcriptionally down-regulate the expression of root epidermal P_i transporters, this regulatory mechanism would be by-passed by constitutive over-expression of *HvPT8*. Therefore, the growth and P response of transgenic AM plants could be expected to reflect this. Phosphate uptake via the direct pathway would be expected to increase in transgenic AM plants. As growth and P_i uptake of transgenic plants was not enhanced relative to transgenic controls these data support previous assertions (Chapters 4 and 5) that suppression of direct P_i uptake in AM plants does not occur via transcriptional regulation of P_i transporters. It would be interesting to confirm this finding using the transgenic barley plants produced by Rae *et al.* (2004) which constitutively over-express HvPT1, the native, root-epidermal P_i transporter that is normally involved in the direct P_i uptake pathway in wild-type plants.

8.6 Future work

Specific areas for future endeavour arising from the work presented herein should include:

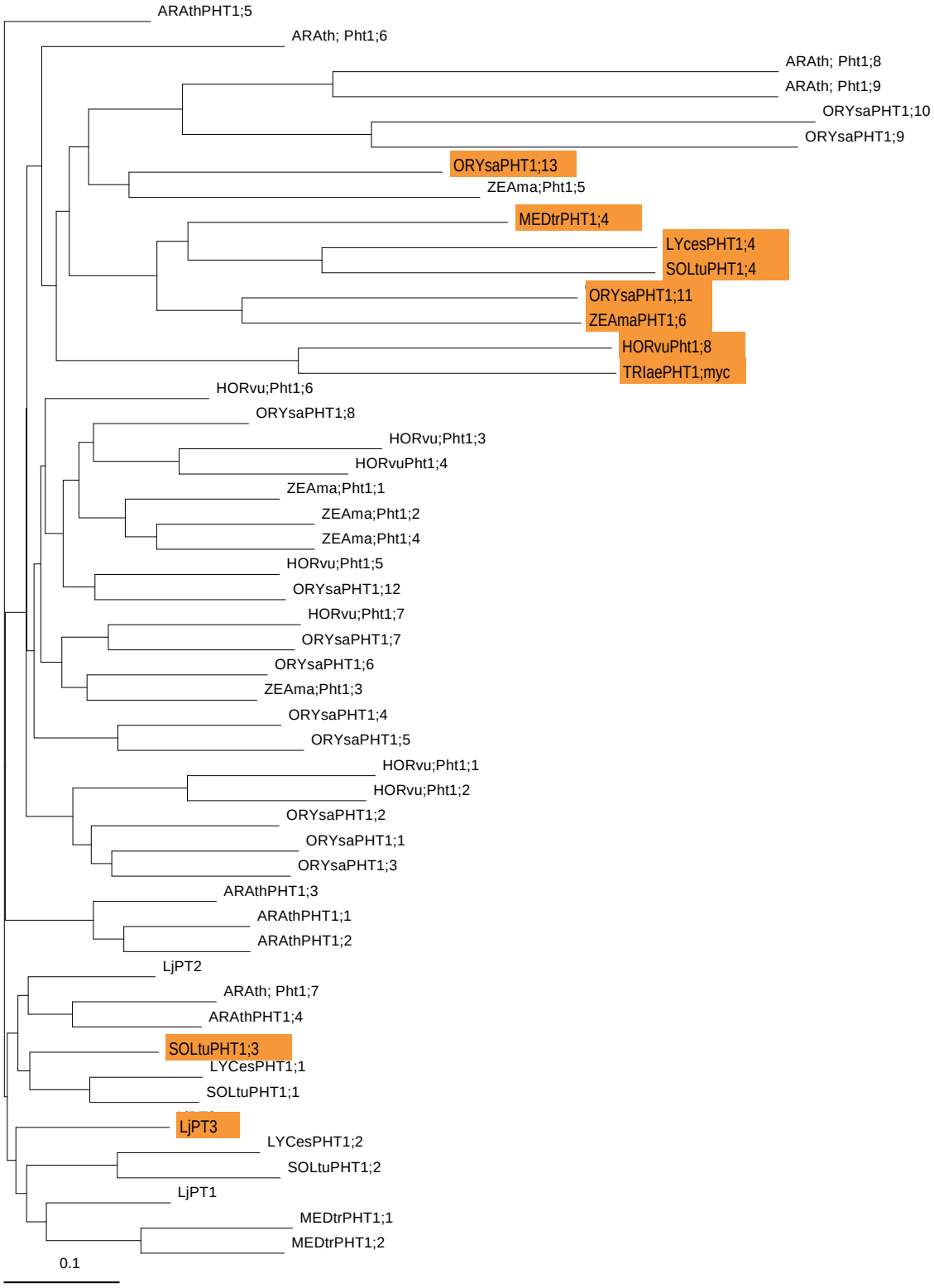
- Quantification of P_i transfer via the AM pathway in transgenic over-expressors of HvPT8, using compartmented pots incorporating ^{32}P label and including a greater range of AM fungi;
- Confirmation of the *HvPT8* expression pattern in a developmental time-course using the fluorescent reporter system developed here and assessment of the precise location of *HvPT8* expression;
- Analysis of transgenic barley lines expressing the RNAi construct to determine; a) the degree of silencing in the twenty putative transformants; b) the AM colonisation phenotype; and c) quantification of the contribution of the AM pathway using ^{32}P labelling in compartmented pots and comparison to *HvPT8* over-expressors;
- Analysis of the response of transgenic barley lines over-expressing *HvPT1* to AM colonisation (these have been kindly supplied by Dr A. Rae, CSIRO Plant Industry, Brisbane) to determine; a) the AM colonisation phenotype; and b) quantification of the contribution of direct and AM P_i uptake pathways using ^{32}P labelling.

8.7 Conclusions

The work presented in this thesis investigated the role of P_i transporters in governing P uptake in an AM plant using combined physiological and molecular approaches which, at the outset of this work, had not been previously reported. This combined approach is critical to furthering our understanding of symbiotic P_i transfer processes, particularly in non-responsive hosts. Non-responsive AM plants include some of our most widely cultivated crop species. These species present a great potential for increasing productivity and or yield through AM symbioses by breeding or engineered traits which increase their responsiveness to AM. The persistence of AM in commercially grown crops and the ability of AM fungi to alter plant nutrient uptake pathways suggest that the role of AM symbioses cannot be ignored and that attempts to characterise or enhance plant nutrient uptake without consideration of AM interactions will be flawed.

The majority of investigations of nutrient uptake and transfer in AM symbioses have focussed on identification and characterisation of genes in individual plant species or single plant:AM fungal interactions, as was attempted here. So far there has been limited focus on differences between plant and fungal species and the resultant effects on symbiotic function. Future research should include comparison between non-responsive and responsive plant species with the aim of elucidating the mechanisms underlying functional differences in these AM interactions. The question of why the direct and AM pathways of P_i uptake are not additive in non-responsive plants should be a key research focus. If the epidermal pathway is not switched off when the AM pathway is operating in a non-responsive species, I hypothesise that a positive MGR would result and agricultural benefits follow. Exploring this issue should be a focus for future research.

Appendix 1: Unrooted phylogenetic tree of PhT1 transporters



Unrooted phylogenetic tree of plant PhT1 transporters commonly referred to in this thesis. The tree was generated by alignment of full-length protein sequences in AlignX (Vector NTI Advance 10.3.0). AM-inducible P_i transporters are shaded in orange. For references see Table 1.1.

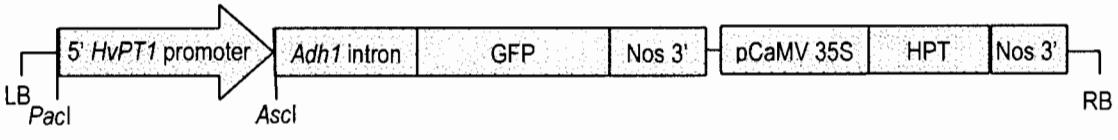
Appendix 2: Vectors used in plant transformation

pWBVec8 supplied by Dr. P Schunmann, CSIRO Plant Industry, Canberra, Australia.

NOTE: This figure is included on page 124 of the print copy of the thesis held in the University of Adelaide Library.

Originally constructed by MB Wang, pWBVec8 is a binary vector designed for cereal transformation and incorporates a hygromycin (*Hpt*) selectable marker gene under the control of the CaMV35S promoter and *Nos* terminator.

The construct supplied by Dr Schunmann contained the following cassette inserted at the *Not1* cloning site (Schunmann *et al.*, 2004).



pWBvec8 is transformed into *E. coli* or *Agrobacterium* and transformed colonies are selected on LB/spectinomycin or LB/rifampicin/spectinomycin, respectively (see Appendix 3).

pPZPUbi supplied by Dr K. Oldach, ACPFG, Adelaide, Australia.

NOTE: This figure is included on page 125 of the print copy of the thesis held in the University of Adelaide Library.

Originally constructed by Hajdukiewicz *et al.* (1994)

The vector was adapted by Dr K. Oldach to include the maize *Ubiquitin1* promoter and *Nos* terminator for constitutive over-expression in cereals. It includes spectinomycin and hygromycin resistance. GOI are cloned into the multi-cloning site (MCS) and expression is driven by the maize *Ubiquitin1* promoter.

Clones were sequenced with the PZPseqF/R primer pair.

Primer	Sequence
PZPseq F	CCATGTTATCACATCAATCC
PZPseq R	GCCCTTTTAAATATCCGTTA

pPZPUbi is transformed into *E. coli* or *Agrobacterium* and transformed colonies are selected on LB/spectinomycin or LB/rifampicin/spectinomycin, respectively (see Appendix 3).

Appendix 3: Preparation of competent cells and culture media

3.1 High-efficiency calcium chloride competent *E.coli* cells (DH5α) (Inoue *et al.*, 1990)

Frozen stock of DH5α cells was thawed on ice then 30-50 µL used to inoculate 2 x 250 ml SOB medium (see below for composition). Cultures were incubated overnight in an orbital shaker (200-250 rpm, 23°C). 250 mL SOB was added per flask, cultures were divided into 4 x 1 L flasks and incubated, shaking for a further 2-3 h. Cultures were then transferred to ice (10 min) and transferred from 2 x 1 L flasks to 6 x pre-cooled 500 mL centrifuge bottles ~1/2 full (autoclaved). These were centrifuged (6000 rpm, 10 min, 4°C). The S/N was poured off and the pellet was resuspended in 5 mL ice-cold TB (see below). Cell suspensions were combined and topped up with TB to ~half full (100 mL). This was incubated on ice for 10 min then centrifuged (6000 rpm, 10 min, 4°C). The S/N was poured off and the cell pellet was resuspended in the small volume of remaining S/N and transferred to a falcon tube. This cell suspension was made up to 20 mL with 7% DMSO in ice-cold TB (1.5 mL DMSO in 18.5 mL ice-cold TB) then incubated in an ice bath (10 min) prior to dispensing aliquots (200 µL) into pre-cooled eppendorf tubes. Eppendorf tubes were chilled immediately on dry ice and stored at –80°C for future use.

SOB Medium for the preparation of competent host cells prior to transformation; (g L^{-1}) bacto-tryptone, 20; bacto-yeast extract, 5; NaCl, 0.5; 250 mM KCl, 10 mL L^{-1} (added while stirring). Adjust pH to 7.0, make up to volume and autoclave to sterilise. Prior to use add 5 mL L^{-1} 1M $MgSO_4$ + 5 mL 1M $MgCl_2$ (filter sterilised).

TB (transformation buffer); (mM) PIPES, 10; $CaCl_2$, 15; KCl, 250, Adjust pH to 6.7 and add $MnCl_2$ (55mM). Sterilise by filtration through a pre-rinsed 0.45 µm filter. Store at 4°C.

Following transformation of DH5α according to Section 2.14.5, cells were recovered on SOC media and cultured on LB selection media prepared as follows:

SOC Media prepared as for SOB media with the addition of 20 mL L^{-1} 1M glucose.

LB (Luria–Bertani) broth; (g L^{-1}) bacto-tryptone, 10; bacto-yeast extract, 5; NaCl, 10, Adjust the pH to 7 with 5 N NaOH, adjust the volume to 1 L with distilled H_2O and sterilise by autoclaving.

LB plates Add 15 g bacto-agar prior to autoclaving

Spectinomycin was prepared by dissolving in H_2O and filter sterilising. Following autoclaving LB was cooled to 50°C and spectinomycin was added to a final concentration of 50 µg mL^{-1}.

3.2 Freeze-thaw competent *Agrobacterium tumefaciens* (AGL-1)

Stock *Agrobacterium* culture was inoculated in 5-10 mL TYNG/rifampicin (see below) and cultured overnight. A 1 mL aliquot was transferred to 30 mL TYNG/rifampicin and cultured for 4-6 h at 28^{o}C until cloudy. Cultures were chilled on ice for 10 min then centrifuged (4500 rpm, 10 min, 4^{o}C). The S/N was poured off and the cell pellet was resuspended in 500 µL ice-cold $CaCl_2$ (20 mM). Aliquots (100 µL) were transferred to pre-cooled eppendorf tubes, frozen in liquid N_2 and stored at -80^{o}C.

TYNG medium; (g L^{-1}) bacto-tryptone, 10; yeast extract, 5; NaCl, 5; $MgSO_4.7H_2O$, 0.2; Adjust the pH to 7.5, adjust the volume to 1 L with distilled H_2O and sterilise by autoclaving.

Rifampicin was prepared by dissolving in H_2O and filter sterilising. Following autoclaving TYNG was cooled to 50^{o}C and rifampicin was added to a final concentration of 50 µg mL^{-1}.

Following transformation of AGL-1 as outlined in Section 2.14.5, cells were recovered on TYNG medium and cultured on LB/rifampicin/spectinomycin selection media. The concentration of both rifampicin and spectinomycin was 50 µg mL^{-1}.

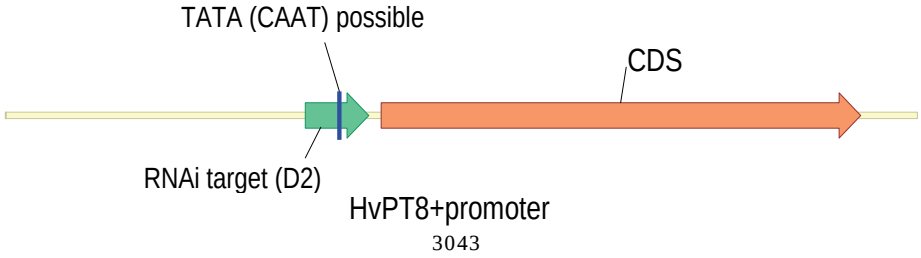
3.3 Media used in Agrobacterium-mediated transformation of barley

Callus induction medium: Based on the recipe of Wan & Lemaux (1994). This medium is composed of MS macro-nutrients (Murashige & Skoog, 1962), FHG micro-nutrients (Hunter, 1988), supplemented with 30 g L^{-1} maltose, 1 mg L^{-1} thiamine-HCl, 0.25 g L^{-1} myo-inositol, 1 g L^{-1} casein hydrolysate, 0.69 g L^{-1} L-proline, 2.5 mg L^{-1} Dicamba, 10 µM CuSO4, 95 µM hygromycin and is solidified with 3.5 g L^{-1} Phytagel (Sigma Chemicals, St. Louis, MO, USA).

Shoot regeneration medium: This medium is based on the FHG recipe of Wan & Lemaux (1994). It contains FHG macro- and micro-nutrients (Hunter, 1988), 1 mg L^{-1} thiamine-HCl, 1 mg L^{-1} BAP, 0.25 g L^{-1} myo-inositol, 0.73 g L^{-1} L-glutamine, 62 g L^{-1} maltose, 10 µM CuSO4, 38 µM hygromycin B, and is solidified with 3.5 g L^{-1} Phytagel.

Appendix 4: Constitutive RNAi knockdown of native HvPT8

The RNAi (Waterhouse *et al.*, 1998) construct targeted a gene-specific 212 bp fragment in the 5' UTR of *HvPT8*. The target region was identified with reference to published methods (Helliwell & Waterhouse, 2003; Reynolds *et al.*, 2004). The 212 bp amplification product (labelled D2 below) incorporated 114 bp upstream of the putative CAAT box and 95 bp downstream, ending 41 bp before the translation start site. NCBI BLAST searches confirmed that this region is specific to *HvPT8*. The RNAi target was amplified with the primers listed below.



Primers used in the amplification of the RNAi target.

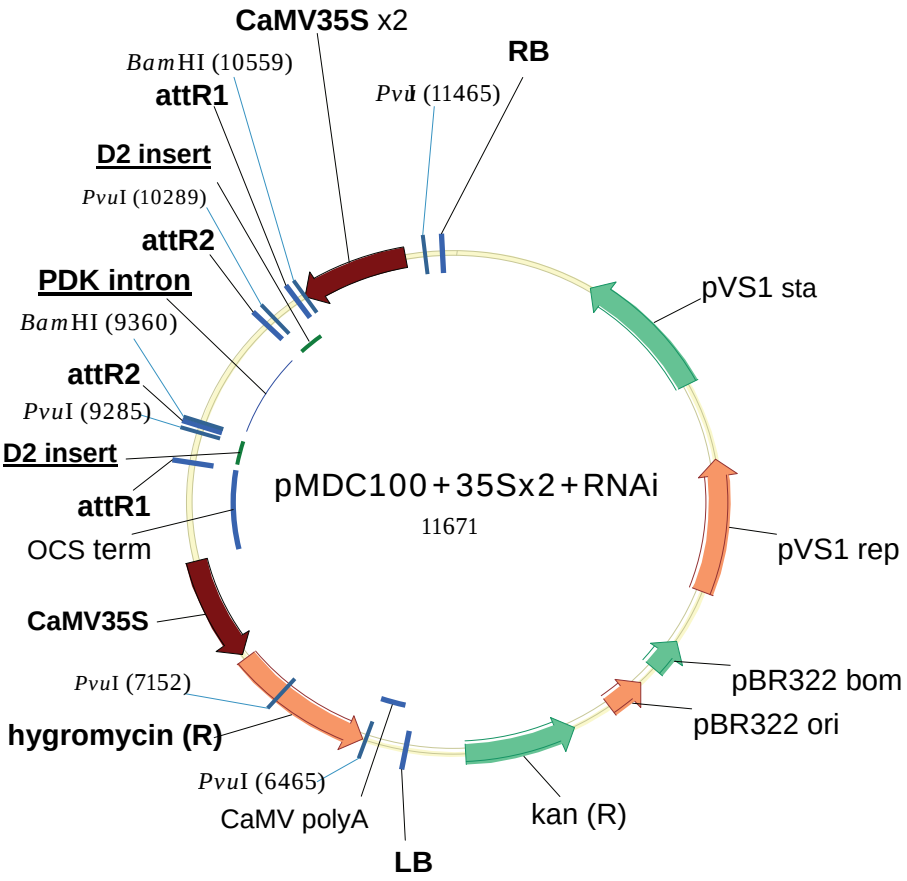
Primer	Sequence	T_m (°C)[1]
PT8RNAi2F	TTTCAGCAAGTTCATTGTCTGG	52
PT8RNAi2R	GCAAAGCAAGATTCAAGACG	58

[1]Primer T_m as reported by the manufacturer (Proligo).

The amplified fragment was cloned into the entry vector PCR8/GW/TOPO (Invitrogen, Mount Waverley, Victoria, Australia), the orientation was checked and the insert was sequenced. The insert was then Gateway cloned into a modified pMDC100 binary vector (Curtis & Grossniklaus, 2003). The original pMDC100 vector was modified by D. Plett (ACPFG, Adelaide, Australia). This modified vector is designed for preparation of double-stranded RNAi constructs and includes two attR recombination sites for Gateway recombination which are oppositely orientated and separated by a 742 bp intron from pyruvate orthophosphate dikinase (pdk) to aid in stability of the inverted repeat DNA (Wesley *et al.*, 2001). The binary vector was transformed into *E. coli* (2.14.5). Individual colonies were amplified in liquid media, plasmid DNA was isolated (2.14.6) and the vector integrity was checked by RE digestion with *Pvu*I or *BamH*I. Plasmids demonstrating the expected fragmentation pattern were sequenced to ensure errors had not been introduced. The final RNAi vector including the D2 insert is illustrated below.

The completed expression vector was transformed into *Agrobacterium* AGL-1 (Section 2.14.5) and subsequently used for the transformation of *Hordeum vulgare* cv. Golden Promise as outlined in

Section 2.14.7. Regenerated transformants were selected on antibiotic selection media for the presence of the T-DNA. Twenty putative independent lines were regenerated, transferred to commercial grade potting mix and grown to maturity in a glasshouse facility. The resultant T1 seed was harvested and awaits analysis.



Structure of the completed RNAi vector for plant transformation. The attR1 and attR2 gateway recombination sites are indicated. The RNAi target, designated 'D2 insert', was directionally cloned so that the sense and antisense fragments were situated at either end of the pyruvate orthophosphate dikinase intron (PDK).

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References

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