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ORIGINAL ARTICLE

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Cereal phosphate transporters associated with the mycorrhizal pathway of phosphate uptake into roots

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A very large number of plant species are capable of forming symbiotic associations with arbuscular mycorrhizal (AM) fungi. The roots of these plants are potentially capable of absorbing P from the soil solution both directly through root epidermis and root hairs, and via the AM fungal pathway that delivers P to the root cortex. A large number of phosphate (P) transporters have been identified in plants; tissue expression patterns and kinetic information supports the roles of some of these in the direct root uptake pathways. Recent work has identified additional P transporters in several unrelated species that are strongly induced, sometimes specifically, in AM roots. The primary aim of the work described in this paper was to determine how mycorrhizal colonisation by different species of AM fungi influenced the expression of members of the *Pht1* gene families in the cereals *Hordeum vulgare* (barley), *Triticum aestivum* (wheat) and *Zea mays* (maize). RT-PCR and in-situ hybridisation, showed that the transporters *HORvu;Pht1;8* (AY187023), *TRIae;Pht1;myc* (AJ830009) and *ZEAma;Pht1;6* (AJ830010), had increased expression in roots colonised by the AM fungi *Glomus intraradices*,*Glomus* sp. WFVAM23 and *Scutellospora calospora*. These findings add to the increasing body of evidence indicating that plants that form AM associations with members of the Glomeromycota have evolved phosphate transporters that are either specifically or preferentially involved in scavenging phosphate from the apoplast between intracellular AM structures and root cortical cells. Operation of mycorrhiza-inducible P transporters in the AM P uptake pathway appears, at least partially, to replace uptake via different P transporters located in root epidermis and root hairs.

Keywords P acquisition · AM colonisation · *Glomus* · *Scutellospora* · *Hordeum vulgare* · *Triticum aestivum* · *Zea mays*

Abbreviations P: Phosphate · AM: Arbuscular mycorrhizal · sp: Species · RT-PCR: Reverse transcriptase polymerase chain reaction

Introduction

Many soils worldwide are phosphate (P) deficient, which has encouraged the use of fertilisers for profitable agricultural production. However, the inefficiencies, expense and environmental issues associated with high fertiliser use have led to a search for technologies that improve P uptake and utilisation by plants, including identification and manipulation of genes involved in P uptake. P is absorbed from the soil solution, where it is present at very low concentrations (usually <10 μM, Mimura 1999; Schatchtman et al. 1998). Plants have evolved several strategies which improve P acquisition, including alterations in root morphology, modification of the soil chemistry around the roots (e.g. by lowering pH or exudation of organic anions) and by the formation of symbiotic associations with arbuscular mycorrhizal (AM) fungi (Comerford 1998; Lynch and Brown 1998; Smith and Read 1997; Trolove et al. 2003).

Arbuscular mycorrhizal symbioses are arguably the most common underground symbiosis, with around 80% of terrestrial plant species potentially able to associate with members of the fungal phylum Glomer-

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omycota, in which AM fungi are now included (Schüssler et al. 2001). Although not always symbiotic, the mutualistic nature of the associations between plants and AM fungi is based largely on the exchange of nutrients, whereby the plant receives soil-derived mineral nutrients (particularly P) from the fungus, while the fungus receives organic carbon compounds from the plant (Smith and Read 1997). AM plants are therefore potentially able to acquire P via two pathways, directly via root epidermal cells and root hairs and via the AM fungi, which deliver P directly to the root cortex. The relative contributions of these two pathways to whole plant uptake and the roles of different plant P transporters in them have only recently begun to be explored in any detail (Smith et al. 2003, 2004).

Where two pathways are possible it has been assumed that the AM fungus makes little contribution and that the direct uptake pathway via root epidermis and root hairs is able to supply all the needs of the plant (Smith et al. 2003). However, use of isotopically labelled P supplied to the external AM mycelium has demonstrated a considerable contribution of the AM pathway to P uptake in both barley (*Hordeum vulgare*, Zhu et al. 2003) and wheat (*Triticum aestivum*, Schweiger and Jakobsen 2000) as well as to other non-responsive species (Smith et al. 2003; 2004). Quantification of AM P uptake in three non-cereal species indicated that the contribution could be very high and was not directly related either to extent of AM colonisation or, more importantly, to the mycorrhizal response of the plants in terms of growth or total P uptake. Non-responsive tomato (*Lycopersicon esculentum*), as well as highly responsive medic (*Medicago truncatula*) and flax (*Linum usitatissimum*) received up to 100% of their P via the AM pathway, although the percentage was strongly influenced by the fungal species (Smith et al. 2003, 2004). These findings are particularly important to agricultural practices since, based on our earlier understanding, the role of AM fungi in non-responsive crops such as many cereals has often been dismissed as being unimportant.

Quantification of the importance of mycorrhizal P uptake has occurred in parallel to the identification of plant P transporters likely to be associated with operation of the mycorrhizal P uptake pathway in a number of plant species. P transporters specifically expressed, strongly upregulated or showing an altered pattern of expression in mycorrhizal roots have been identified in cereals (*Oryza sativa*; Paszkowski et al. 2002), members of the Solanaceae (*Solanum tuberosum*, Rausch et al. 2001; *L. esculentum*, Rosewarne et al. 1999; *Petunia hybrida*, Karandashov et al. 2004) and Leguminoseae (*M. truncatula*, Harrison et al. 2002; *Lotus japonicus*, Karandashov et al. 2004). The expression of *ORYsa;Pht1;11* in rice roots (demonstrated by real-time RT-PCR) was related to the presence of internal mycorrhizal structures and presumed to be involved with delivering P to the plant (Paszkowski et al. 2002). In other plants various techniques have been used to show localisation of expression to plant cells containing intracellular fungal structures. Immuno-localisation of MEDtr;Pht1;4 was clearly to the plant membrane surrounding arbuscules formed by *G. versiforme* in roots of medic (Harrison et al. 2002). Transformation of potato with the promoter of *SORtu;Pht1;3* fused to GUS (β-glucuronidase, Jefferson 1987) demonstrated expression of this gene, again in arbuscule-containing cells (Rausch et al. 2001). Subsequent work indicates that expression is also associated with intracellular AM hyphal coils and not influenced by pathogen infection (Karandashov et al. 2004). Although the gene is very strongly induced in AM roots, expression can also be detected in shoots; this gene is not therefore completely mycorrhiza-specific (Karandashov et al. 2004). *LYCes;Pht1;1* is expressed in both AM and non-mycorrhizal tomato roots, but localisation of expression by in situ hybridisation with RNA probes was apparently changed from the epidermis and root hairs in non-mycorrhizal roots (Daram et al. 1998) to cortical cells containing arbuscules (Rosewarne et al. 1999), suggesting involvement of this gene in mycorrhizal P uptake.

Sequence comparisons of these mycorrhiza-regulated genes indicate that although those from tomato and Medic are similar, the rice gene *ORYsa;Pht1;11* is at a considerable evolutionary distance from them (Paszkowski et al. 2002). Given the synteny between rice and other cereals such as barley and wheat it might be expected that they would also possess mycorrhiza-regulated genes and that these would have close sequence similarity to the rice transporter. Eight genes in the *Pht1* P transporter family have been identified in barley. *HORvu;Pht1;1*,*1;2* and *1;3*/*1;4* are expressed almost exclusively in roots and expression is strongly reduced when plants are grown in high P hydroponic solutions, compared to low P solutions (Schünmann et al. 2004; Smith et al. 1999). *HORvu;Pht1;6* is expressed in shoots of non-mycorrhizal plants (Rae et al. 2003), no information on the expression of other members of the gene family is available, including relation to AM fungal colonisation.

The work described in this paper assessed the expression and localisation of eight barley P transporters (*HORvu;Pht1;8*), one wheat P transporter (*TRIae;Pht1;myc*) and one maize P transporter (*ZEAma;Pht1;6*) in roots to determine their potential involvement in P acquisition via the 'mycorrhizal' P pathway. We capitalised on the availability of constructs of the promoter regions of *HORvu;Pht1;1* and *2* linked to GFP (Schünmann et al. 2004) to assess tissue localisation of expression in transformed barley plants. RT-PCR, real time PCR and in-situ hybridisation were used to investigate expression both of these genes and those for which no GFP-linked constructs were available.

Materials and methods

Soil, soil mixes and phosphorus amendments

Two soils, Millmerran and Ashland, were used to provide conditions suitable for colonisation of plants by the

different AM fungi (Dickson et al. 1999). Soil from Millmerran (Queensland, Australia; ~28°S 151°E) had a pH of 8.5 (H_2O) and bicarbonate extractable P content of approximately 10 mg kg^{-1} (Colwell 1963). The soil from Ashland (Withcott, Queensland, Australia; ~ 28°S 153°E) had a pH of 5.6 (H_2O) and bicarbonate extractable P content of approximately 10 mg kg^{-1} (Colwell 1963). Washed fine quartz sand was mixed with the soil in the ratio of 1 part soil to 9 parts sand. The soil:sand mixes were then sterilised by autoclaving at 121°C for 90 min twice, with an interval of 72 h between autoclavings. Non-draining pots were filled with 2 kg of the soil:sand mix. Two levels of P were prepared: 20 mg and 118 mg kg^{-1} soil:sand mix added as a dry fine powder of $CaH_4(PO_4)_2{\cdot}H_2O$ and mixed thoroughly. These are designated low P and high P treatments (personal communication, FW Smith). Three treatments were established: non-mycorrhizal low P, non-mycorrhizal high P and mycorrhizal low P. Mycorrhizal treatments were established by adding 200 g of soil/root inoculum to 1.8 kg low P pots to give a total 2 kg pot.

Cultures of AM fungi

Three AM fungi were used: *Glomus* sp. WFVAM 23 (formerly called *G. versiforme* see Gao et al. 2001), *Glomus intraradices* Schenck and Smith (DOAM 181602) and *Scutellospora calospora* (Nicolson and Gerdemann) Walker and Sanders (WUM12(2)). *Glomus* sp. WFVAM23 and *G. intraradices* were maintained on *Allium porrum* L. cv Vertina (leek) in Millmerran soil, as they require neutral to alkaline pH soil for successful colonisation, and *S. calospora* was maintained on leek in Ashland soil, as successful colonisation by this fungus occurs in low pH soils (Dickson et al. 1999). These pots are known as nurse pots. Soil/root inoculum was from nurse pots grown for 8 weeks. Barley, wheat or maize plants required to be colonised by AM fungi were grown in established nurse pots to ensure a high percentage of colonisation.

Plants, plant propagation, growth conditions and harvesting

Barley cv. Golden Promise, wheat cv Grebe (wheat) and maize cv Gold Queen (maize) were used in the experiments reported here. Seeds were sterilised by immersion in a bleach solution containing ~5.7% available chlorine for 10 min, followed by three washes in sterile H_2O. Seeds were germinated between two sheets of filter paper (30×30 cm) soaked in 0.5 mM $CaSO_4$, incubated at 4°C for 24 h, then moved to ~26°C under growth lights until germination. Seeds usually germinated in 5 days and were then transplanted into pots or established nurse-pots, one plant per pot.

All plants, except those for production of immature embryos, were grown in the Controlled Environment Facility at CSIRO, St Luica, Queensland, Australia. Growth room conditions were the same as for immature embryo production except that the temperature was 25°C.

Pots were watered to weight with distilled water twice per week to maintain soil moisture at 0.1 g g^{-1} dry soil. This water content represents the amount of water retained by this soil/sand mix after wetting it up and allowing it to free-drain overnight under conditions that prevented evaporative loss. Pots received 10 mL nutrient solution minus P once per week (0.4 mM K_2SO_4, 0.3 mM $MgSO_4{\cdot}7H_2O$, 0.6 mM $Ca(NO_3)_2$, 0.02 mM FeEDTA, 0.8 mM $(NH_4)_2SO_4$, 0.4 mM $NaNO_3$, and 1 mL of the following H_3BO_3 (2.8 mg L^{-1}), $MnCl_2.4H_2O$ (1.81 mg L^{-1}), $ZnSO_4.7H_2O$ (0.22 mg L^{-1}), $CuSO_4.5H_2O$ (0.08 mg L^{-1}) and $NaMoO_4.2H_2O$ (0.025 mg L^{-1})).

Analysis of mycorrhizal colonisation

Root samples for assessment of mycorrhizal colonisation were cleared in KOH and stained with trypan blue by a modification of the method of Phillips and Hayman (1970), that eliminated the use of phenol (Cavagnaro et al. 2001). Assessment of colonisation was done by the magnified line intersect method (McGonigle 2000).

Phosphate analysis

Weighed subsamples of dried root material were ashed at 220°C for ~20 h. Ashed samples were resuspended in 20 mL 0.1N HCl. P concentrations in extracts were detemined by the molybdenum blue method of Watanabe and Olsen (1965), with absorbances read at 650 nm on a GBC 911 UV-Visible Spectrophotometer (GBC Scientific Equipment, Dandenong, Victoria, Australia).

RNA extractions

Large-scale RNA preparations were extracted from plant material through a caesium chloride pad by the method of Chirgwin et al. (1979). Small-scale RNA preparations utilised the QIAGEN RNeasy Plant Mini Kit, supplied by QIAGEN Pty Ltd (Clifton Hill, Victoria, Australia). The small-scale extractions included the QIAGEN 'on the column' RNase-Free DNase in order to remove any remaining genomic DNA.

Production of *HORvu;Pht1;1* and *HORvu;Pht1;2* transgenic barley plants

Plasmids containing the promoter region of barley P transporters, *HORvu;Pht1;1* or *HORvu;Pht1;2* controlling expression of green fluorescent protein gene (GFP) were provided by Dr P H D Schünmann (CSIRO Plant

Industry, Canberra, Australia) (Schünmann et al. 2004). These constructs were transformed into *Agrobacterium tumefaciens* AGL1 by electroporation (Bio-Rad Gene Pulser) and transformed cells were selected on YEP media (Chanda et al. 1994) containing rifampicin and spectinomycin.

Barley (cv Golden Promise) grown for transformation studies was grown in 2 kg draining pots filled with Searles Peat 80 potting mix and fertilised with Osmocote (pots, planters and indoors 6 month controlled release, as per manufacturers recommendations, Scotts Australia Pty Ltd, Baulkham Hills, New South Wales, Australia). These pots of barley were grown in a plant growth room at 12°C with 16 h photoperiod (500 μmol m^2 s^{-1} photon flux density) and 65% humidity. Immature embryos isolated from developing grains (Patel et al. 2000) were transformed by co-cultivation with *A. tumefaciens* strain AGL1 harbouring binary plasmids (Tingay et al. 1997). Transformed embryonic calli was selected by resistance to hygromycin and regenerated plantlets were grown for up to 8 weeks before being transferred to soil and grown to maturity. Plantlets (T_0) regenerated from a single transformed embryonic callus (line) were classified as replicates.

Reporter gene analysis of transgenic barley

Core samples of roots were taken and washed to remove soil prior to visualisation. GFP fluorescence was observed using a Leica MZ6 dissecting microscope with the GFP PLUS fluorescence module and a Leica TCS SP2 Confocal System on an upright Leica DMRXE microscope for Laser Scanning Confocal Microscopy (Leica AG, Heerbrugg, Switzerland).

RT-PCR analysis of barley P transporters in mycorrhizal and non-mycorrhizal roots

RNA was extracted from root core samples as described above. Approximately 5 μg of total RNA was used as a template for first-strand cDNA synthesis, using random primers and a Superscript First Strand cDNA Synthesis Kit (Invitrogen, Invitrogen, California, USA) according to the manufacturer's instructions.

One μL of first strand cDNA was then used for PCR using gene-specific primers for each of the 8 barley P transporters and wheat and maize P transporters studied. Primers specific for 6 of the 8 barley *Pht1* P transporters, their sequences, $MgCl_2$ concentrations and expected RT-PCR products are given in Table 1. *HORvuPht1;3* and *1;4* show 100% homology throughout the coding region so the primers will amplify both genes. All primers were tested with gDNA (from the appropriate plant), at various $MgCl_2$ concentrations, prior to RT-PCR analysis. PCR was performed with EXPAND High Fidelity polymerase (Roche Diagnostics Australia, Castle Hill, New South Wales, Australia) according to the manufacturer's instructions. Thermal cycling consisted of an initial denaturation at 94°C for 5 min, followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 60 s, and then an additional 20 cycles during which the extension time was increased by 5 s per cycle, followed by a final extension at 72°C for 7 min. RT-PCR reactions were visualised on 1.2% agarose gels (Sambrook et al. 1989).

Identifying and cloning 'mycorrhizal' P transporters from wheat and maize

Primers designed to *HORvu;Pht1;8* gene were used to amplify a homologue from wheat in RT-PCR analysis. The RT-PCR amplification: primers, sense 5′ ATGGCACGGCAGCAGCTGCA and anti-sense 5′ CTACAAGGGAAGAACCTTGG, $MgCl_2$ concentration of 2.5 mM and an extension time of 3 min in the thermal cycle program. Following amplification the PCR product was cloned into pGEMT-easy (Promega, Annandale, New South Wales, Australia), transformed into *E. coli* DH10B by electroporation in 0.1 cm cuvettes, sequenced with ABI PRISM® Big Dye™ Terminator Ready Reaction Cycle Sequencing Kit version 3 (Applied Biosystems, Quantum Scientific, Milton, Queensland, Australia) and sequence analysis with programs on the Australian National Genomic Information Service (http://www.angis.org.au). The same procedure was used to clone the maize P transporter with the following primers: sense 5′ GACACCTGCCTTACATTGCC and anti-sense 5′ CTGCTTACTCGATCACGCATGC.

Pht1 Family Topology

The computer program TMHMM version 2, available on http://www.cbs.dtu.dk/services/TMHMM-2.0/, was used to determine the 2-D topology of HORvu;Pht1;8, TRIae;Pht1;myc and ZEAma;Pht1;6.

Real-Time RT-PCR analysis of *HORvu;Pht1;1*,*HORvuPht1;2* and *HORvu;Pht1;8*

Samples were prepared as for RT-PCR analysis and a 'no-RT' control for each sample was included to detect the presence of gDNA. The first strand cDNA synthesis involved two reactions, with and without the addition of RT-polymerase.

The Taqman Sequence Detection System 7700 (Applied Biosystems, Victoria, Australia) and corresponding computer programs were used for the design of primers (Table 1), real-time RT-PCR reaction and detection of product. The SYBR Green PCR Master Mix, Micro-Amp 96-well reaction plate and Optical Caps were supplied by Applied Biosystems. The PCR reactions were set up according to the SYBR Green PCR