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Functional characterisation and transcript analysis of an alkaline phosphatase from the arbuscular mycorrhizal fungus *Funneliformis mosseae*

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ABSTRACT

Alkaline phosphatases (ALP) in arbuscular mycorrhizal (AM) fungi have been suggested to be involved in transfer of phosphate from the mycorrhizal fungus to the host plant, but exact mechanisms are still unknown, partially due to the lack of molecular information. We isolated a full-length cDNA (Fm*ALP*) from the AM fungus *Funneliformis mosseae* (syn. *Glomus mosseae*) showing similarity with putative ALP genes from *Rhizophagus intraradices* (syn. *Glomus intraradices*) and *Gigaspora margarita*. For functional characterisation Fm*ALP* was expressed heterologously in the yeast *Pichia pastoris*. The recombinant FmALP protein had a pH optimum of 9.5, and catalysed the hydrolysis of glycerolphosphate and, to a lesser extent of glucose-1- and 6-phosphate, confirming it to be an alkaline phosphatase belonging to the family of alkaline phosphomonoesterases (EC 3.1.3.1). FmALP did not catalyse the hydrolysis of ATP or polyP. Relative Fm*ALP* transcript levels were analysed in intra- and extraradical hyphae isolated from *F. mosseae* infected ryegrass (*Lolium perenne*) using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Fm*ALP* was highly expressed in intraradical hyphae at low P_i supply, and its expression was repressed by high P_i supply. Taken together this study provides evidence for mycorrhizal alkaline phosphatases playing a role in P mobilisation from organic substrates under P starvation conditions.

1. Introduction

Arbuscular mycorrhizal (AM) fungi form symbiotic associations with most land plants and promote growth of their hosts through enhanced uptake of nutrients, particularly phosphorus (P) in exchange for carbon and energy (Pfeffer et al., 1999; Smith and Read, 1997). AM fungi take up inorganic phosphate (P_i) from soil through extraradical hyphae and transfer the P_i to the host plants (Smith and Gianinazzi-Pearson, 1988; Smith and Read, 1997). P_i efflux from interfacial arbuscules, distinctive organs formed in mycorrhizal roots, has been shown to be catalysed by mycorrhiza-specific P_i transporters that are localised around arbuscules (Harrison et al., 2002; Paszkowski et al., 2002; Rausch and Bucher, 2002). Nevertheless, the mechanisms of P metabolism in AM fungi, particularly in intraradical tissues, are still speculative. It is generally thought that the P_i absorbed from soil by AM fungi via extraradical hyphae is condensed into polyphosphate (polyP) and translocated to intraradical mycelia, then hydrolysed and released at interfacial arbuscules (Cox et al., 1980; Ezawa et al., 2003; Funamoto et al., 2007; Javot et al., 2007).

Alkaline phosphatase (ALP) in AM fungi is an enzymatic marker specific to the symbiotic status (Saito, 1995) and localised in intraradical hyphae and fine structural (mature) arbuscules (Ezawa et al., 1995; Gianinazzi et al., 1979). Histochemical evidence of strong ALP activity in intraradical tissues has been seen to be indicative of ALPs from AM fungi to be involved in P_i efflux processes from arbuscules (Ezawa et al., 1995; Gianinazzi et al., 1979) and intraradical hyphae (Kojima and Saito, 2004). It has been speculated that ALPs might be involved in hydrolysis of polyP (Funamoto et al., 2007) and organic phosphorus compounds (Kojima and Saito, 2004) in intraradical mycelia. ALPs might also be involved in soil organic P mineralisation by extraradical hyphae (see review Joner et al., 2000). However, biochemical studies revealed that the ALPs in AM fungi were most likely involved in metabolism of organic phosphomonoesters rather than other phosphate compounds (such as polyP or ATP). For example, an earlier study by Gianinazzi-Pearson and Gianinazzi (1978) showed that *Funneliformis mosseae* specific ALP, which was biochemically isolated from *F. mosseae* (syn. *Glomus mosseae*; Schüßler and Walker, 2010) infected onion roots, had a marked affinity for naphthyl- and phenyl-phosphates and glycerolphosphate, but it was incapable of breaking down other complex phosphate esters. Ezawa et al. (1999) showed that ALP extracted from *Glomus etunicatum* intraradical hyphae had increased affinity for sugar phosphates and did not hydrolyse polyP. These controversies regarding ALP

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catalysed hydrolysis of P compounds in the mycorrhizal symbiosis have hindered our understanding of the physiological function of ALPs in AM fungi, and the identification and characterisation of ALP genes from AM fungi might shed more light on their biological function.

Alkaline phosphatases are found in organisms from all three kingdoms and ALP genes from several species have been molecularly characterised. For instance, the ALPs in *Saccharomyces cerevisiae* and *Escherichia coli* have been characterised for their substrate specificities and kinetic parameters (Coleman, 1992; Kaneko et al., 1987). In contrast, molecular information for ALP genes from symbiotic AM fungi is relatively sparse. Currently, there are only two putative ALP gene sequences reported from the AM fungi *Rhizophagus intraradices* (syn. *Glomus intraradices*; Gi*ALP*; Accession no. AB114298) and *Gigaspora margarita* (Gm*ALP*; Accession no. AB114299) (Aono et al., 2004), but their enzymatic activities have not been confirmed so far. Here, we report a fungal ALP gene isolated from ryegrass roots infected with the AM fungus *F. mosseae* (Fm*ALP*) using RACE (Rapid Amplification of cDNA ends) techniques. We heterologously expressed the FmALP protein in the *Pichia pastoris* expression system (Cregg et al., 2009; Macauly-Patrick et al., 2005) and analysed substrate specificities of the recombinant FmALP protein. We also analysed the expression of Fm*ALP* in intra- and extraradical hyphae of *F. mosseae* infected ryegrass (*Lolium perenne*) grown at low and high P_i supply in a three compartment system (Chen et al., 2001) using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

2. Materials and methods

2.1. Isolation of F. mosseae alkaline phosphatase mRNA (FmALP)

To prepare cDNA libraries, total RNA was isolated from *L. perenne* roots inoculated with *F. mosseae* (INVAM collection strain UK115, USA) using TRIzol (Invitrogen NZ Ltd., Auckland, NZ). RACE cDNA libraries were synthesised following the manufacturer's recommendations using the SMART™ RACE cDNA Amplification Kit (Clontech, Norrie Biotech, Auckland, NZ). Fm*ALP* specific primers (5′-AAGCATCGACCTCCACCAAACA-3′; 5′-CCGTATCACCATGCTACACCAGCTTC-3′) were designed based on the *R. intraradices* Gi*ALP* mRNA sequence (Accession no. AB114298; Aono et al., 2004) and used to amplify 5′- and 3′-fragments of Fm*ALP* cDNAs using the Advantage 2 PCR Kit (Clontech, Norrie Biotech, Auckland, NZ). The PCR products of 5′- and 3′-end fragments were cloned (TOPO TA Cloning kit, Invitrogen NZ Ltd., Auckland, NZ) and sequenced (Allan Wilson Centre, Massey University, Palmerston North, NZ). The full length mRNA sequence was validated further by PCR using primers designed to the 5′- and 3′-termini of Fm*ALP* (TripleMaster® PCR System, Eppendorf, Thermo Fisher Scientific Inc., Auckland, NZ). The mRNA sequence of Fm*ALP* has been deposited in the NCBI database (Accession number JX997747).

Bioinformatics analyses of the FmALP protein sequence were performed with the software programmes BlastP (Altschul et al., 1990) and ClustalW (Larkin et al., 2007). The secondary protein structure was determined using a PROSITE programme of Expasy and TMpred (Sigrist et al., 2002). Phylogenetic analysis of fungal ALP proteins was performed using NCBI Blast pairwise alignments by the neighbour joining method (Fig. S1).

2.2. Heterologous expression of FmALP protein in P. pastoris

For heterologous expression of FmALP in *P. pastoris* we used the EasySelect™ *Pichia* Expression Kit (Invitrogen, Auckland, NZ). To clone Fm*ALP* into the *pPICZα-B* expression vector, the sequence was amplified with the forward primer 5′-AGctgcagAATCGGATCCAGCAGAAGC-3′ and the reverse primer 5′-GTtctagaTCAATGTGAGTGTGAGGGTGATG-3′ (lower case letters indicate *PstI* and *XbaI* restriction sites, respectively), and fused in-frame with the α-factor secretion signal of the vector for extracellular expression. The *pPICZα-B* expression vector contains the promoter of alcohol oxidase 1 (AOX1) to drive heterologous expression in *Pichia* upon induction with methanol. The vector also contains a c-myc epitope and a hexameric His-tag between the *Xba1* site and the stop codon, potentially allowing the detection of the expressed recombinant protein using c-myc epitope-specific antibodies, and the purification of the recombinant protein using Ni-columns. The correct insertion of Fm*ALP* was confirmed by sequencing. The *PemI*-linearised plasmids of the empty vector *pPICZα-B* (control) and the recombinant Fm*ALP*/*pPICZα-B* constructs were transformed into the *P. pastoris* strains X-33 or GS115 by electroporation according to the manufacturer's instructions. These strains are Mut + phenotypes showing high growth in the presence of methanol due to the insertion of the plasmid 5′ to the intact AOX1 locus and the gain of P_{AOX1} (for details see the manual from the EasySelect™ *Pichia* Expression Kit).

To express FmALP protein, cells from a single colony of *P. pastoris* containing either the empty vector *pPICZα-B* or Fm*ALP*/*pPICZα-B* were inoculated into 500 ml liquid BMGY medium (1% yeast extract, 2% peptone, 1% glycerol, 400 μg/l biotin, 0.1 M potassium phosphate, 1.34% yeast nitrogen base (YNB), pH 6.0), respectively, and the cultures were grown at 30 °C with vigorous shaking overnight. After the cultures reached an OD_{600nm} of approx. 2, the cells were collected (centrifuged 5 min at 1500*g*) and resuspended in 500 ml with an OD_{600nm} of up to 1 in BMMY medium (1% yeast extract, 2% peptone, 400 μg/l biotin, 0.1 M potassium phosphate, 1.34% YNB, pH 6.0) containing 0.5% (v/v) methanol as an inducer and grown in the same conditions as described above. In order to maintain the induction, absolute methanol was fed daily to 0.5% (v/v) for 2 days, after which culture supernatants and cells were collected as described above. We monitored the protein expression level by assaying ALP activity against *p*-nitrophenyl phosphate substrate (PNPP, Sigma–Aldrich NZ Ltd., Auckland, NZ) and by analysing proteins isolated from subsamples by SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis).

2.3. Isolation of recombinant FmALP protein

In the present experiment ALP activity was much higher in the intracellular protein fraction from cell lysates compared to its activity in excreted protein fractions from culture supernatants (for details see Section 3.2). We therefore used the unpurified crude intracellular proteins for subsequent enzymatic assays of FmALP activity with proteins of *P. pastoris* transformed with the empty vector (eV) isolated as controls.

To isolate proteins, cells of *P. pastoris* expressing FmALP or eV were harvested by centrifugation (1500*g*, 5 min). One part of the cell pellet was directly lysed using breaking buffer (50 mM Na_2HPO_4 pH 7.4, 1 mM EDTA, 5% glycerol) as suggested in the EasySelect™ *Pichia* Expression kit, and the obtained recombinant protein was used to assay FmALP activity against PNPP substrate. The other part of the cell pellet was pre-washed using sterile distilled H_2O to remove phosphate originating from the BMMY culture medium with the P_i absence in the cell solution monitored by determining PO_4^{3-} concentration using the colorimetric ascorbic acid method (Murphy and Riley, 1962). The washed cells were then lysed in PO_4^{3-}-free lysis buffer (25 mM Tris/HCl pH 7.5, 1 mM EDTA, 0.1% Triton X-100, and 2 mM $MgCl_2$), and proteins were collected and used to assay FmALP activity against other phosphate-releasing substrates (see Section 2.5). In order to minimise the effect of untargeted small (<10 kDa) protein molecules, the obtained recombinant proteins were further concentrated using an Amicon®