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Detection of the 3-phosphoglycerate kinase protein of *Glomus mosseae*

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Abstract The *Glomus mosseae* 3-phosphoglycerate kinase (*PGK*) gene encodes a polypeptide of 416 amino acids. A synthetic peptide was designed to the C-terminus of the polypeptide for the production of a polyclonal antibody. The antibody was tested against the synthetic peptide in an immuno-dot blot and was then used to investigate the asymbiotic and symbiotic accumulation of the PGK protein. Western blot analysis revealed that a polypeptide of approximately 45 kDa accumulated in *G. mosseae*-colonised tomato roots; this is similar to the theoretical molecular weight of 44.764 kDa. The protein was not detected in non-mycorrhizal roots. Quantitative immuno-dot blotting revealed that the polypeptide accumulated in germinating spores and hyphae of *G. mosseae* and also in tomato roots colonised by *G. mosseae.* The amount detected in the mycorrhizal root system was significantly higher than that found in germinating sporocarps. The variation in the levels of glycolytic activity in the symbiotic and asymbiotic developmental stages of *G. mosseae* is discussed.

Key words *Glomus mosseae* · Phosphoglycerate kinase · Polyclonal antibody

Introduction

Arbuscular mycorrhizal (AM) fungi (Order Glomales) are an integral and important component of most terrestrial ecosystems (Harley and Smith 1983). In the past, molecular study of fungal genes expressed during asymbiotic and symbiotic stages of development was hampered by lack of knowledge of the physiology and reproductive strategies of these fungi. The problem was further compounded by the inability to grow these fungi in culture independently of the host plant. Over the past few years, there has been a concerted effort to isolate and study genes from AM fungi (Kaldorf et al. 1994, 1998; Harrison and Van Buuren 1995; Franken et al. 1997; Burleigh and Harrison 1998; Harrier et al. 1998; Butehorn et al. 1999; Lanfranco et al. 1999a, b; Requena et al. 1999; Ferrol et al. 2000). However, study of specific proteins corresponding to functional metabolic genes from AM fungi utilising antibodies has been limited. Antibodies to fungal proteins have been used in attempts to identify fungal species (Aldwell et al. 1983, 1985; Kough et al. 1983; Wilson et al. 1983; Aldwell et al. 1985; Wright et al. 1987; Hahn et al. 1993; Hahn and Hock 1994; Göbel et al. 1995) and/or to detect AM fungi in plant root and soil systems (Aldwell and Hall 1986; Wright and Morton 1989; Friese and Allan 1991; Cordier et al. 1996). There is, however, a lack of data on proteins corresponding to functional metabolic genes (Anstrom et al. 1994; reviewed in Hahn et al. 1994; Gianinazzi-Pearson and Gianinazzi 1995) The majority of research has focussed on antibodies to determine structural modifications related to infection and development. With the advent of molecular biology and the isolation of AM fungal genes, opportunities are now available for the production of polyclonal antibodies to specific AM fungal proteins.

In a previous study, the cDNA of the 3-phosphoglycerate kinase (*PGK*) gene was isolated from the AM fungus *Glomus mosseae* (Harrier et al. 1998). Expression studies utilising reverse transcriptase/polymerase chain reaction (RT-PCR) successfully detected the *PGK* gene transcript from germinated spore tissue and *G. mosseae*-colonised plant root tissue. However, the detection of an mRNA transcript corresponding to a gene of interest does not indicate that a functional protein is synthesised. Therefore, in this present study we aimed to produce a polyclonal antibody targeted to the

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G. mosseae PGK (GmPGK) protein and to establish whether it was also present in the asymbiotic and symbiotic stages of *G. mosseae* development. Quantitative evaluation of the GmPGK protein levels will allow a better understanding of carbon utilisation in the symbiotic and asymbiotic development of AM fungi.

Materials and methods

Plant growth conditions

Seeds of tomato (*Lycopersicon esculentum* Mill.) cv. Moneymaker were surface sterilised and germinated (Somasegaran and Hoben 1985). The germinated seedlings were transferred into pots containing Terragreen (Oil-Dri UK, Cambridge, UK) and were inoculated with 50 sporocarps of the arbuscular mycorrhizal fungus *G. mosseae* (Nicol. & Gerd.) Gerdemann & Trappe [Banque Européene des Glomales Isolate 12 (BEG12)]. The plants were grown in a controlled environment with a 12-h day length and at a temperature of 25 °C ± 2 °C. Control treatments were not inoculated. Plants were grown for 12 weeks before harvest. Roots and shoots were excised and cooled in liquid nitrogen before storage at –80 °C. Root tissue samples were stained for the presence of AM fungi according to Grace and Stribley (1991) and estimation of mycorrhizal colonisation was by the method described by Trouvelot et al. (1986). The results are expressed as percentage colonised root cortex (M%) and the intensity of arbuscule development (A%) within the mycorrhizal root system.

Fungal material

Sporocarps utilised throughout this work were recovered from pot cultures of *G. mosseae* maintained in the collection of the Department of Biotechnology, Scottish Agricultural College, Edinburgh, Scotland. Sporocarps were also obtained from Biorize, Dijon, France. Germinated sporocarps and hyphal material were obtained by utilising the membrane sandwich method of Giovannetti et al. (1993). Tomato seedlings established in the membrane sandwich system were grown under the environmental conditions detailed above.

Peptide design and antibody production

A peptide was selected from the deduced amino acid sequence of the GmPGK cDNA on the basis of hydrophobicity, hydrophilicity, surface exposure, β-turn formation and antigenicity: CELLEGKDLPGVSALSSK. This peptide was chosen from position 400– 416 of the C-terminus of the protein. It has a region of turn at position 407 and is probably surface exposed as it is the terminal of the protein. A cysteine residue was added at the N-terminal of the peptide to allow for the conjugation of keyhole limpet hemacyanin (KLH). The peptides were conjugated to KLH because of their small size and were produced by Sigma Genosys Biotechnology Ltd.. The Scottish Antibody Production Unit (SAPU) immunised a rabbit for the production of a polyclonal antibody with 100 μg of peptide and with three subsequent booster injections. Pre-immune serum was taken from the rabbit before the first immunisation and then at 35, 47 and 81 days after the first immunisation.

Immunodepletion experimentation

Immunodepletion experiments were performed as detailed by Cao et al. (1999). Synthetic peptide was mixed with varying volumes (0.2 V, 0.4 V,1.0 V) of serum, whereas equalised protein extracts from experimental tissues were mixed with an appropriate volume of serum. Preliminary experiments were performed in which the volume of immune serum added to the experimental tissue protein samples was varied, to ensure that these conditions were saturating for the amount of protein present (data not shown).

Protein extraction

Soluble protein was extracted as described by Forbes et al. (1998) from germinating sporocarps (including hyphal material of *G. mosseae*), non-mycorrhizal and *G. mosseae*-colonised tomato root tissue. The protein content was determined (Bradford 1976) using bovine serum albumin (BSA) as a standard.

Western blot analysis

Ten micrograms of soluble protein from mycorrhizal and non-mycorrhizal root tissue was utilised for the western blotting. Soluble protein samples were separated on a precast 12% Tris-glycine 1.0-mm gel (Novex) for 2 h at 25 mA, 135 V, under non-denaturing conditions in a Novex X Cell II, Mini-Cell. Gels were run in duplicate: one gel was used to detect polypeptides by silver staining and the other gel was electroblotted. Freshly electrophoresed proteins were electroblotted for 2 h at 145 mA, 35 V and 4 °C per gel onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a Novex X Cell II Blot module. The efficiency of the transfer was evaluated by colloidal gold total protein stain (Bio-Rad).

Non-specific binding was blocked by immersing the membranes in Tris buffered saline (TBS) containing 1% western blocking reagent (Amersham, Pharmacia Biotech, UK) for 1 h at room temperature. The primary antibody (rabbit anti GmPGK) was added to the buffer (concentration 1:5000) and incubated overnight on a rotating platform at room temperature. The membranes were rinsed twice with TBS containing 0.1% Tween-20 and 0.5% blocking reagent. Membranes were incubated with the secondary antibody (goat anti-rabbit -peroxidase conjugate) in TBS containing 1% blocking reagent at room temperature for 1 h (concentration 1:16,000). Membranes were rinsed for 15 min, three times in TBS containing 0.1% Tween-20. The immunodetection for both immuno-dot blotting and western blotting was revealed using ECL western blotting detection reagents (Amersham, Pharmacia Biotech, UK) according to the manufacturer's recommendations. The apparent molecular weights (MW) of the polypeptides were estimated in relation to those of standard proteins (Novex SeeBlue Pre-stained protein Standard).

Quantitative immuno-dot blotting

The amount of fungal material present in the *G. mosseae*-colonised root system was determined by utilising the monoclonal antibody F5G5 which is specific to *Glomus* species. Aliquots (2.5 μg) of protein from germinated sporocarps and dilutions of the protein samples of the mycorrhizal root system were dot blotted onto Hybond-C+ membrane using the Bio-Dot Microfiltration Apparatus (Biorad). Non-specific binding was blocked by immersing the membranes in TBS containing 2% BSA (Fraction V, Sigma) for at least 1 h at room temperature. The blocking buffer was removed and the primary antibody (F5G5) specific to the fungal material was added undiluted and incubated overnight on a rotating platform at room temperature.

The membranes were rinsed twice with TBS containing 0.01% Tween-20 and 2% BSA reagent. Membranes were incubated with the secondary antibody (goat anti-mouse -peroxidase conjugate) in TBS containing 1% BSA at room temperature for 1 h (concentration 1:4000). Subsequent washing and detection of the membrane was as detailed above. The signals were quantified using the Biometra IS500 Image Analysis System (Flowgen). Equalised amounts of protein from the mycorrhizal plant and fungal materi-

Fig. 1 Screening of a polyclonal antibody against the synthetic peptide: (i) no primary antibody, (ii) pre immune serum at a concentration of 1:5000, (iii) *Glomus mosseae* 3-phosphoglycerate kinase (GmPGK) antibody at a concentration 1:20,000, (iv) GmPGK antibody at a concentration 1:10,000, (v) GmPGK antibody at a concentration 1:5,000, (vi) GmPGK antibody at a concentration 1:500

al were applied in the dot-blotting procedure. In addition, 1 ng of synthetic peptide was blotted onto the membranes being utilised for the immuno-dot blots as a positive control.

Analysis of variance was carried out on the quantified signals and all statistical analyses were made using the GENSTAT statistical package.

Results and discussion

Following analysis of the GmPGK cDNA deduced amino acid sequence for hydrophobicity, hydrophilicity and antigenicity, a region from position 400 to 416 of the polypeptide was chosen to design a synthetic polypeptide for use in polyclonal antibody production. The polyclonal antibody from the 81-day sample reacted the strongest with synthetic peptide in a dot-blot (Fig. 1, iii–vi). The peptide did not react with the pre-immune serum (Fig. 1, ii). The latter confirms that there was no cross-reaction with any rabbit antibodies prior to injection with the synthetic peptide designed to the GmPGK. Furthermore, immunodepletion experiments utilising the synthetic peptide (Fig. 2, iv–vi) demonstrated that the polyclonal antibody produced was specific to the synthetic peptide designed from the GmPGK deduced amino acid sequence.

Soluble proteins were extracted from non-inoculated and *G. mosseae*-inoculated tomato roots (M% was 64.2 and A% was 34.3) and separated on a 12% Tris-glycine polyacrylamide gel and silver stained (Fig. 3i). After transfer of the proteins to PVDF membrane and incubation of the GmPGK polyclonal antibody, a polypeptide was detected in mycorrhizal roots only (Fig. 3, ii). This confirms that there was no cross-reaction between the GmPGK antibody and plant-derived proteins. The apparent MW of the GmPGK polypeptide was approximately 45 kDa, which was similar to the theoretical MW of 44.764 kDa (Harrier et al. 1998). Moreover, PGKs have been isolated from many species and have been shown to consist of a single polypeptide with a MW around 45 kDa: human (Michelson et al. 1985), horse (Banks et al. 1979), mouse (Mori et al. 1986), *Trypanosoma brucei* (Osinga et al. 1985) and *Saccharomyces cerevisiae* (Perkins et al. 1983). The PGK monomeric enzyme has been shown to have a highly conserved primary structure and is composed of N- and C-terminal domains connected by a well-conserved hinge region (Blake 1997; Bernstein et al. 1997). PGK is a hinge-bending enzyme whereby substrate-induced effects combine synergistically to induce major conformational changes that bring together the two ligand-binding sites to allow a direct phosphotransfer reaction to occur (Blake 1997; Bernstein et al. 1997).

To determine whether there was any quantitative change in the levels of GmPGK during the asymbiotic and symbiotic stages of the *G. mosseae* life cycle, a method was devised to determine the fungal content of the *G. mosseae*-colonised root system utilising the monoclonal antibody F5G5. Monoclonal antibodies have been produced against particular antigens of AM fungi, for example, spore and hyphal surface antigens, in order to produce antibodies which would specifically recognise AM fungi at the genus or species level (Wright and Morton 1989; Wright et al. 1987; Hahn et al. 1993; Hahn and Hock 1994; Göbel et al. 1995). This procedure utilised a monoclonal antibody to quantify the amount of fungal material in a mycorrhizal root system, in order to equalise amounts of fungal material when comparing symbiotic and asymbiotic samples. An overview of the fungal quantification procedure utilised here is given in Figure 4. Soluble proteins were extracted from germinated sporocarps, non-colonised and *G. mosseae*-colonised tomato root systems (M% and A% as detailed above). A known amount (2.5 μg) of fungal protein material was spotted onto a nylon membrane along with dilutions of the protein samples isolated from a mycorrhizal root system. The membranes were then incubated with the antibody F5G5. The rela-

Fig. 2 Immunodepletion of GmPGK polyclonal antibody against the synthetic peptide: (i) no primary antibody, (ii) pre immune serum at a concentration of 1:5,000, (iii) GmPGK antibody at a concentration of 1:5,000, (iv) peptide immunodepleted with 0.2 V of PGK antibody, (v) peptide immunodepleted with 0.4 V of PGK antibody, (vi) peptide immunodepleted with 1.0 V of PGK antibody

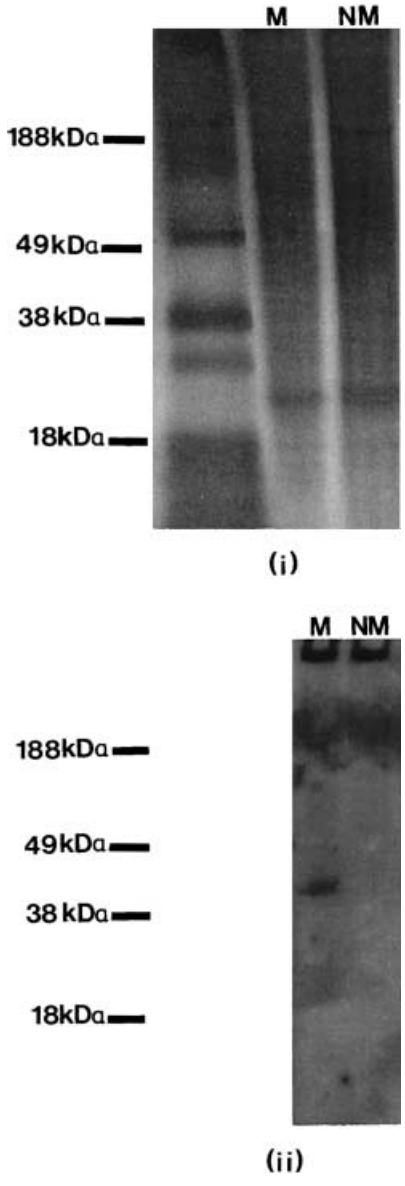

Fig. 3 Western blot analysis of the *G. mosseae* PGK protein. Ten micrograms of soluble proteins extracted from mycorrhizal (*M*) and non-mycorrhizal (*NM*) root systems were separated by native PAGE, silver stained (i) and western blotted with the *G. mosseae* PGK polyclonal antibody (ii). *Left lane* Molecular weight markers

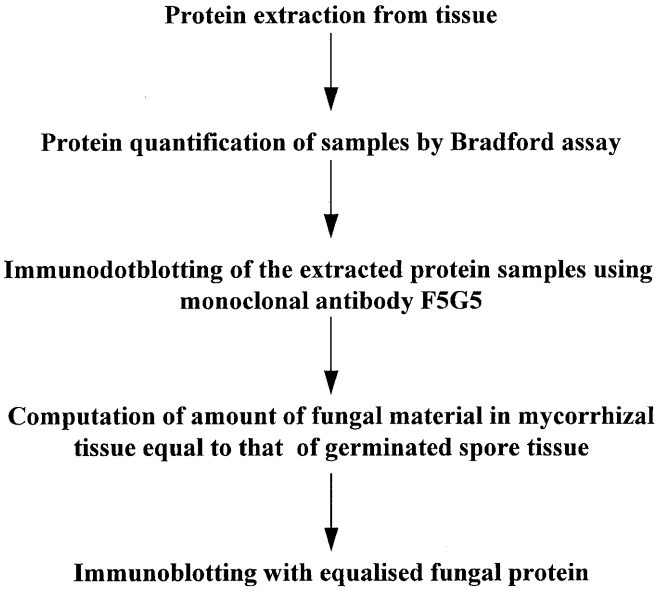

Fig. 4 Procedure developed for equalising the amount of protein from a *G. mosseae*-colonised tomato root system and isolated fungal material

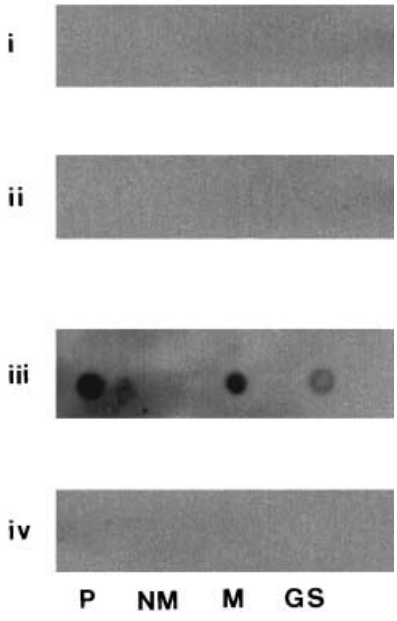

Fig. 5 Protein dot blot experiments: (i) control, performed by omitting the PGK antibody; (ii) control, performed by using pre-immune serum from rabbit; (iii) detection of PGK protein with GmPGK antibody (equalised amounts of fungal protein and 1 ng of synthetic peptide were blotted onto the membranes); (iv) immunodepletion of synthetic peptide and experimental samples (*GS* germinated sporocarps including hyphae of *G. mosseae, NM* uncolonised and *M G. mosseae*-colonised tomato root system, *P* synthetic peptide corresponding to that used for immunisation of rabbit)

tive amounts present in the *G. mosseae*-colonised tomato root system were computed from the signals detected.

Immuno-dot blotting with equalised fungal protein and the GmPGK antibody was used to establish whether the GmPGK protein was present in both asymbiotic and symbiotic stages of the *G. mosseae* life cycle. No signal was obtained in the control incubated with no primary antibody (Fig. 5, i), in the membrane incubated with pre-immune serum (Fig. 5, ii) or in the immunodepleted samples (Fig. 5, iv). The latter demonstrates that the signal detected in the immuno-dot blots is not non-specific antibody binding. However, when the membrane containing protein samples which were not immunodepleted were incubated with the GmPGK antibody (Fig. 5, iii), PGK protein was detected in germi-

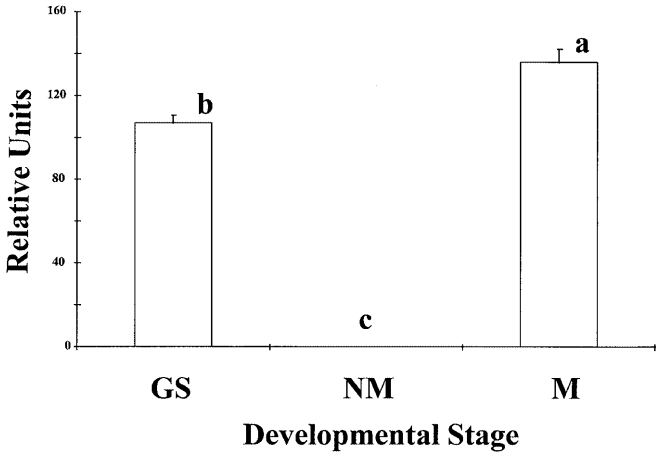

Fig. 6 Levels of PGK protein in the different stages of symbiotic and asymbiotic development of the AM fungus *G. mosseae*. The membranes were exposed to chemiluminescent film for 15 s prior to development of the film and the signal intensity was determined using the Biometra IS500 Image Analysis System. Data are means ± standard error and letters denote significant differences ($P<0.05$) between samples (*GS* germinated sporocarps including hyphae of *G. mosseae*, *NM* uncolonised and *M G. mosseae*-colonised tomato root system)

nated sporocarps and in *G. mosseae*-inoculated tomato roots. Moreover, significant differences ($P<0.05$) in the levels of detection of Gm PGK were obtained, with the highest found in *G. mosseae*-colonised tomato root systems (Fig. 6). These results consolidate the data of Harrier et al. (1998) as RT-PCR successfully detected the *PGK* gene transcript from germinated spore tissue and *G. mosseae*-colonised plant root tissue. Our data also confirm that the GmPGK protein is present within these asymbiotic and symbiotic stages and indicate that the fungus is glycolytically active during these stages. Furthermore, the different levels of GmPGK protein suggest that glycolytic activity differs between the symbiotic and asymbiotic stages of fungal development. The higher levels of GmPGK protein in the symbiotic state is probably due to the significant carbon flow taking place from the plant to the fungus via the internal fungal structures (Shachar-Hill et al. 1995; Smith and Read 1997; Pfeffer et al. 1999). A subsequent higher level of conversion of the carbon sources to carbohydrates and lipids within fungal structures (Pfeffer et al. 1999) would require enhanced glycolytic activity.

Other glycolytic enzymes have been assayed during symbiotic and asymbiotic growth of *G. mosseae* (Macdonald and Lewis 1978) and *Gigaspora margarita* (Saito 1995). Hexokinase, which catalyses the first step in glycolysis, was detected in intraradical hyphae of *G. margarita* but not in germinated spores (Saito 1995). Other glycolytic enzyme activities detected during asymbiotic and symbiotic growth of AM fungi are phosphofructokinase (EC 2.7.1.11) (Saito 1995) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (Macdonald and Lewis 1978).

In conclusion, the detection of the protein corresponding to GmPGK, the RT-PCR detection of the PGK gene transcript (Harrier et al. 1998), and assays of other glycolytic enzymes (Macdonald and Lewis 1978; Saito 1995) provide convincing evidence of glycolytic activity in AM fungi during asymbiotic and symbiotic growth. Evidence from other fungal systems suggests that the *PGK* gene is transcriptionally rather than translationally regulated (Takaya et al. 1994; Le Dall et al. 1996). Isolation of the GmPGK gene promoter region and subsequent testing of this promoter will help to establish whether carbon source regulates the expression of this gene in AM fungi.

The detection and quantification of functional AM fungal proteins is a prerequisite for understanding not only the carbon metabolism of these fungi but also factors which influence asymbiotic and symbiotic development. Future work aims to investigate whether the *PGK* gene transcripts and corresponding protein can be used as a functional marker for glycolytic activity in AM fungi, and thus provide a useful tool for ecophysiological studies.

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