Mycorrhiza (2002) 12:219–222
DOI 10.1007/s00572-002-0173-x

SHORT NOTE

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A mycorrhiza-responsive protein in wheat roots

Received: 13 November 2001 / Accepted: 25 March 2002 / Published online: 1 May 2002

Abstract A small protein, designated Myk15, was found to be strongly induced in wheat (*Triticum aestivum*) roots colonized by the arbuscular mycorrhizal fungus *Glomus intraradices*. This protein, which is most abundant in root fractions characterized by strong mycorrhizal colonization, has been characterized using two-dimensional polyacrylamide gel electrophoresis and microsequencing. It has an apparent molecular mass of 15 kDa and an isoelectric point of 4.5. The *N*-terminal sequence has high similarity to a peptide sequence deduced from an expressed sequence tag (EST) clone derived from *Medicago truncatula* roots colonized by *G. intraradices*. This EST clone is predicted to code for a protein with a similar size and isoelectric point as Myk15. The *N*-terminus of the deduced *M. truncatula* protein contains a highly hydrophobic stretch of 24 amino acid residues preceding the region with high similarity to the Myk15 *N*-terminus. This hydrophobic stretch is predicted to form a transmembrane α-helix and may correspond to a cleavable targeting domain.

Keywords One-dimensional polyacrylamide gel electrophoresis · Two-dimensional polyacrylamide gel electrophoresis · Arbuscular mycorrhiza · *Triticum aestivum* · *Medicago truncatula*

Introduction

The molecular basis of functional mycorrhizal symbiosis is virtually unknown. One approach used to understand the temporal and spatial control of mycorrhiza-responsive genes is the analysis of protein patterns in mycorrhizal roots, which reflect global gene expression. This has extensively been performed in the past (e.g. Dumas-Gaudot et al. 1994; Simoneau et al. 1994; Samra et al. 1997; Benabdellah et al. 1998; Dassi et al. 1999). In most cases, however, these studies did not lead to new insights into the metabolic regulation of the root-fungus interactions. This will most likely change in the near future because a growing number of expressed sequence tag (EST) clones will be available for the mycorrhizal model plant *Medicago truncatula*. This data will facilitate the analysis of proteins in amounts not suitable for conventional microsequencing techniques (Dumas-Gaudot et al., in press). For wheat (*Triticum aestivum*), similar approaches are not feasible and only a few studies using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of root proteins have been described for this plant (Ouerghi et al. 2000).

In the course of our studies on the molecular regulation of arbuscular mycorrhizas, we observed the massive accumulation of a small mycorrhiza-responsive polypeptide, named Myk15. In the present communication we report on the characterization of this protein.

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Materials and methods

Plant material and arbuscular mycorrhizal fungus inoculation

Wheat plants (*T. aestivum* L. cv. Caprimus) were grown from grains (Walz, Stuttgart) in growth chambers in 0.5-l plastic pots (1 plant pot^{-1}) filled with expanded clay (Lecaton, 2–5 mm particle size; Fibo Exclay Deutschland, Pinneberg). The plants were inoculated with the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith by the application of propagules in expanded clay (isolate 49, provided by H. von Alten from the collection of the Institut für Pflanzenkrankheiten und Pflanzenschutz der Universität Hannover, Germany). Details of plant growth conditions have been described previously (Maier et al. 1995). Approximate values of colonization frequency (%) were estimated by counting the proportion of 30 root pieces (2 cm) with mycorrhizal structures (arbuscules, vesicles, spores) after staining with trypan blue in lactophenol according to Phillips and Hayman (1970).

Dissection of root systems and protein extraction

The root systems were either dissected according to Fester et al. (1999) or extracted in toto. Dissection concentrated on two easily

distinguishable root fractions: young roots (about 2 weeks old) with few signs of beginning mycorrhizal colonization, and lateral roots from 3- to 4-week-old roots with established fungal colonization. For further characterization of the root fractions see Fester et al. (1999). Dissected roots from several plants were pooled in order to obtain enough material for protein extraction. Non-mycorrhizal roots served as controls.

Protein extraction was performed with at least 200 mg fresh roots and at 4°C throughout the whole procedure. Roots were ground in a mortar in liquid nitrogen and the homogenate suspended in 100 mM *N*[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (pH 7.2), 250 mM sorbitol, 5 mM mercaptoethanol and 0.1% (w/v) phenylmethylsulphonyl fluoride. The suspension was filtered through one layer of Miracloth, the filtrate centrifuged first for 5 min at 1,500 *g* and then for 1 h at 100,000 *g*. The supernatant of this centrifugation step (100 *S* supernatant) contained the soluble protein fraction that was further analysed.

The protein content of the soluble fraction was determined according to Bradford (1976). Then proteins were precipitated according to Petersen (1977) by adding 100 µl sodium desoxycholate and 120 µl trichloroacetic acid to 1 ml protein solution and incubating for 20 min on ice. After centrifugation (10 min, 5,000 *g*) the pellet was washed twice with acetone (–20°C). The pellets were dried in vacuo and finally suspended in 8 M urea containing 0.5% (w/v) Triton X-100, 0.5% (v/v) Pharmalyte 3–10 and 0.2% (w/v) dithiothreitol.

Electrophoretic methods

The first dimension of 2D-PAGE was performed according to the Pharmacia manual using Immobiline DryStrip gels (pH 3–10, length 11 cm; Pharmacia), a 2117 Multiphor electrophoresis unit (LKB, Pharmacia) and a 2297 Macrodrive 5 constant power supply (LKB, Pharmacia). The second dimension was performed using either ExcelGel precast gels gradient 8–18% (Pharmacia) or the Protean II xi Cell (Bio-Rad) and the discontinuous buffer system according to Laëmmli (1970). The separating gel contained 12% (w/v) acrylamide, with an acrylamide/bisacrylamide ratio of 37.5:1. First dimensions were washed as described in the Pharmacia manual, layered between the two glass plates (0.75-mm spacers), before preparing the second dimension. Gels were stained as described in the PlusOne Silver Staining Kit, Protein manual from Pharmacia. Molecular weights and isoelectric points (pI values) were determined using the 2D sodium dodecyl sulphate (SDS)-PAGE standards from Bio-Rad.

1D-PAGE [separating gels with 15% (w/v) acrylamide, acrylamide/bisacrylamide 37.5:1] was performed according to Laëmmli (1970) using Hoefer Mighty Small SE250 apparatus. Proteins were blotted to a polyvinylidene difluoride (PVDF) membrane using a Mini Trans Blot Cell (Bio-Rad) and 49 mM TRIS, 39 mM glycine (pH 9–9.4), 0.037% (w/v) sodium lauryl sulphate (SDS) and 20% (v/v) methanol by applying 100 V for 1 h. Blots were stained by 0.1% Coomassie Brilliant Blue in 50% (v/v) methanol, 10% (v/v) acetic acid and destained by applying the same solution without Coomassie Brilliant Blue. Bands were excised and subjected to sequencing.

Protein sequencing and database analysis

For *N*-terminal sequencing, the protein blotted to PVDF was analysed in an Applied Biosystems 494A sequencer with standard pulsed liquid cycles. For internal sequences, Coomassie-stained SDS-PAGE bands were digested with modified trypsin (Promega) in 25 mM ammonium bicarbonate at 37°C overnight. Peptides were separated on a reversed-phase capillary column (Aquapore OD, 0.5 mm×150 mm) and fractions were spotted onto Biobrene-treated glass fibre filters and sequenced on an Applied Biosystems 494A sequencer. Sequences were analysed using the HUSAR package from the Biocomputing Service Group at the Deutsches Krebsforschungszentrum, Heidelberg (http://genius.embnet.dkfz-heidelberg.de/).

Results and discussion

1D-PAGE revealed a protein of 15 kDa, named Myk15, which accumulated dramatically in mycorrhizal (70% colonization) wheat root systems (Fig. 1A, lanes 1 and 3), whereas only small amounts were observed in non-mycorrhizal roots (Fig. 1A, lanes 2 and 4). After 1D-PAGE and blotting onto a PVDF-membrane, Myk15 was subjected to *N*-terminal sequencing as well as to tryptic digestion and sequencing of the tryptic fragments. We obtained an *N*-terminal sequence with 27 amino acids and an internal stretch of 16 amino acids (Fig. 2).

Repeated 2D-PAGE of the soluble protein fraction from arbuscular mycorrhizal roots showed that Myk15 had a molecular mass of 15 kDa and an isoelectric point at pH 4.5. Only minor traces of other polypeptides were visible at the given molecular mass (Fig. 1C), corresponding to the detection of a single *N*-terminal sequence after 1D-PAGE. With reference to 1D-PAGE as well as 2D-PAGE, the electrophoretic mobility of Myk15 showed some variability, probably due to strong variations in local protein concentrations. Given the observation of small amounts of Myk15 in non-mycorrhizal wheat roots after 1D-PAGE (Fig. 1A, lanes 2 and 4) and 2D-PAGE (Fig. 1B), a fungal origin of the protein is unlikely. This point could be further substantiated by amplifying DNA fragments corresponding to Myk15 or to the respective *M. truncatula* EST clone from non-mycorrhizal plant tissue. So far, however, reverse transcription-PCR experiments using various primer combinations and starting materials have failed.

2D-PAGE analysis showed other differences in the protein patterns of mycorrhizal and non-mycorrhizal roots, which were difficult to document in a reproducible way and were not further investigated. After dissecting the wheat root system in strongly colonized fractions and those showing first signs of mycorrhization according to Fester et al. (1999), it was found that Myk15 was particularly abundant in the strongly colonized root parts (Fig. 1A, lane 3).

The *N*-terminal sequence of Myk15 showed 56% identity and 70% similarity to an EST clone (MtBC37G05, GenBank accessions AL386938, AL386939) from an EST library prepared from *M. truncatula* roots colonized by *Glomus intraradices* (http://sequence.toulouse.inra.fr/Mt/public/EST_sequencing.html, see Fig. 2). Sequence similarity starts at position 30 of the protein deduced from this EST clone. A region showing 31% identity and similarity to the internal sequence of Myk15 starts at position 90. The protein sequence deduced from the *M. truncatula* EST clone preceding position 30 is predicted to form a transmembrane α-helix and to serve for targeting the protein to the ER or to mitochondria (PSORT program of the HUSAR package). A similar targeting domain may precede the *N*-terminus found for Myk15 from wheat and might be cleaved during protein targeting and maturation, leading to a soluble protein. The protein deduced from the EST clone MtBC37G05 consists of 155 amino acids and is predicted to have a molecular

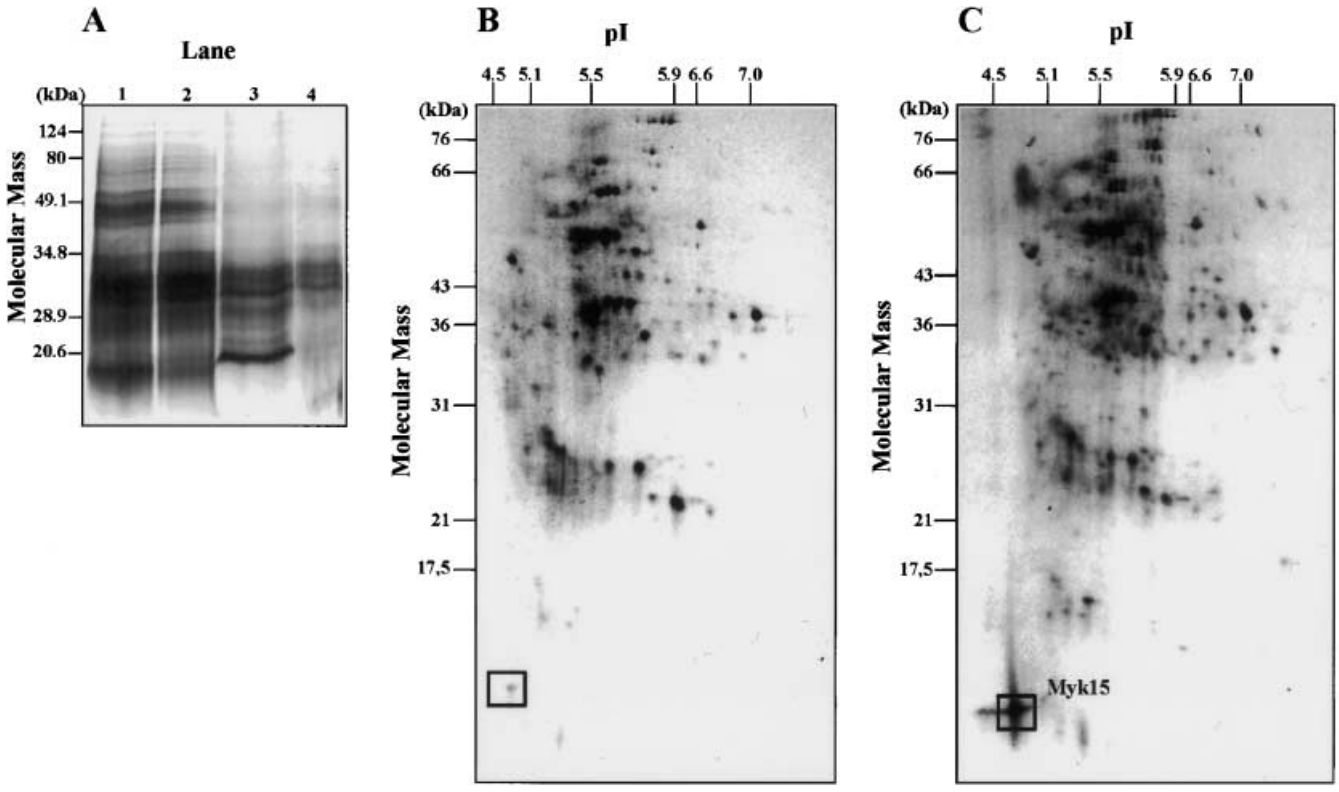

Fig. 1A–C Accumulation of Myk15 in mycorrhizal wheat (*Triticum aestivum*) roots. **A** Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showing accumulation of a small polypeptide in mycorrhizal roots (*lane 1*, 70% mycorrhizal colonization) versus non-mycorrhizal roots (*lane 2*). Protein extracts loaded in *lanes 3 and 4* have been extracted from root fractions collected according to Fester et al. (1999) representing strongly colonized (*lane 3*) regions of the wheat root system and the corresponding regions from non-mycorrhizal plants (*lane 4*). *Lanes 1, 2 and 3* were loaded with 8 µg protein, *lane 4* with <4 µg due to a very low protein concentration. **B**, **C** Silver-stained two-dimensional (2D)-PAGE (second dimension: ExcelGel precast gel gradient 8–18%, Pharmacia) of 2.5 µg soluble protein from non-mycorrhizal **(B)** and mycorrhizal **(C)** roots. The position of Myk15 (molecular mass of 15 kDa and pI of 4.5) is indicated by a *rectangle*. Other differences in the protein patterns have not been further analysed. Variations in the electrophoretic mobility of Myk15 (1D-PAGE and 2D-PAGE) are probably due to the presence of different amounts of low molecular compounds in the various root fractions **(A)** and to strong variations in local protein concentrations **(B**, **C)**

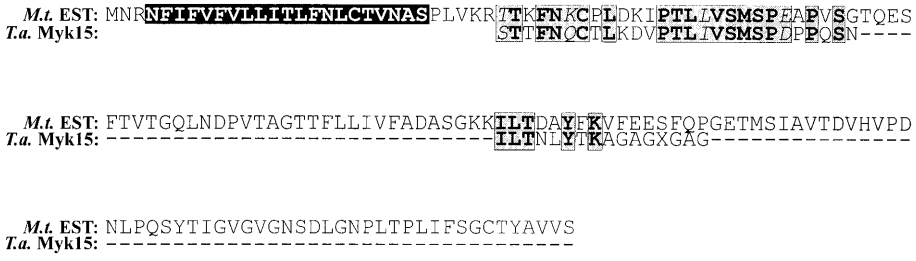

Fig. 2 Alignment of *N*-terminal and internal peptide sequences of Myk15 (*T.a. Myk15*) with a peptide sequence deduced from an expressed sequence tag clone (MtBC37G05) derived from mycorrhizal *Medicago truncatula* roots (*M.t. EST*). The putative transmembrane α-helix is shaded in *black*, similar regions to the *N*-terminal and internal sequence are shaded in *grey*. Identical amino acids are given in *bold*, similar ones in italics

mass of 16.7 kDa and an pI of 4.8. Assuming a cleavage of the protein between positions 29 and 30 (in accordance with the *N*-terminus found for Myk15), the mature protein from *M. truncatula* would consist of 126 amino acids and is predicted to have a molecular weight of 13.3 kDa and pI of 4.2.

In summary, Myk15 exhibits sequence homology as well as a similar molecular mass and pI to EST clone MtBC37G05 from *M. truncatula*, which is expressed under the same physiological conditions, i.e. during colonization of the plant root by *G. intraradices*. It is interesting to note that there are reports of other mycorrhiza-responsive proteins with similar characteristics. For example, in roots of *Lycopersicon esculentum* a protein of 18 kDa and pI of 4.82 has been found (Benabdellah et al. 1998). In *Pisum sativum* a protein of 14 kDa and pI of 5.2 was detected (Samra et al. 1997). In mycorrhizal wheat roots, Myk15 was found to accumulate in considerable amounts in a root fraction which was characterized by extensive mycorrhizal colonization, vesicle for-

mation and by massive accumulation of apocarotenoids (Fester et al. 1999). Studies have been initiated to immunolocalize Myk15 in mycorrhizal wheat roots with the aim of evaluating the possible role of this protein in the plant-fungus association.

Acknowledgements Our work is supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

References

Benabdellah K, Azcón-Aguilar C, Ferrol N (1998) Soluble and membrane symbiosis-related polypeptides associated with the development of arbuscular mycorrhizas in tomato (*Lycopersicon esculentum*). New Phytol 140:135–143

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Dassi B, Samra A, Dumas-Gaudot E, Gianinazzi S (1999) Different polypeptide profiles from tomato roots following interactions with arbuscular mycorrhizal (*Glomus mosseae*) or pathogenic (*Phytophthora parasitica*) fungi. Symbiosis 26:65–77

Dumas-Gaudot E, Guillaume P, Tahiri-Alaoui A, Gianinazzi-Pearson V, Gianinazzi S (1994) Changes in polypeptide patterns in tobacco roots colonized by two glomus species. Mycorrhiza 4: 215–221

Dumas-Gaudot E, Bestel-Corre G, Gianinazzi S (in press) Proteomics, a powerful approach towards understanding functional plant root interactions with arbuscular mycorrhizal fungi. In: Pandalai SG (ed) Recent research developments in plant biology. Research Signpost, Trivandrum, India

Fester T, Maier W, Strack D (1999) Accumulation of secondary compounds in barley and wheat roots in response to inoculation with an arbuscular mycorrhizal fungus and co-inoculation with rhizosphere bacteria. Mycorrhiza 8:241–246

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Maier W, Peipp H, Schmidt J, Wray V, Strack D (1995) Levels of a terpenoid glycoside (blumenin) and cell wall-bound phenolics in some cereal mycorrhizas. Plant Physiol 109:465–470

Ouerghi Z, Rémy R, Ouelhazi L, Ayadi A, Brulfert J (2000) Two-dimensional electrophoresis of soluble leaf proteins, isolated from two wheat species (*Triticum durum* and *Triticum aestivum*) differing in sensitivity towards NaCl. Electrophoresis 21: 2487–2491

Petersen GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:581–587

Samra A, Dumas-Gaudot E, Gianinazzi S (1997) Detection of symbiosis-related polypeptides during the early stages of the establishment of arbuscular mycorrhiza between *Glomus mosseae* and *Pisum sativum* roots. New Phytol 135:711–722

Simoneau P, Louisy-Louis N, Plenchette C, Georges Strullu D (1994) Accumulation of new polypeptides in Ri T-DNA-transformed roots of tomato (*Lycopersicon esculentum*) during the development of vesicular-arbuscular mycorrhizae. Appl Environ Microbiol 60:1810–1813