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Intraradical hyphae phosphatase of the arbuscular mycorrhizal fungus, *Gigaspora margarita*

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Abstract An alkaline phosphatase in the intraradical hyphae of arbuscular mycorrhizal fungi was found to be closely related to an improvement of plant growth. To detect the phosphatase activity in a crude extract of mycorrhizal roots, phosphatase isozymes in mycorrhizal and non-mycorrhizal onion roots were compared with those in *Gigaspora margarita* by electrophoresis. A mycorrhiza-specific band was found when the phosphatase was stained under alkaline conditions. To clarify the origin of this phosphatase, the phosphatase extracted from intraradical hyphae was also compared with the phosphatase from mycorrhizal roots by electrophoresis. The intraradical hyphae was isolated from mycorrhizal roots by enzyme digestion followed by Percoll gradient centrifugation. The soluble protein was extracted from the hyphae by ultra-sonication after treatment with chitinase. A phosphatase in the hyphal soluble protein showed a similar, but slightly higher, relative mobility on the gel, compared with the mycorrhiza-specific phosphatase from roots. By adding the hyphal extract to the root extract, the relative mobility of the mycorrhiza-specific phosphatase was slightly changed and became identical to that of the phosphatase in the hyphae. This indicated that the specific band of phosphatase found in the crude extract from mycorrhizal roots was of intraradical hyphal origin.

Key words Mycorrhiza-specific phosphatase · Intraradical hyphae · *Gigaspora margarita* · Onion

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Introduction

The metabolism of phosphorus in the hyphae of arbuscular mycorrhizal (AM) fungi has been studied (Capaccio and Callow 1982; Gianinazzi-Pearson and Gianinazzi 1983; Smith and Gianinazzi-Pearson 1988), but the biochemical mechanism of phosphorus transfer from fungi to host is still unclear (Smith and Read 1996; Smith and Gianinazzi-Pearson 1988; Gianinazzi 1991; Schwab et al. 1991).

Cyto-/histochemical observations showed that an alkaline phosphatase was localized in the arbuscule and intraradical hyphae (Gianinazzi et al. 1979; Smith and Gianinazzi-Pearson 1990; Tisserant et al. 1993; Ezawa et al. 1995; Saito 1995). The alkaline phosphatase in intraradical hyphae was found to be related to the stimulation of the growth of plants when infected by AM fungi (Tisserant et al. 1993). Measurement of this phosphatase activity would be a good index of the mycorrhizal effect on host plant growth.

A mycorrhiza-specific phosphatase was first reported by Gianinazzi-Pearson and Gianinazzi (1976, 1978) who, by using electrophoresis, found an alkaline phosphatase specific to mycorrhizal colonization in onion roots symbiotic with *Glomus mossae*. But it was uncertain whether this phosphatase originated from the intraradical hyphae of AM fungi or the host plant. Phosphatase expressed only during AM infection was also found by Ezawa et al. (1994a and b) in marigold roots colonized by *Glomus etunicatum*. It was partially purified, and the authors suggested that it was a non-specific acid phosphatase based upon its specificity for certain substrates and inhibitors. Furthermore, it seemed to be of host origin judged by its N-terminal amino acid sequence (Ezawa et al. 1996). Thus, the alkaline phosphatase in intraradical hyphae has neither been identified in the soluble fraction of mycorrhizal roots, nor enzymatically characterized. Therefore, in the present study, we sought a mycorrhiza-specific phosphatase in root extracts by using electrophoresis, and we hoped to identify this as the intraradical hyphal phosphatase of AM fungi.

Materials and methods

Mycorrhizal roots

Non-mycorrhizal onion plants in low- or high-phosphate medium and mycorrhizal ones in low-phosphate medium were grown for 6 or 9 weeks in a growth cabinet with a 14 h day at 23 °C, a 10 h night at 20 °C, and a light intensity of 465 μ E m^{-2} s^{-1}. Onion seeds (*Allium cepa* L. cv. Sensyucyukouki) were surface sterilized in 0.5% (w/v) NaOCl solution for 1 h, and germinated on moist filter paper. After about 14 days, seedlings were transplanted to a pot and were inoculated with spores of an isolate of *Gigaspora margarita* Becker and Hall (MAFF 520054) to give the mycorrhizal treatment (Saito 1995). The low-phosphate medium was a mixture of sand, soil and a horticultural medium ("Engei-Baido", Kureha Chemical Co., Japan) in the ratio of 5:4:1 (in volume) with $CaCO_3$ added at a rate of 1 g l^{-1}. The high-phosphate medium was solely comprised of the horticultural medium. The soil was collected from the surface layer of a Haplumbrept at the experimental farm of the National Grassland Research Institute, Tochigi, Japan. The soil was poor in available phosphate (11 mg soluble phosphate-phosphorus kg^{-1} dry soil extracted in dilute H_2SO_4). The horticultural medium was rich in available phosphate (350 mg phosphate-phosphorus kg^{-1} extracted in dilute H_2SO_4). The available phosphate of soils was measured by the Truog method (Nanjo 1986). Before use the sand and horticultural medium were autoclaved for 20 min at 120 °C and the soil was autoclaved for 1 h.

Six or 9 weeks after transplanting, the roots were washed out from the medium and their fresh weights measured. Shoot fresh weights were also measured. Mycorrhizal infection was evaluated by the method of Giovanetti and Mosse (1980).

Electrophoresis and phosphatase active stain of soluble protein from onion roots

The roots were cut into 1 cm to 2 cm segments and stored at –80 °C until analysis. The roots were first powdered with liquid nitrogen in a mortar on ice. Soluble protein was then extracted with 50 mM TRIS buffer (pH 7.5) containing 1 mM $MgCl_2$ and 0.5% Triton X-100 at the rate of 3 ml g^{-1} root by homogenizing the roots with sea sand. The homogenate was centrifuged at 8000 *g* for 30 min to obtain a supernatant for the experiments. The soluble protein extract was concentrated with Minicon (Amicon Co., USA) and loaded for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) or native PAGE using 10% gel (Laemmli 1970). The electrophoresis was run at 4 °C to maintain the enzyme activities. To detect the phosphatase activities at different pH, the gel was first equilibrated at room temperature for 30 min with the following buffer: 50 mM TRIS/citric acid (pH 8.5), 50 mM TRIS/maleic acid/NaOH (pH 7.0), or 100 mM acetic acid/sodium acetate (pH 5.5). The gel was placed in the staining solution (1 mg ml^{-1} Fast Blue RR salt, 4 mM α-naphthylphosphate, 1 mM $MgCl_2$ in one of the above buffers) and incubated at 35 °C for 30 min with continuous shaking. The reaction was stopped by substitution with a destaining solution [water:ethanol:acetic acid in a ratio of 6:3:1 (v/v/v)]. Molecular weights of phosphatases were estimated by comparing their electrophoretic response with those of low molecular weight protein standards (Pharmacia, Sweden). Protein concentrations in the extracts were determined using a protein assay kit (Bio-Rad, USA) with bovine serum albumin as the standard.

Extraction of soluble protein of the intraradical hyphae isolated from the mycorrhizae

Intraradical hyphae was collected from mycorrhizae according to Saito (1995). Soluble protein of the intraradical hyphae was extracted as follows. The hyphae were suspended in cell-wall-degrading solution [50 mM acetic acid buffer (pH 5.5), 5 mg ml^{-1} Fungase type II (Nagase Biochemical Co., Japan), 300 mM mannitol] and incubated at 30 °C with shaking (120 strokes min^{-1}) for 1 h. After centrifugation at 10 000 *g* for 10 min, the hyphae were washed with a buffer [10 mM TRIS/HCl buffer (pH 7.4), 300 mM mannitol, 1 mM dithiothreitol (DTT)] and centrifuged again. The washed hyphae were suspended with 50 mM TRIS/HCl buffer (pH 7.5) containing 1 mM $MgCl_2$ and 0.5% (w/v) Triton X-100 and treated with ultra-sonication (Branson sonifier 250) i.e. less than 10 W for about 100 s. After centrifugation at 8 000 *g* for 10 min, the supernatant was collected and used as the soluble protein extract from the intraradical hyphae.

Phosphatase assay

Phosphatase activity in the soluble protein extract was measured at pH 7.0 in a 50 mM TRIS/maleic acid/NaOH buffer according to Gianinazzi-Pearson and Gianinazzi (1976).

Results

The growth of mycorrhizal onions at 6 weeks after transplanting was comparable with that of non-mycorrhizal onions in the high-phosphate medium (Table 1). At 9 weeks, mycorrhizal plants had grown a little more than non-mycorrhizal plants in the high-phosphate medium. Non-mycorrhizal plants in low-phosphate medium grew poorly.

The soluble protein contents in roots of mycorrhizal plants was much higher than those of non-mycorrhizal plants, while the specific activity of phosphatase at pH 7.0 on a protein basis in mycorrhizal roots was much less than those in non-mycorrhizal ones. The phosphatase activities based on root weight were comparable in the three treatments because of the higher protein contents in mycorrhizal roots (Table 2).

Figure 1 shows the phosphatase activities of soluble proteins of onion roots in SDS-PAGE at three different pH levels. Since the band patterns at 6 weeks did not differ from those at 9 weeks, only the results of 9 weeks are presented. At any pH level, the band patterns of phosphatase from non-mycorrhizal onion roots were almost the same irrespective of the phosphate concentration of the medium. In mycorrhizal roots, however, the specific phosphatase band at higher molecular weight range was found at pH 7.0 and pH 8.5 (Fig. 1). Similarly, the results of native-PAGE

Table 1 Growth and mycorrhizal colonization of onion plants. *+P* High phosphate medium, *–P* low phosphate medium, *LSD* least significant difference

Treatment	Root fresh weight (g/plant)		Shoot fresh weight (g/plant)		Infection (%)	
weeks	6	9	6	9	6	9
Mycorrhizal	0.22	0.73	0.24	0.78	56	60
Non-mycorrhizal (*+P*)	0.18	0.47	0.17	0.44		
Non-mycorrhizal (*–P*)	0.14	0.13	0.14	0.15		
LSD (*P*<0.05)	0.02	0.28	0.05	0.34		

Table 2 Phosphatase activity of onion root. *+P* High phosphate medium, *–P* low phosphate medium

Treatment	Phosphatase activity	
	(unit/g root)[a]	(unit/mg protein)
6 weeks		
Mycorrhizal	1.29	0.349
Non-mycorrhizal (*+P*)	1.09	0.451
Non-mycorrhizal (*–P*)	0.886	0.882
9 weeks		
Mycorrhizal	1.02	0.337
Non-mycorrhizal (*–P*)	1.14	0.807
Non-mycorrhizal (*–P*)	1.22	0.880

[a] One unit represents the activity required to degrade 1 μmol *p*-nitrophenyl phosphate in 1 min

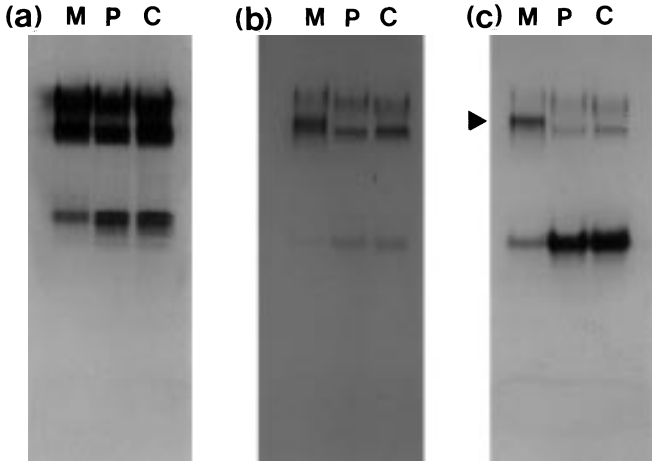

Fig. 1 a–c Phosphatase activities of mycorrhizal and non-mycorrhizal onion roots inoculated with *G. margarita*. Root soluble extracts were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained in the following buffer: **a** 100 mM acetic acid/sodium acetate (pH 5.5), **b** 50 mM TRIS/maleic acid/NaOH (pH 7.0), **c** 50 mM TRIS/citrate (pH 8.5) respectively. *M* Mycorrhizal, *P* non-mycorrhizal in the high phosphate medium, *C* non-mycorrhizal in the low phosphate medium

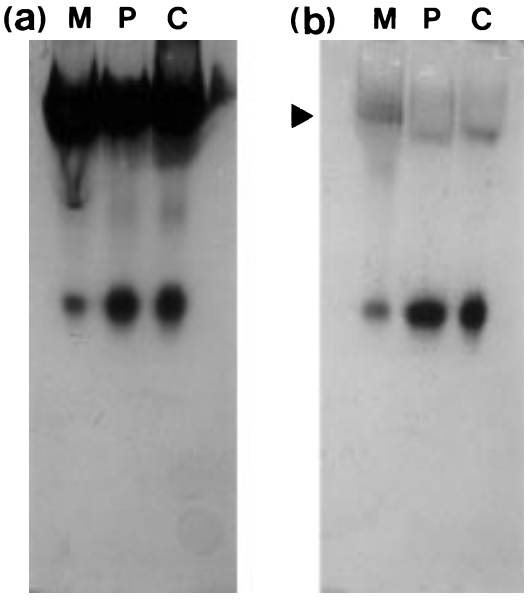

Fig. 2 a, b Phosphatase activities of mycorrhizal and non-mycorrhizal onion roots inoculated with *G. margarita*. Root soluble extracts were separated by Native PAGE and stained in the following buffer: **a** 100 mM acetic acid/sodium acetate (pH 5.5), **b** 50 mM TRIS/citrate (pH 8.5), respectively. *M* Mycorrhizal, *P* non-mycorrhizal in the high phosphate medium, *C* non-mycorrhizal in the low phosphate medium

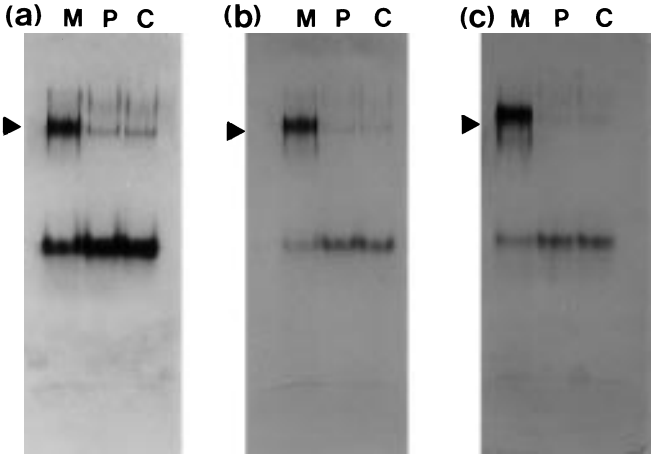

Fig. 3 a–c Effect of K_2HPO_4 and $MgCl_2$ on phosphatase activities of mycorrhizal and non-mycorrhizal onion roots inoculated with *G. margarita*. Root soluble extracts were separated SDS-PAGE and stained in 50 mM TRIS/citrate buffer (pH 8.5): **a** control, **b** 1 mM K_2HPO_4, **c** 10 mM $MgCl_2$. *M* Mycorrhizal, *P* non-mycorrhizal in the high phosphate medium, *C* non-mycorrhizal in the low phosphate medium

showed that a band specific to mycorrhizal roots was found at pH 8.5, but that there was no difference in the band pattern of phosphatases from non-mycorrhizal roots regardless of the level of phosphate in the medium (Fig. 2).

Characteristics of the phosphatase band specific to mycorrhizae at pH 8.5 was examined by SDS-PAGE in the staining solution added with 10 mM $MgCl_2$ or 1 mM K_2HPO_4 (Fig. 3). The activity of the mycorrhiza-specific phosphatase band was not affected by phosphate and a little enhanced by 10 mM $MgCl_2$. K_2HPO_4 and $MgCl_2$ inhibited other phosphatase bands to some degree.

The intraradical hyphae of *Gigaspora margarita* collected from onion mycorrhizae were almost free from cellular debris of host plant tissue when examined using a microscope. The amount of the collected hyphae was too small to be homogenized with liquid nitrogen. We employed the hyphal-wall digestive enzyme, fungase, in isotonic solution (0.3 M mannitol) and the following ultra-sonication step was carried out in hypotonic buffer. After a 1 h enzyme treatment, the hyphae were a little fragmented and showed no wall degradation when examined under an optical microscope. By cyto-/histochemical staining we observed that phosphatase activity was still present in the hyphae at this stage. The ultra-sonication of the treated hyphae in hypotonic buffer disrupted hyphal cells (Fig. 4) and little phosphatase activity was found in the hyphal

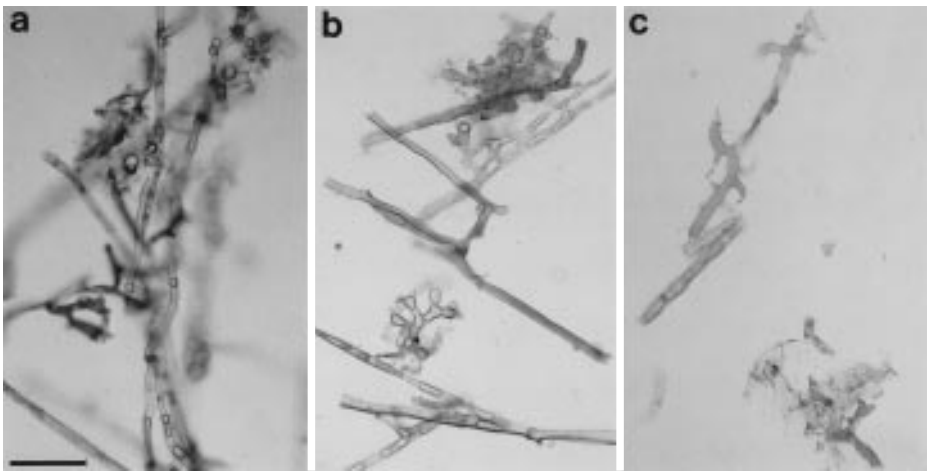

Fig. 4 a–c Intraradical hyphae of *G. margarita*. **a** Isolated hyphae, **b** treated with Fungase, **c** treated with Fungase and ultrasonication. *Bar* = 50 μm

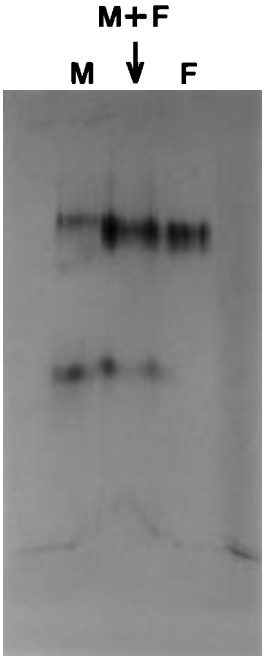

Fig. 5 Phosphatase activities of mycorrhizal onion roots and separated intraradical hyphae. The soluble extracts were separated by SDS-PAGE and stained in 50 mM TRIS/citrate buffer (pH 8.5). *M* Mycorrhizal, *F* separated intraradical hyphae, *M+F* mixture of both kept overnight at 4 °C

debris. This procedure enabled efficient extraction of soluble protein from a small quantity of hyphae.

The phosphatases in the soluble protein from the intraradical hyphae were examined by SDS-PAGE and subsequent active staining. Hyphal phosphatase at pH 8.5 showed a similar band pattern to those of mycorrhizal roots in the higher molecular weight range (Fig. 5). The relative migration of the hyphal phosphatase was slightly greater than that of the mycorrhiza-specific phosphatase in roots. The presumed molecular weight of the former phosphatase was 73 kDA, while that of the latter was 90 kDA. Migration of the hyphal phosphatase activity in the gel was stimulated by 10 mM $MgCl_2$, as occurred for the mycorrhiza specific phosphatase (data not shown). To clarify the difference between the mycorrhizal specific phosphatase and the hyphal phosphatase, the hyphal protein was mixed with the mycorrhizal root protein and kept overnight at 4 °C. The mixture was then run on SDS-PAGE and the phosphatase stained. When the mixture was run on SDS-PAGE immediately, after mixing the broad band which indicated the two phosphatases was found on the gel (data not shown). Overnight, however, the phosphatase band identical to that of the mycorrhizal root phosphatase disappeared, and only the band identical to the hyphal phosphatase remained (Fig. 5).

Discussion

In this experiment we succeeded in efficiently extracting soluble protein from a small amount of intraradical hyphae of *Gigaspora margarita* by using an enzyme treatment followed by ultra-sonication. Ultra-sonication has often been employed for the degradation of hyphal cells (Polacheck and Rosenberger 1975). However, the intraradical hyphae could not be degraded by ultra-sonication alone. In some fungi, disrupting the protoplasts by mechanical means is a more efficient method for extracting protein from a small amount of hyphae. Since the components of fungal cell walls differ among species, the protoplasts preparation of fungal cells needs to follow a protocol which depends on the species. Novozyme containing glucanase and chitinase has been used for preparing protoplasts (Barret et al. 1989; Yanai et al. 1990). We, however, used Fungase which contained chitinase produced by *Trichoderma harzianum*, because chitin is present, whereas $\beta(1\rightarrow3)$ glucan is absent in the hyphal wall of *Gigaspora* sp. (Bonfante-Fasolo et al. 1988; Grandmaison et al. 1988; Gianinazzi-Pearson et al. 1994). Fungase treatment for 1 h neither degraded the hyphal wall completely nor changed the hyphal phosphatase activity detected by cyto-/histochemistry, but it did facilitate the disruption of treated hyphae with further ultrasonication. This implied that the Fungase treatment caused some damage to hyphal walls. The protocol using the Fun-

gase treatment followed by ultra-sonication did not produce protoplasts from *Gigaspora margarita* hyphae, but was effective in extracting protein from a small amount of hyphae by disrupting damaged cell walls.

This study was an attempt to detect the hyphal phosphatase of an AM fungus in symbiosis with onion roots by electrophoresis. A mycorrhiza-specific band of phosphatase was found on the gel when the phosphatase was stained under alkaline conditions (Figs. 1, 2), and compares with the band of phosphatases derived from *Glomus mossae* (Gianinazzi-Pearson and Gianinazzi 1976). Alkaline phosphatase activity was cyto-/histochemically demonstrated to be localized in arbuscules and intraradical hyphae (Tisserant et al. 1993; Ezawa et al. 1995; Saito 1995), thus implying that the mycorrhiza-specific phosphatases are probably of intraradical hyphal origin. However, this study showed that the relative migration of the mycorrhiza-specific phosphatase during electrophoresis was slightly different from that of the hyphal phosphatase (Fig. 5). We found that the relative migration of the mycorrhizal phosphatase was identical to that of the hyphal phosphatase after mixing the protein from the roots and intraradical hyphae (Fig. 5). This result indicates that the mycorrhiza-specific phosphatase in the root extract originated from the intraradical hyphae. Many phosphatases are known to be glycosylated. Such sugar residues might be hydrolyzed with some enzymes contained in the hyphal extract but not with those in the root extract. This might cause the change in the relative migration of the mycorrhiza-specific phosphatase on the electrophoresis gel (Duff et al. 1994). Although it is unknown why the relative migration of the mycorrhizal-specific phosphatase was different at the many steps of the treatment, the results shown in Fig. 5 support the opinion that the mycorrhiza-specific band of phosphatase from root originated from that of the intraradical hyphae of the AM fungi.

The purification of the phosphatase and its characterization would provide greater understanding with respect to the mechanisms of the phosphorus transfer from AM fungi to host plant. However, it is very difficult to obtain a large mass of intraradical hyphae for the purification of the hyphal phosphatase, though a large mass of root extract may be obtained. Therefore, the hyphal phosphatase band from the mycorrhizal root extracts must be a useful index through the purification processes of the enzyme.

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References

Barret V, Lemke PA, Dixon RK (1989) Protoplast formation from selected species of ectomycorrhizal fungi. Appl Microbiol Biotechnol 30:381–387

Bonfante-Fasolo, Vian B, Testa B (1986) Ultrastructural localization of chitin in the cell wall of a fungal spore. Biol Cell 57:265–270

Capaccio LCM, Callow JA (1982) The enzymes of polyphosphate metabolism in vesicular-arbuscular mycorrhizas. New Phytol 91:81–91

Duff SMG, Sarth G, Plaxton WC (1994) The role of acid phosphatases in plant metabolism. Physiol Plant 90:791–800

Ezawa T, Yoshida T (1994a) Acid phosphatase specific to arbuscular mycorrhizal infection in marigold and possible role in symbiosis. Soil Sci Plant Nutr 40:655–665

Ezawa T, Yoshida T (1994b) Characterization of phosphatase in marigold roots infected with vesicular-arbuscular mycorrhizal fungi. Soil Sci Plant Nutr 40:255–264

Ezawa T, Saito M, Yoshida T (1995) Comparison of phosphatase localization in the intraradical hyphae of arbuscular mycorrhizal fungi, *Glomus* spp. and *Gigaspora* spp. Plant Soil 176:57–63

Ezawa T, Saito M, Hayatsu M, Yoshida T (1996) Characterization and localization of phosphatases in the plant and fungal symbionts of arbuscular mycorrhiza. Abstract, 1st Int Conf Mycorrhizae, Univ California, Berkely, p 48

Gianinazzi S (1991) Vesicular-arbuscular (endo-) mycorrhizas: cellular, biochemical and genetic aspects. Agric Ecosys Environ 35:105–119

Gianinazzi S, Gianinazzi-Pearson V, Dexheimer J (1979) Metabolism of vesicular-arbuscular mycorrhiza. III. Ultrastructural localization of acid and alkaline phosphatase in onion roots infected by *Glomus mosseae* (Nicol & Gred). New Phytol 82:127–132

Gianinazzi-Pearson V, Gianinazzi S (1976) Enzymatic studies on metabolism of vesicular-arbuscular mycorrhiza. I. Effect of mycorrhizal formation and phosphorus nutrition on soluble phosphatase activities in onion roots. Physiol Veg 14:833–841

Gianinazzi-Pearson V, Gianinazzi S (1978) Enzymatic studies on metabolism of vesicular-arbuscular mycorrhiza. II. Soluble alkaline phosphatase specific to mycorrhizal infection in onion roots. Physiol Plant Pathol 12:45–53

Gianinazzi-Pearson V, Gianinazzi S (1983) The physiology of vesicular-arbuscular mycorrhizal roots. Plant Soil 71:197–209

Gianinazzi-Pearson V, Lemoine MC, Arnould C, Gollotte A, Morton JB (1994) Localization of $\beta(1\rightarrow3)$ glucans in spore and hyphal walls of fungi in the Glomales. Mycologia 86:478–485

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol 84:489–500

Grandmaison J, Benhamou N, Furlan V, Visser SA (1988) Ultrastructural localization of *N*-acetylglucosamine residue in the cell wall of *Gigaspora margarita* throughout its life cycle. Biol Cell 63:89–100

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

Nanjo M (1986) Available phosphate. In: Japanese Society of Soil Science and Plant Nutrition (ed) Standard soil analysis (in Japanese). Hakuyusya Press, Tokyo, pp 127–130

Polacheck Y, Rosenberger RF (1975) Autolytic enzymes in hyphae of *Aspergillus nidulans*: their action on old and newly formed walls. J Bacteriol 121:332–337

Saito M (1995) Enzyme activities of intraradical hyphae and germinated spores of arbuscular mycorrhizal fungus, *Gigaspora margarita* Becker & Hall. New Phytol 129:425–431

Schwab SM, Menge JA, Tinker (1991) Regulation of nutrient transfer between host and fungus in vesicular-arbuscular mycorrhizas. New Phytol 117:387–398

Smith SE, Gianinazzi-Pearson V (1988) Physiological interactions between vesicular-arbuscular mycorrhizal plants. Annu Rev Plant Physiol Plant Mol Biol 39:221–244

Smith SE, Gianinazzi-Pearson V (1990) Phosphate uptake and arbuscular activity in mycorrhizal *Allium cepa* L.: effects of photon irradiance and phosphate nutrition. Aust J Plant Physiol 17:177–188

Smith SE, Read DJ (1996) Mycorrhizal symbiosis, 2nd edn. Academic Press, London, pp 379–408

Tisserant B, Gianinazzi-Pearson V, Gianinazzi S, Gollotte A (1993) *In planta* histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. Mycol Res 97:245–250

Yanai K, Horiuchi H, Takagi M, Yano K (1990) Preparation of protoplasts of *Rhizopus niveus* and their transformation with plasmid DNA. Agric Biol Chem 54:2689–2696