Title	Release of acid phosphatase from extraradical hyphae of arbuscular mycorrhizal fungus Rhizophagus clarus
Author(s)	Sato, Takumi; Ezawa, Tatsuhiro; Chenga, Weiguo; Keitaro, Tawaraya
Citation	Soil Science and Plant Nutrition, 61(2), 269-274 https://doi.org/10.1080/00380768.2014.993298
Issue Date	2015-03
Doc URL	http://hdl.handle.net/2115/61161
Rights	This is an Accepted Manuscript of an article published by Taylor & Francis in Soil science and plant nutrition on [date of publication], available online: http://www.tandfonline.com/10.1080/00380768.2014.993298
Type	article (author version)
File Information	Sato_SSPN.pdf

Release of acid phosphatase from extraradical hyphae of arbuscular mycorrhizal

fungus *Rhizophagus clarus*

Takumi SATO[1, 2], Tatsuhiro EZAWA[3], Weiguo CHENG[1], Keitaro

TAWARAYA[1*]

[1]Faculty of Agriculture Yamagata University, Tsuruoka, 997-8555, Japan

[2]The United Graduate School of Agricultural Sciences, Iwate University, Morioka,

020-8550, Japan

[3]Graduate School of Agriculture, Hokkaido University, Sapporo, 060-8589, Japan

*Corresponding author: Faculty of Agriculture, Yamagata University, Tsuruoka

997-8555, Japan.

tawaraya@tds1.tr.yamagata-u-ac.jp,   Telephone: +81-235-28-2870;   Facsimile:

+81-235-25-8578)

Running title: Acid phosphatase from extraradical hyphae

Abstract

Arbuscular mycorrhizal (AM) fungi enhance plant uptake of available phosphorus (P) from soil through their extraradical hyphae. The mechanism underlying this P uptake enhanced by AM fungi is the increase in the surface area for absorption of available P. Little is known about utilization of unavailable P by AM fungi. We investigated whether extraradical hyphae of AM fungi release ACP. Sterilized Andosol was packed in pots that were separated into the mycorrhizal and hyphal compartments with a nylon net of 30 μm pore size. Seeds of *Allium fistulosum* L. were inoculated or uninoculated with the AM fungus *Rhizophagus clarus*. Mullite ceramic tubes were buried in the soil of each compartment, and soil solution was collected. *A. fistulosum* L. and *Linum usitatissimum* L. inoculated with *R. clarus* were grown in sand culture and *in vitro* monoxenic culture, respectively. Uninoculated *A. fistulosum* L was grown in hydroponic culture to collect root exudate. The soil solution, hyphae extracts, root extract, and root exudates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Shoot P concentration, shoot P content, and shoot dry weight were higher in the inoculated treatment than in the uninoculated treatment. Activity staining of the gel revealed that ACP activity at 187 kDa was observed the soil solution in the inoculation treatment and hyphal extract collected from sand culture and *in vitro* monoxenic culture, but neither in the exudate of non-mycorrhizal roots grown in the hydroponic culture nor in the root extracts irrespective of mycorrhizal status. Those results provide strong evidence that the corresponding activity in the soil solutions in soil culture is of *R. clarus* CK001 origin. These findings suggested that the fungus releases ACP from extraradical hyphae into the hyphosphere.

Key words: arbuscular mycorrhizal fungi, hyphal exudate, soil solution, acid phosphatase, compartment pot

INTRODUCTION

Phosphate fertilizer is produced from phosphate rock. Despite the fact that phosphate

fertilizer demand is projected to increase, global phosphate fertilizer production is

estimated to peak around 2030, and phosphate rock may be depleted 50-100 years later

(Cordell *et al*. 2009). Two strategies in tackling depletion of phosphate rock are

considered. One is to develop plants that can recycle phosphorus (P) more efficiently

within their body. The other one is to develop plants that can take up phosphate more

efficiently from the soil. Concentration of plant-available phosphate i.e. free inorganic

orthophosphate (P_i) in the soil is usually very low in most ecosystems, which frequently

limits plant growth. On the other hand, large parts of phosphate in the soil are present as

plant-unavailable forms: sparingly soluble inorganic phosphate and organic phosphate.

In particular, organic phosphate accounts for 20-80% of total phosphate in the soil

(Dalal 1977), and thus conversion (hydrolysis) of organic phosphate into plant-available

Pi is a key to sustainable food production.

 Plants and fungi possess many genes encoding acid phosphatase (EC 3.1.3.2, ACP) in

the genomes, e.g., 29 genes in the plant *Arabidopsis thaliana* (Wang *et al*., 2014) and

at least 7 genes are expressed in *R. clarus* (e.g. Kikuchi and Ezawa, 2012, unpublished

data). Secretion of ACP into rhizosphere/hyphosphere, however, has been

demonstrated only in limited ranges of plant (e.g., Tadano and Sakai 1991; Tarafdar

and Claassen 1988) and fungi (e.g., Crowther *et al*. 2011; Tarafdar *et al*. 1988; van

Aarle and Plassard 2010). In plants, secreted ACP hydrolyzes organic phosphate,

increases the Pi pool in the rhizosphere, and thus enhances P_i uptake (Wasaki *et al.*

2009; Maruyama *et al.* 2012; Robinson *et al.* 2012).

 AM fungi associate with 80% of land plants and enhance P_i uptake of plants from the

soil through their extraradical hyphae (Smith and Read 2008). It has been considered that the increase in surface area for P_i uptake is the main mechanism underlying the enhanced Pi uptake in the associations. In addition to the mechanism, the involvement of AM fungal ACP in the enhanced Pi uptake has also been proposed. Tarafdar and Marschner (1994) employed the compartment culture system in which the mycorrhizal (i.e. roots + hyphae) and hyphal compartments were separated by a 30-μm nylon mesh and observed higher ACP activity in the hyphal compartment in the presence of an AM fungus *Glomus mosseae*, suggesting that the fungus secreted ACP from extraradical hyphae. Koide and Kabir (2000) demonstrated hydrolysis of organic phosphate in the hyphal compartment of the *in vitro* two-compartment monoxenic culture of *G. intraradices* with a concurrent increase in root P content, suggesting that plants can access to organic phosphate via fungal ACP secretion. Joner and Johansen (2000) found that fungal ACP was mostly associated with the cell wall and released ACP was undetectable, which was also reproduced by Olsson *et al.* (2002).

In plants, not only cell wall-bound ACP but also released ACP plays a significant role in organic P hydrolysis in the rhizosphere. In *Arabidopsis thaliana,* two purple acid phosphatases encoded by *AtPAP12* and *AtPAP26* are released from the roots (Tran *et al.*, 2010), and the corresponding knockout mutants showed poorer growth than the wild type in the presence of organic P (fructose-6-phosphate) as the sole P source in the medium, suggesting that ACP released from the roots greatly contributes to plant growth (Wang *et al.*, 2014). Although these observations led us to hypothesize that AM fungi also release ACP into the soil solution, a technical breakthrough was necessary for the collection and detection of released ACP.

The exudation of organic acid by extraradical hyphae of an AM fungus has been

successfully demonstrated in the soil solution collected using mullite ceramic tubes in a two-compartment culture system (Tawaraya *et al.*, 2006). In the present study, this collection technique was employed for the qualitative assessments of AM fungal ACP exuded in the soil solution to test the hypothesis that AM fungi exude soluble ACP from extraradical hyphae.

MATERIALS AND METHODS

Two-compartment culture for collection of soil solution

Andosol was collected from a native pasture in Haguromachi, Yamagata prefecture, Japan. The soil was air-dried in glass house, sieved on a 2 mm sieve, and steam-sterilized twice at 80°C for 45 min. Chemical properties of the soil were as follows: pH (H_2O), 4.78; Organic carbon, 10.9%; total nitrogen, 0.88%; Truog-P, 5.02 mg P kg soil^{-1}; cation exchange capacity, 43.2 cmol (+) kg soil^{-1}. The soil was fertilized with ammonium sulfate, potassium sulfate, and superphosphate at rates of 1.00 g nitrogen (N), 0.83 g potassium (K), and 0.15 g P kg^{-1} soil, respectively, and pH was adjusted to pH 5.1 with calcium carbonate at a rate of 4.19 g kg^{-1} soil. The AM fungus *Rhizophagus clarus* (T.H. Nicolson & N.C. Schenck) C. Walker & A. Schüßler strain CK001 was propagated with Welsh onion (*Allium fistulosum* L. cv. Motokura), sorghum (*Sorghum bicolor* (L.) Moench. cv. New sorghum 2 gou), and white clover (*Trifolium repens* L. cv. California ladino) for 3 months, and the soil that includes spores, extraradical hyphae, and the roots was used as inoculum. The two-compartment pots were prepared as described by Tawaraya *et al.* (2006). A nylon bag (top 140 mm, bottom 80 mm, height 90 mm) was prepared with a 30 μm nylon net (NY30HD, Sefar Inc., Heiden, Switzerland), though which only fungal hyphae are able to pass, but not

plant roots, filled with soil-inoculum layers as follows: 20 g of the sterilized in the bottom, 10 g of the inoculum as the second layer, 20 g of the sterilized soil as the third layer, 10 g of the inoculum as the fourth layer, and then covered with 40 g of the sterilized soil (mycorrhizal compartment). For the uninoculated treatment, no inoculum layers were incorporated in the compartment. The nylon bag was placed at the center of a 500 mL plastic pot (11.5× 9.5 cm), and the remaining space was filled with 300 g of the sterilized soil (hyphal compartment). Each six mullite ceramic tubes (50 × 2.5 mm, 1DH-1525, Sakaguchi E.H. VOC Corp., Tokyo, Japan) connected to Teflon tubes (150 × 1.5 mm) were embedded in the mycorrhizal and hyphal compartments at intervals of 2 and 3 cm in a circular pattern, respectively (Tawaraya *et al.* 2006). There were five replications of each treatment. Twelve seeds of Welsh onion were sown in the mycorrhizal compartment at 1 cm depth, irrigated with deionized water to maintain the water potential at −0.03 Mpa, and grown in a 16 h photoperiod (150 μmol m^{-2} s^{-1}) at 27˚C/25˚C (light/dark) in a growth chamber. The pots were irrigated with deionized water every other day. Each pot was covered until germination, and plants were thinned to six per pot. A plastic syringe (5 mL plastic syringe, TOP Co., Ltd., Tokyo, Japan) was connected to a Teflon tube, and 1 mL of soil solution was collected into the syringe by pulling the piston several times. The solution was collected from all tubes 40, 45, 50, and 55 days after sowing, stored at -30˚C, and pooled before concentration. Shoots and roots were harvested 55 days after sowing. Shoots were weighted after drying at 70˚C for 72 h and were used for plant P assessment. Roots were washed with tap water, wiped with a paper towel, weighed, and used for enzyme preparation and assessment of mycorrhizal colonization.

Sand culture for preparation of extraradical hyphae

Welsh onion inoculated with *R. clarus* CK001 was grown on sterilized (121°C, 45 min.) sea sand in a glass house. A nutrient solution (40 mg N L^{-1} ($NH_4 NO_3$), 20 mg N L^{-1} ($NaNO_3$), 60 mg K L^{-1} (K_2SO_4), 80 mg Ca L^{-1} ($CaCl_2$), 40 mg Mg L^{-1} ($MgSO_4$), 2 mg Fe L^{-1} ($FeSO_4$), 1 mg Mn L^{-1} ($MnSO_4$), 0.01 mg Cu L^{-1} ($CuSO_4$), 0.005 Mo mg L^{-1} [$(NH_4)_6Mo_7O_{24}$], 0.4 mg B L^{-1} (H_3BO_3), 0.2 mg Zn L^{-1} ($ZnCl_2$), and 1 mg P L^{-1} (NaH_2PO_4)) (Wagatsuma *et al.* 1988) was applied to plants every other day. Extraradical hyphae were collected 45 days after sowing by wet sieving, wiped with a paper towel, weighed, stored at -30°C, and used for enzyme extraction.

In vitro **monoxenic culture for preparation of extraradical hyphae**

Agrobacterium rhizogenes-transformed roots (hairy roots) of flax (*Linum usitatissimum* L.) was purchased from Glomeromycota *in vitro* collection (http://www.mycorrhiza.be/ginco-bel/) were inoculated with surface-sterilized *R. clarus* CK001 spores and grown on modified Strullu–Romand (MSR) medium (Declerck *et al.* 1998) for 60 days in 9 cm Petri dishes according to Chabot *et al.* (1992). After removing the roots with tweezers, the culture medium of 30 Petri dishes was transferred to 1 L of 10 mM sodium citrate (pH 6.0) to solubilize the Gellan Gum medium (Doner and Becard 1991). The extraradical hyphae were collected on a 30 μm nylon net, washed with sterilized water, wiped with a paper towel, weighed, and used for enzyme extraction.

Hydroponic culture for collection of root exudate

Seeds of Welsh onion were sown on moist vermiculite spread. One week after sowing,

30 seedlings were carefully lifted out of the vermiculite, fixed with a sponge in a paper cup filled with 200 mL of the nutrient solution without P, and grown in the growth chamber (light 16 h / dark 8 h, 150 μmol m^{-2} s^{-1}, 27°C (light)/ 25°C (dark)). The pH of the nutrient solution was adjusted daily to 5.0 ± 0.05 with 0.5 M H$_2$SO$_4$ and 0.5 M NaOH. The nutrient solution was aerated with an air pump and replaced weekly. Thirteen days after transplanting, the seedlings were transferred to 50 ml of sterile deionized water and incubated for 12 h under the same conditions to collect root exudate, and then the solution was stored at –30°C and used for qualitative analysis.

Enzyme preparation

Soil solution and root exudates were first concentrated over 200-fold using Amicon® Ultra-15 centrifugal filter units (Millipore, MA, U.S.A.), further to 1800- and 500-fold, respectively, using Microcon® YM-30 centrifugal filter units (Millipore Corporation, MA, U.S.A.), and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Roots and extraradical hyphae were ground using mortar and pestle at 0°C with an equal volume (wt/vol) of 100 mM borate buffer (pH 8.8) with 10 μL mL^{-1} protease inhibitor (Plant ProteaseArrest™, Takara Bio Inc., Shiga, Japan). The resulting slurry was transferred to a 1.5 mL tube and centrifuged at 15,000 × g for 30 min at 4°C. The supernatant of root extract was transferred to a new tube and subjected to SDS-PAGE. The supernatant of hyphal extract was concentrated 24 fold using Microcon® YM-30 centrifugal filter units and subjected to SDS-PAGE.

Qualitative analysis of acid phosphatase

SDS-PAGE was performed as described by Ezawa and Yoshida (1994). Ten microliter each of the soil solution, root exudate, hyphal extract and root extract were loaded on a 10% SDS-polyacrylamide gel without denaturing and electrophoresed. The gel was gently shaken in washing buffer [50 mL of 100 mM acetate/sodium buffer (pH 5.0) containing 0.5 mL of Triton-X] for 30 min, and ACP activity on the gel was visualized by the azo dye method (Scandalios 1969). The molecular weight of protein was estimated based on mobility relative to those of the protein standards (Takara bio inc. Shiga, Japan) on the gel.

Assessment of plant P and mycorrhizal colonization

Ground shoots were digested in a HNO_3-$HClO_4$-H_2SO_4 (5:2:1) solution. The P content in the digested solution was determined colorimetrically by vanadomolybdate-yellow assay (Olsen and Sommers, 1982).

Percent AM colonization was determined using the gridline intersect method (Giovannetti and Mosse 1980) after root staining with 500 mg L^{-1} aniline blue solution (Tawaraya *et al*. 1998). One hundred root segments from each replicate bag were observed under a microscope (magnification, $\times$ 100).

ANOVA with Tukey-HSD test ($p < 0.05$) was performed with Kaleida Graph 4.0j (HULINKS Inc., Tokyo, Japan).

RESULTS

Qualitative characterization of acid phosphatase released from mycorrhiza

AM colonization was observed in the inoculated treatment 55 days after sowing, but not in the uninoculated treatment. Shoot P concentration, P content, and dry weight

were higher in the inoculated treatment than in the uninoculated treatment (Table 1).

Activity staining of the gel revealed that ACP activity at 187 kDa was observed exclusively in the presence of the fungus in the solutions obtained from the mycorrhizal and hyphal compartments in the soil culture (Fig. 1a). This activity was also detected in the extracts of extraradical hyphae grown both in the sand culture and the *in vitro* monoxenic culture, but neither in the exudate of non-mycorrhizal roots grown in the hydroponic culture nor in the root extracts irrespective of mycorrhizal status (Figs. 1b and c). ACP activity at 41 kDa was observed in all samples irrespective of mycorrhizal status, except for the root extracts.

DISCUSSION

Release of ACP from extraradical hyphae of AM fungus *R. clarus*

The detection of the ACP activity of 187 kDa in the *in vitro* culture provides strong evidence that the corresponding activity in the soil solutions in soil sand culture is of *R. clarus* CK001 origin, suggesting that the fungus releases ACP from extraradical hyphae into the hyphosphere. This finding is further supported by the absence of the activity in the root exudate and extract. The detection of the ACP activity in the *in vitro* culture also excludes the possibility that the activity was of other microorganisms present in the rhizosphere/hyphosphere.

We demonstrated for the first time using mullite ceramic tubes in conjunction with SDS-PAGE that AM fungi release ACP from the extraradical hyphae to the hyphosphere. Our results has been suggested that a large part of extracellular ACP is bound to the cell wall in AM fungi, because released ACP fraction collected from the culture medium i.e. polysaccharide gel in the *in vitro* culture (Koide and Kabir, 2000;

Olsson *et al*., 2002) or that collected by 1 h incubation of hyphae in a buffer (Joner and Johansen, 2000) showed little activity.   The successful detection of released ACP activity in the present study was likely to be achieved by the concentration of the activity up to three orders of magnitude by ultrafiltration.

Although, fungi have several ACP orthologus on genome (for example, at least 7 genes are expressed in *R. clarus*, e.g. Kikuchi and Ezawa, 2012, unpublished data), molecular characterization has been rarely reported. Additionally, mobility on the PAGE analysis is modified by glycosylation (Weber and Pitt 1997). Thus, estimation of gene is impossible. The identification of the ACP of 187 kDa is necessary using immunological approach and genetic information of known ACPs.

Although ACP of 41 kDa was detected in all soil solution irrespective of mycorrhizal status, it seems unlikely that the activity is solely of the host plant Welsh onion. This is because the activity was detected not only in the hyphal extract in the sand culture but also that in the *in vitro* culture, in which different host plants, onion for the sand culture and flax for the *in vitro* culture, were used to grow the fungus, raising two possibilities; the two plants exude ACPs of similar molecular weight that migrate at similar rates on the gel, or onion and the fungus possess ACPs of similar molecular weight, which were in either case indistinguishable in electrophoresis. The ACP of 41 kDa was detected in root exudates, but not root extracts. The reason of incompatible result might be different mobility caused by formation of dimer (Ozawa *et al*., 1995) or glycosylation (Weber and Pitt 1997). Further biochemical characterization and identification of genes encoding these ACPs would be necessary to test the possibilities.

 Our results indicate that the AM fungus *R. clarus* CK001 releases ACP from its

extraradical hyphae. It has been considered that increase in surface area for P_i uptake by extraradical hyphae is the main mechanism underlying the enhanced P_i uptake in the mycorrhizal plants. The present study can propose a new mechanism that extraradical hyphae of AM fungi hydrolyze soil organic phosphate and improve P acquisition. The hydrolysis of soil organic phosphate with released ACP and uptake of the resultant P_i by extraradical hyphae remain to be clarified.

ACKNOWLEDGMENTS

REFERENCES

Chabot S, Becard G. & Piche Y. 1992: Life cycle of *Glomus intraradix* in root organ culture. *Mycologia* **84,** 315–321.

Cordell D, Drangert J O, White S 2009: The story of phosphorus: global food security and food for thought. *Glob. Environ. Chang.*, **19,** 292-305.

Crowther T W, Jones T H, Boddy L, Baldrian P 2011: Invertebrate grazing determines enzyme production by basidiomycete fungi. *Soil Biol. Biochem.,* **43,** 10 2060-2068.

Dalal R C 1977: Soil organic phosphorus. *Adv. Agron.,* **29,** 83-117.

Declerck S, Strullu D G, Plenchette C 1998: Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. *Mycologia,* **90,** 579–585.

Doner LW Becard G 1991: Solubilization of gellan gels by chelation of cations. *Biotechnol. Tech.,* **5:** 25–29

Ezawa T and Yoshida T 1994: Characterization of phosphatase in marigold roots
infected with vesicular-arbuscular mycorrhizal fungi. *Soil Sci. Plant Nutri.* **40,**
255-264.

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

Joner E J, Johansen A 2000: Phosphatase activity of external hyphae of two arbuscular
mycorrhizal fungi. *Mycol. Res.,* **104,** 81-86.

Koide R T, Kabir Z 2000: Extraradical hyphae of the mycorrhizal fungus *Glomus
intraradices* can hydrolyse organic phosphate. *New Phytol.,* **148,** 511-517.

Maruyama H, Yamamura T, Kaneko Y, Matsui H, Watanabe T, Shinano T, Osaki M,
Wasaki J 2012: Effect of exogenous phosphatase and phytase activities on organic
phosphate mobilization in soils with different phosphate adsorption capacities. *Soil
Sci. Plant Nutr.,* **58,** 41-51.

Olsen S R and Sommers L E 1982: Phosphorus. In Methods of soil analysis. Part 2.
Chemical and microbiological properties, ed. A. L. Page, 403–430. Madison, WI:
American Society of Agronomy.

Olsson P A, van Aarle I M, Allaway W G, Ashford A E, Rouhier H 2002: Phosphorus
effects on metabolic processes in monoxenic arbuscular mycorrhiza cultures. *Plant
physiol.,* **130,** 1162-1171.

Ozawa K, Osaki M, Matsui H, Honma M, Tadano T. 1995: Purification and properties
of acid phosphatase secreted from lupin roots under phosphorus-deficiency
conditions. *Soil Sci. Plant Nutr.,* **40,** 461-469.

Robinson W D, Park J, Tran H T, Del Vecchio H A, Ying S, Zins J L, Patel K,
McKnight T D, Plaxton W C 2012: The secreted purple acid phosphatase isozymes

AtPAP12 and AtPAP26 play a pivotal role in extracellular phosphate-scavenging by *Arabidopsis thaliana*. *J. Exp. Bot.,* **63,** 6531-6542.

Scandalios J G 1969: Genetic control of multiple molecular forms of enzymes in plants: A review. *Biochem. Genet.,* **3,** 37-79.

Smith S E, Read D J 2008: *Mycorrhizal symbiosis*, 3rd ed. Academic Press, London, UK

Tarafdar J C, Claassen N 1988: Organic phosphorus compounds as a phosphorus source for higher plants through the activity of phosphatases produced by plant roots and microorganisms. *Biol. Fertil. Soils,* **5,** 308-312.

Tarafdar J C, Marschner H 1994: Phosphatase activity in the rhizosphere and Hyphosphere of VA mycorrhizal wheat supplied with inorganic and organic phosphorus. *Soil Biol. Biochem.,* **26,** 387-395.

Tawaraya K, Hashimoto K, Wagatsuma T 1998: Effect of root exudate fractions from P-deficient and P-sufficient onion plants on root colonisation by the arbuscular mycorrhizal fungus Gigaspora margarita. *Mycorrhiza,* **8,** 67–70.

Tawaraya K, Naito M, Wagatsuma T 2006: Solubilization of insoluble inorganic phosphate by hyphal exudates of arbuscular mycorrhizal fungi. *J. Plant Nutr.,* **29,** 657-665.

Tran H T, QIAN W Q, Hurley B A, She Y M, Wang D W, Plaxton W C 2010: Biochemical and molecular characterization of AtPAP12 and AtPAP26: the predominant purple acid phosphatase isozymes secreted by phosphate-starved *Arabidopsis thaliana. Plant Cell Environ.,* **33,** 1789-1803.

van Aarle I M, Plassard C 2010: Spatial distribution of phosphatase activity associated with ectomycorrhizal plants is related to soil type. *Soil Biol. Biochem.,* **42,** 324-330.

Wagatsuma T, Kawashima T, Tawaraya K 1988: Comparative stainability of plant root cells with a basic dye (methylene blue) in association with aluminum tolerance. *Commun. Soil Sci. Plant Anal.*, **19,** 1207-1215

Wang LS, Lu S, Zhang Y, Li Z, Du XQ, Liu D 2014: Comparative genetic analysis of Arabidopsis purple acid phosphatases AtPAP10, AtPAP12, and AtPAP26 provides new insights into their roles in plant adaptation to phosphate deprivation. *J. Integr. Plant Biol.*, **56,** 299-314

Wasaki J, Maruyama H, Tanaka M, Yamamura T, Dateki H, Shinano T, Ito S, Osaki M 2009: Overexpression of the *LASAP2* gene for secretory acid phosphatase in white lupin improves the phosphorus uptake and growth of tobacco plants. *Soil Sci. Plant Nutr.,* **55,** 107-113.

Weber RWS, Pitt D 1997; Purification, characterization and exit routes of two acid phosphatases secreted by *Botrytis cinerea. Mycol. Res.*, **101,** 1431-1439.

Table 1 AM colonization, shoot P concentration, shoot P content, and shoot dry weight of Welsh onion without (-) or with (+) AM fungus *R. clarus* CK001 inoculation 55 days after sowing. S.E. indicates standard error. Values followed by a different letter are significantly different ($P<0.05$).

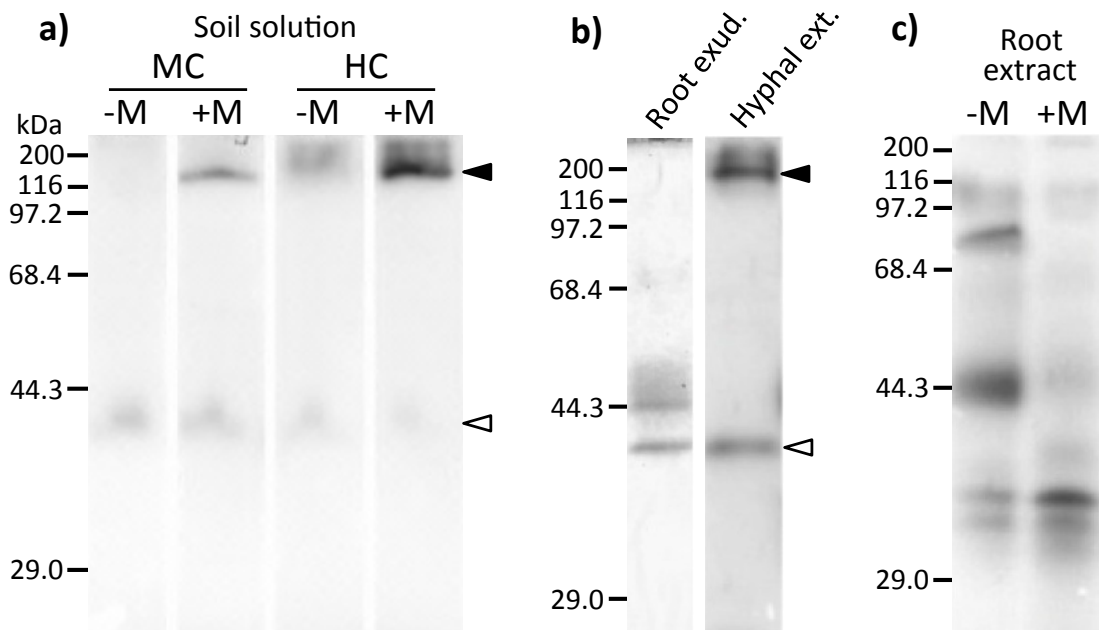| AMF	AM colonization (%)	± S.E.		Shoot P concentration (mg P g^{-1})	± S.E.		Shoot P content (mg P pot^{-1})	± S.E.		Shoot dry weight (mg pot^{-1})	± S.E.	
-	0	± 0	b	0.825	± 0.086	b	0.0473	± 0.0044	b	58.0	± 3.7	b
+	87	± 2	a	2.422	± 0.261	a	0.7686	± 0.0320	a	325.2	± 24.2	a

Fig. 1 SDS-PAGE analysis of ACP activity of 1800-fold concentrated soil solution collected from the mycorrhizal compartment (MC) and hyphal compartment (HC) of Welsh onion inoculated with (+M) or without (-M) *R. clarus* CK001 (a), 24-fold concentrated extraradical hyphal extract from *R. clarus* CK001 collected from sand culture (Hyphal ext. sand) or *in vitro* monoxenic culture (Hyphal ext. *in vitro*), 500-fold concentrated root exudates (Root exud.) from Welsh onion grown on P-free nutrient solution (b), and root extract from Welsh onion inoculated with (+M) or without (-M) AM fungus *R. clarus* CK001 (c). Black and white arrows indicate fungal ACP activity.