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Phosphatase activities of arbuscular mycorrhizal intraradical and extraradical mycelium, and their relation to phosphorus availability

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We investigated the influence of changes in external phosphorus (P) concentration on the proportion of phosphatase-active structures of the arbuscular mycorrhizal fungus *Gigaspora margarita* associated with *Allium cepa*. The P treatment was started when mycorrhizal colonisation had been established, and plant systems were harvested three times after the start of the P treatment. Higher shoot dry weights and P contents were observed in the high-P treated plants at the last harvest. We did not find any change in the proportion of phosphatase-active extraradical mycelium following P treatment. However, the proportion of alkaline phosphatase-active mycelium was positively correlated for extraradical and intraradical mycelium. Also, the proportion of alkaline phosphatase-active arbuscules seemed to increase with the shoot fresh weight, whereas the proportion of acid phosphatase-active arbuscules decreased with higher shoot P concentration and dry weight. We have shown experimentally that the intraradical mycelium of *G. margarita*, but not the extraradical mycelium, responds metabolically to plant P concentration, and possibly also to external P availability.

INTRODUCTION

Decreased soil phosphorus (P) may lead to an increase in the colonisation of plants by arbuscular mycorrhizal (AM) fungi (Abbott, Robson & de Boer 1984, Thomson, Robson & Abbott 1991, Tawaraya *et al.* 1994). The AM fungal association often improves plant P uptake under P-limited conditions (Smith & Read 1997, Dickson, Smith & Smith 1999a, b). It has recently been shown that a phosphate transporter gene in the extraradical mycelium of *Glomus intraradices* is expressed at low phosphate concentration in the growth medium (Maldonado-Mendoza, Dewbre & Harrison 2001). When grown under P-limited conditions the production of phosphatase enzymes by many fungi increases (Grotelueschen *et al.* 1994, Kaffman *et al.* 1994, O'Neill *et al.* 1996), although such regulation has not been demonstrated for mycorrhizal fungi. The acid phosphatase (ACP) of intraradical AM fungal hyphae is located mainly in the vacuoles and sometimes in arbuscules (Gianinazzi, Gianinazzi-Pearson & Dexheimer 1979, Ezawa, Saito & Yoshida 1995, Saito 1995). It has been hypothesized that ACP could be involved in the hydrolysis of vacuolar polyphosphate after which inorganic

P could be released by the fungus into the plant–fungal interface (Ezawa, Smith & Smith 2001). The presence of ACP activity has also been demonstrated in extraradical mycelium (van Aarle, Olsson & Söderström 2001) and alkaline phosphatase (ALP) activity has been found in both intraradical (Gianinazzi *et al.* 1979, Smith & Gianinazzi-Pearson 1990, Tisserant *et al.* 1993) and extraradical AM fungal mycelium (Boddington & Dodd 1999, van Aarle *et al.* 2001). It is assumed that the ALP of the intraradical mycelium is involved in the transfer of P from the fungus to the plant (Tisserant *et al.* 1993), since a relationship has been found between the ALP of the intraradical mycelium and P transfer (Tisserant, Gianinazzi & Gianinazzi-Pearson 1996).

It has been suggested that the location of phosphatases may reflect the site for P transfer (Ezawa *et al.* 1995), but no experimental data on the relationship between external P concentration and the proportion of phosphatase-active extraradical and intraradical AM fungal mycelium are available. In the present work we studied the influence of external P availability on the relative amount of phosphatase-active structures of an AM fungus, in order to illustrate the P metabolism of an AM fungal symbiosis. We compared extraradical and

intraradical mycelium associated with plants established under P-limited conditions, and then exposed these plants either to increased P availability or to P depletion. We then examined whether the proportions of phosphatase-active mycelium and arbuscules were related to P availability or to fungal P transfer.

MATERIALS AND METHODS

Experimental set-up and harvesting

Seeds of *Allium cepa* (cv. 'Senshuchukou') were surface-sterilized and incubated for 12 d in the dark at 23 °C during the daytime and 20 ° during the night. 50 ml tubes (disposable polypropylene syringes without a plunger: Iwaki, Japan) were painted black to prevent light transmission and filled with 75 g of washed autoclaved river sand. One seedling was transplanted into each tube and, for the mycorrhizal treatment, 30 spores of *Gigaspora margarita* (MAFF 520054) were placed below the root at the time of transplantation. Spores were collected from a soil culture with *Sorghum bicolor* as host. To all tubes, 10 ml of a nutrient solution with 1 mg P l⁻¹ (low-P treatment) was added containing (mg l⁻¹): NH₄NO₃ (114.3), NaNO₃ (121.3), NaHPO₄·12H₂O (11.6), K₂SO₄ (133.7), CaCl₂ (221.5), MgSO₄·7H₂O (202.8), Fe-EDTA (15.1), MnCl₂·4H₂O (3.6), CuSO₄·5H₂O (0.039), Na₂MoO₄·2H₂O (0.0126), H₃BO₃ (2.29) and ZnCl₂ (0.417), and the pH was adjusted to approx. 6.5. The tubes were kept in a growth cabinet (23/20 °, 14 h photoperiod and a photosynthetic photon flux density of 465 µmol m⁻² s⁻¹) and watered twice a week with 10 ml of the nutrient solution.

Three mycorrhizal and three non-mycorrhizal plants were harvested 37 d after transplantation. The fresh weight of the plant and the dry weight of the shoot were recorded. For the mycorrhizal plants, the extraradical mycelium was collected from the sand. After staining with trypan blue (adapted from Phillips & Hayman 1970), root colonisation and arbuscule formation were assessed with the magnified intersections method (McGonigle *et al.* 1990).

The remaining mycorrhizal plants were randomly divided into two groups, each group receiving a different kind of P treatment. One group was watered twice a week with the nutrient solution containing no P (no-P treatment), while the other group was watered with the nutrient solution containing a five times increased P concentration (5 mg P l⁻¹; high-P treatment). At 42, 49 and 56 d after transplantation, 3 mycorrhizal plants from each P treatment were harvested. Two non-mycorrhizal plants were watered with the low-P solution and harvested 56 d after transplantation.

Collection of intraradical mycelium

Fresh root samples (cleaned from debris, extraradical mycelium and spores) were kept on ice, cut into pieces 5 mm in length and placed in 10 ml of a freshly prepared

filtered enzyme digestion solution, as described by Saito (1995). The samples were placed under vacuum for 8 min, covered with Parafilm, and then incubated at 30 ° for 2 h on a reciprocal shaker at 120 strokes min⁻¹. The digested root samples were collected on a 45 µm nylon mesh, washed clean with a washing buffer (Saito 1995) and kept on ice. The root cortex was opened under a dissecting microscope and removed with two pairs of fine forceps. The intraradical mycelium was carefully separated from the stele and transferred to fresh washing buffer.

Enzyme histochemical staining

The intraradical and extraradical mycelium was subjected to histochemical assessment of ACP and ALP activity. For ALP activity a 0.1 M Tris-HCl buffer (pH 8.5) with 1.1 mg ml⁻¹ α-naphthyl phosphate Na salt (Sigma) and 1.0 mg ml⁻¹ fast blue RR (Sigma) was used, while for ACP activity the Tris-HCl buffer was replaced by a 0.1 M sodium acetate buffer (pH 5.5). The samples were incubated for 1 h at 30 °, washed with deionised water, counterstained with acid fuchsin in lactic acid and stored in the stain (Saito, Stribley & Hepper 1993). The samples were mounted on microscope slides with lactoglycerol. Fast blue RR precipitation of the samples was assessed with a compound light microscope (Nikon Eclipse E800) with a bright field view at 400 times magnification. For each sample, approx. 200 hyphal intersections were assessed. Intersections of hyphae or arbuscules were classified as active or non-active, depending on whether they exhibited fast blue RR precipitation or not (see Fig. 1). The proportion (%) of ACP- and ALP-active hyphae and arbuscules was determined. Images were taken with a SPOT RT camera (Diagnostic Instruments, Burrough, MN).

P determination

Dried shoot material (10–50 mg) was digested (with H₂SO₄ and H₂O₂) at 200 ° according to the method of Mizuno & Minami (1980). Colorimetric determination of phosphate was carried out according to Watanabe & Olsen (1965) with a spectrophotometer by measuring the absorbance at 710 µm 15 min after mixing the samples with the chromophoric solution (ammonium molybdate, potassium antimony tartrate and ascorbic acid).

Statistical analysis

Means, standard errors and linear regression coefficients were calculated. To test for effects of P treatment (no-P and high-P) the data were subjected to a 1-tailed *t* test with unequal variances, for shoot dry weight and shoot P content, and a 2-tailed *t* test with unequal variances for the proportion of phosphatase-active structures. To compare the proportion of ACP-active structures with that of ALP-active structures, the data were subjected to a 2-tailed paired *t* test with unequal variances.

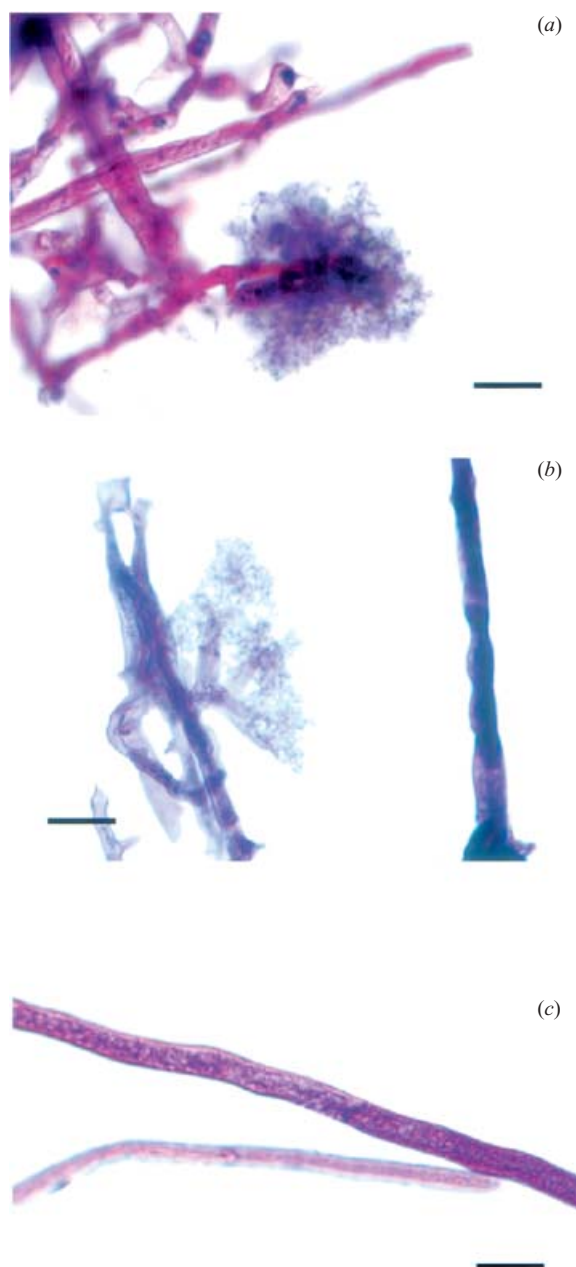


Fig. 1. Extraradical and intraradical mycelium of *Gigaspora margarita* collected from enzymatically digested roots of *Allium cepa*. The mycelium was stained with fast blue RR to visualise alkaline (ALP) and acid (ACP) phosphatase activity (dark precipitate) and counterstained with acid fuchsin (magenta). In (a) intraradical hyphae with low and an arbuscule with high ALP activity are shown; in (b) the intraradical hyphae show high and the arbuscule low ACP activity. Extraradical mycelium with some ACP activity is shown in (c). Bars = 20 μ m.

RESULTS

Growth and phosphatases

At the final harvest (56 d), the shoot dry weight of the high-P treated plants was significantly higher than that of the no-P treated plants (Table 1). High-P treated plants also had a higher shoot weight than the

non-mycorrhizal plants at 56 d. The P content of the shoot increased with time, and a greater ($P=0.056$) increase was observed for the high-P treated plants (Table 1).

Root colonisation and arbuscule formation by *Gigaspora margarita* were 33 and 24%, respectively, at the start of the P treatment. After a further slight increase in colonisation, both root colonisation and arbuscule formation seemed to stabilise. Mean values for root colonisation were between 40 and 55% of the total root length, while arbuscules were present in 29–49% of the root length. No significant differences were found between the kind of P treatment or time of harvest.

The proportions of phosphatase-active extraradical and intraradical mycelium were estimated after staining with the fast blue RR substrate (Table 2). The staining of the intraradical mycelium was not affected by enzymatic digestion of the roots. The fast blue RR precipitation in arbuscules was mostly confined to the trunk hyphae. Arbuscules with fast blue RR precipitation in the fine arbuscular branches (Fig. 1a) were rarely seen.

Apart from the proportion of ACP-active arbuscules at 49 d, no difference was found in the proportions of phosphatase-active intraradical mycelium between the two kinds of P treatment. The proportions of both ACP- and ALP-active intraradical mycelium decreased with time. In the intraradical mycelium, the proportion of ACP-active mycelium was significantly higher than the ALP-active proportion (Table 2), and the hyphae were more intensely stained (compare hyphae in Figs 1a and 1b).

No difference could be seen in the proportion of phosphatase-active extraradical mycelium, between the two kinds of P treatment. Extraradical mycelium did not show strong fast blue RR precipitation (Fig. 1c), and only a small proportion of the mycelium showed any precipitation. In contrast to the intraradical mycelium, the proportion of ALP-active mycelium was significantly higher than the ACP-active proportion (Table 2).

The proportion of ALP-active extraradical mycelium was correlated with the proportion of ALP-active intraradical mycelium (Fig. 2). This correlation was positive for both kinds of P treatment (significant correlations), and was higher for the no-P than for the high-P treated plants. Due to the low proportion of ACP-active extraradical mycelium, no correlation with ACP in the intraradical mycelium could be observed.

Response to phosphorus availability

The proportion of ACP-active intraradical mycelium in the no-P treated plants showed a significant negative correlation to both the shoot P concentration (Fig. 3) and to the shoot dry weight (data not shown). The proportion of ACP-active arbuscules also showed a significant negative correlation with the shoot P concentration, but no correlation with shoot dry weight was found. For the high-P treated plants no clear trends were observed. No correlations were found regarding the

Table 1. Shoot dry weight and P content at sequential harvests of mycorrhizal and non-mycorrhizal *Allium cepa*. Standard errors (SE) are given in parentheses. The no-P (watered with nutrient solution without P) and high-P (watered with nutrient solution with 5 mg P l⁻¹) treatment was initiated at 37 days on plants previously treated with low-P (1 mg P l⁻¹) solution. NM, non-mycorrhizal plants; Myc, mycorrhizal plants.

		Shoot					
		Dry weight (mg)			P content (µg)		
	Days	No-P	Low-P	High-P	No-P	Low-P	High-P
NM	37		22.1 (2.4)			10.5 (0.9)	
	56		31.2 (0.8)			16.4 (1.4)	
Myc	37		29.7 (3.6)			20.6 (3.2)	
	42	33.2 (1.8)	ns	28.3 (5.3)	22.3 (0.9)	ns	17.1 (6.2)
	49	41.9 (1.7)	ns	42.0 (4.8)	31.5 (4.1)	ns	36.3 (6.6)
	56	44.6 (4.7)	*	62.4 (4.6)	37.5 (9.4)	[*]	61.2 (5.6)

* Significant difference at the 5% level between the no-P and the high-P treated plants (1-tailed *t* test with unequal variances).

[*] Significant difference at the 10% level.

ns, No significant difference.

Table 2. Proportion (%) of phosphatase-active hyphae, arbuscules and ERM as indicated by fast blue RR staining. The intraradical hyphae and arbuscules were collected after enzymatic digestion of the roots. The SE is given in parentheses. P treatment as described in Table 1. Arbs, arbuscules; ERM, extraradical mycelium; IRM, intraradical mycelium.

		Proportion of phosphatase active structures ¹ (%)					
		ACP ²			ALP		
	Days	No-P	Low-P	High-P	No-P	Low-P	High-P
IRM-Hyphae ***	37		80.4 (3.8)			39.7 (17.0)	
	42	88.0 (4.4)		88.5 (4.6)	53.6 (8.8)		58.8 (10.5)
	49	79.5 (2.4)		67.6 (3.1)	53.0 (10.7)		38.1 (17.3)
	56	69.8 (10.2)		70.5 (7.1)	26.3 (3.2)		28.7 (4.4)
IRM-Arbs ***	37		92.1 (6.2)			40.5 (24.3)	
	42	89.3 (3.8)		94.0 (2.3)	35.0 (17.5)		50.9 (7.9)
	49	71.1 (6.9)		64.9 (10.5)	44.6 (13.2)		36.2 (4.3)
	56	80.6 (10.0)		75.7 (5.4)	9.8 (7.4)		31.5 (10.0)
ERM **	37		0.4 (0.4)			1.7 (0.9)	
	42	3.7 (3.5)		0.0 (0.0)	07.6 (1.5)		12.6 (3.7)
	49	0.3 (0.3)		0.8 (0.3)	11.0 (2.3)		4.1 (2.4)
	56	1.1 (0.5)		2.2 (0.8)	4.9 (1.6)		4.2 (1.8)

¹ Proportion (%) of phosphatase-active hyphal intersections calculated as the percentage of active hyphae from the total amount of hyphae counted in the specific subsample that was stained for the specified phosphatase activity. A similar procedure was followed for arbuscules and ERM.

² ACP and ALP indicate staining for acid phosphatase and alkaline phosphatase, respectively. Bold numbers indicate that a significant difference (5% level) was found between the P treatments.

** Significant difference between ACP and ALP staining at the 1% level (2-tailed paired *t* test).

*** Significant difference at the 0.1% level.

proportion of ALP-active intraradical mycelium or arbuscules, or the proportion of ACP- and ALP-active extraradical mycelium.

The shoot P content was positively correlated to the shoot fresh weight (significant correlation, Fig. 4) after 19 d of P treatment (56 d of plant growth), and the proportion of ALP-active intraradical mycelium appeared to increase with higher shoot fresh weight (not significant; data not shown). Also, the proportion of ALP-active arbuscules seemed to increase with higher shoot fresh weight (not significant; Fig. 4). In earlier harvests (at 37, 42 and 49 d), the proportion of ALP-active structures was constant and no correlation with shoot fresh weight was observed.

DISCUSSION

We have presented experimental data on the metabolic response of an AM fungal symbiosis, established under P-limited conditions, to altered external P concentrations. This is the first study in which the relative amount of ACP- and ALP-active structures in both the extraradical and intraradical mycelium has been assayed in relation to a change in external P availability.

Except in one case, we were not able to show any direct change in the relative amount of phosphatase-active mycelium following P treatment for *G. margarita* (Table 2). However, the correlation between the proportion of ALP-active arbuscules and shoot fresh

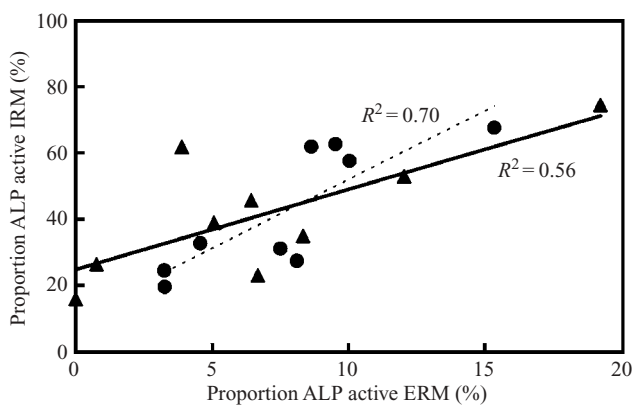


Fig. 2. The relationship between the proportion of ALP-active intraradical (IRM) and extraradical (ERM) mycelium. ---●---●---, no-P treatment; —▲—▲—, high-P treatment; triplicate samples were used. P treatment as described in Table 1.

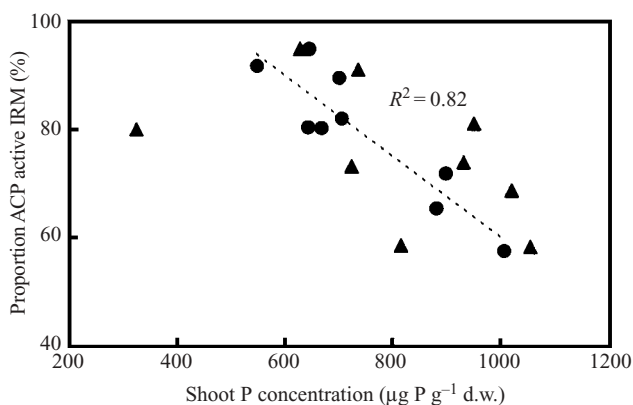


Fig. 3. Proportion of ACP-active intraradical mycelium (IRM) in relation to shoot P concentration. ---●---●---, no-P treatment; ▲, high-P treatment ($R^2=0.25$; no regression line shown); triplicate samples were used. P treatment as described in Table 1.

weight was positive, as was that for shoot P content and shoot fresh weight (Fig. 4). This suggests that the proportion of ALP-active arbuscules is related to the fungal P status or to fungal P transfer to the plant. However, there was a lag phase before the response in ALP was observed. Correlations between ALP and plant P content have been demonstrated previously (Tisserant *et al.* 1996). The proportion of ALP-active intraradical mycelium was not as strongly correlated to shoot fresh weight as the arbuscular proportion, which could be an effect of the central role of arbuscules for P transfer.

Under P-deprived conditions, the proportion of ACP-active intraradical mycelium was correlated to shoot P concentration (Fig. 3) and plant growth. This may suggest that under P limitation, in *G. margarita*, the host P status affected the proportion of ACP-active intraradical mycelium. Ezawa *et al.* (2001) suggested that a non-specific ACP could be involved in the hydrolysis of polyphosphate in intraradical hyphae before inorganic P is released into the plant–fungal interface by

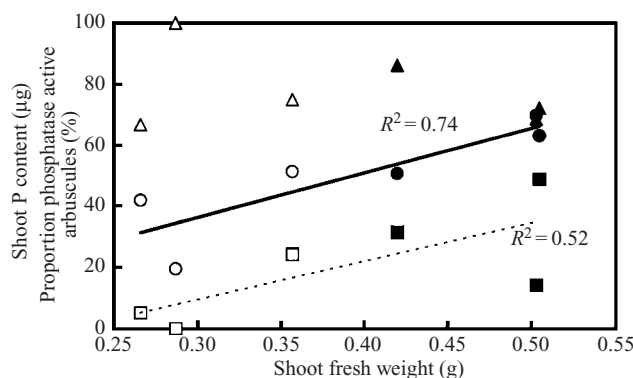


Fig. 4. Shoot P content and the proportion of ACP- and ALP-active arbuscules as a function of the shoot fresh weight of mycorrhizal plants at 56 d. The mycorrhizal plants had been exposed to no-P (open symbols) or to high-P (solid symbols) for 19 d. —○—○—, shoot P content; △ △, proportion of ACP-active arbuscules ($R^2=0.12$; no regression line shown), ---□---□---, proportion of ALP-active arbuscules; triplicate samples were used. P treatments as described in Table 1.

the fungus. In this case, increased ACP activity in the intraradical mycelium could lead to an increase in P transfer from fungus to plant.

All these results indicate that the intraradical mycelium of *G. margarita* responded metabolically to P treatment. The influence of the plant P concentration on the fungal metabolic activity may also be of importance. Such an influence has been found earlier by Thomson *et al.* (1991) and Tisserant *et al.* (1996) who showed that increased plant P led to a decrease in AM fungal growth and activity. However, the lack of correlation between the proportion of phosphatase-active extraradical mycelium and shoot growth or P content supports observations by Boddington & Dodd (1999) that the proportion of ALP-active extraradical mycelium was unaffected by P treatment.

In the extraradical mycelium a large part of the phosphatase activity is associated with the cell walls (Joner & Johansen 2000), whereas in the intraradical mycelium it is mainly located in the acid vacuoles (Gianinazzi *et al.* 1979, Ezawa *et al.* 1995, 2001). A different location and proportion of ALP- and ACP-active structures could indicate that these enzymes have different roles in P metabolism. In the present study we showed that the intraradical mycelium had a higher proportion of phosphatase-active hyphae than the extraradical mycelium (Fig. 1, Table 2). This has previously been observed for ALP in a *Glomus* species (Zhao *et al.* 1997, Kjoller & Rosendahl 2000). The proportion of ALP-active extraradical mycelium could be correlated to the proportion of ALP-active intraradical mycelium (Fig. 2). Furthermore, while in the intraradical mycelium the proportion of ACP-active structures was highest, in the extraradical mycelium the proportion of ALP-active hyphae was highest. This suggests that in the extraradical mycelium the ALPs might be involved in hyphal P acquisition. The high proportion of ACP-active structures in the intraradical

mycelium indicates that one or several ACPs might be involved in P transfer processes.

Our results indicate that mycorrhizal plants, which have previously been limited in P, can respond metabolically to either the external P availability or the plant P concentration. This study emphasizes the importance of studying not only the amount of AM fungal mycelium but also the proportion of active mycelium. Furthermore, we showed that the extraradical and the intraradical mycelium should not be considered as one entity when studying the transfer of nutrients.

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