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**Title:** Polyphosphate has a central role in the rapid and massive accumulation of phosphorus in extraradical mycelium of an arbuscular mycorrhizal fungus

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## Introduction

Arbuscular mycorrhizal (AM) fungi form symbiotic associations with most land plants and promote growth of the host through enhanced uptake of phosphate (Pi) (Smith & Read, 2008). It has been well documented that the high-affinity Pi transporters on the plasma membrane of extraradical hyphae play a main role in Pi uptake from soil (Harrison & Van Buuren, 1995; Maldonado-Mendoza *et al.*, 2001). On the other hand, the mycorrhiza-specific plant Pi transporters localized on the periarbuscular membrane are responsible for the uptake of Pi released from arbuscules (Rausch *et al.*, 2001; Maeda *et al.*, 2006; Javot *et al.*, 2007). Despite increasing knowledge of the membrane transport systems in the symbiotic associations, information about the mechanism of Pi-translocation through AM fungal hyphae is quite limited. Evidence that AM fungi accumulate polyphosphate (polyP) in hyphae was first obtained more than three decades ago (Callow *et al.*, 1978). PolyP is a linear chain of three to thousands Pi-residues linked by high-energy phosphoanhydride bonds and has been found in nearly all classes of organisms (Kornberg *et al.*, 1999). The compound has many functions in the cell, including as a Pi-reservoir, an alternative energy source of ATP and a metal chelator (Kornberg *et al.*, 1999). Although polyP is suggested to be involved in long-distance Pi-translocation through hyphae in AM associations (Cox *et al.*, 1980; Ezawa *et al.*, 2002), the physiological roles and behavior of the compound in

the fungi are largely unknown. It has been reported that the compound consists of only a small part of total cellular phosphorus (P) in AM fungi: the proportions of polyP to total P were estimated as 16% in *Glomus mosseae* (Capaccio & Callow, 1982) and 5-17% in *Gigaspora margarita* (Solaiman *et al.*, 1999). These estimations suggest that simply Pi and/or other P-compounds may play a more significant role in P-storage/translocation in the fungi. On the other hand, Pi taken up by hyphae was converted to polyP quite rapidly (Ezawa *et al.*, 2004; Viereck *et al.*, 2004), and the rate of polyP accumulation was comparable to that of a polyP-hyperaccumulating bacterium found from activated sludge (Ezawa *et al.*, 2004). These observations led us to hypothesize that the fungi could potentially accumulate much larger amounts of polyP than previously reported. In particular, Viereck *et al.* (2004) provided a comprehensive view of the relative dynamics of various (soluble) P-compounds in an AM fungal mycelium using the *in vivo* <sup>31</sup>P-NMR technique and suggested that polyP might be the largest P-storage in the fungi. However, NMR-invisible P-compounds such as long-chain polyP and structural P might be present in the cell, and the absolute (potential) pool size of the cell for polyP has not been estimated so far. Therefore, further quantitative study on the dynamic of polyP in AM fungi with respect to total cellular P is required. In addition, it was predicted that the maximum pool size for polyP would be demonstrable in P-starved AM fungi, which could accumulate polyP as rapidly as a polyP-hyperaccumulator (Ezawa *et al.*, 2004). In the present study, the

dynamics of polyP, total P and Pi were investigated in an AM fungus grown under P-starvation conditions to clarify the significance of polyP in P-storage/translocation in AM fungal associations.

## Materials and methods

### Culture conditions

*Lotus japonicus* L. cv. Miyakojima MG-20 (National Bioresource Project Legume Base, <http://www.legumebase.agr.miyazaki-u.ac.jp/index.jsp>) were sown on a moistened filter paper in a Petri dish and germinated at 25°C for 2 days in the dark. Three seedlings were transplanted to the mycorrhizal compartment (MC) of a dual mesh bag culture system in a 230 mL plastic pot (7.6 cm in diam) and inoculated with *Glomus* sp. HR1 (MAFF 520076) at 500 spores pot<sup>-1</sup>. The dual mesh bag system consisted of two main compartments, a MC and hyphal compartment (HC) that were separated by a cone-shaped dual nylon mesh bag (37 µm pore size, Nippon Rikagaku Kikai, Tokyo, Japan) (Fig. S1). The MC was defined as the region inside of the inner mesh bag [31 mL in vol, 5.2 × 4.5 (W × H) cm], and the HC (159 mL in vol) was defined as the region outside of the outer mesh bag [6.8 × 5.9 (W × H) cm]. The medium in these compartments was autoclaved river sand. In between the inner and

outer mesh bags, autoclaved subsoil with a high-P absorption coefficient (2.8 g P<sub>2</sub>O<sub>5</sub> kg<sup>-1</sup> soil, pH 5.0) was layered as a P-diffusion barrier (10 mm in width). The pore size of the nylon mesh was small enough to prevent *L. japonicus* roots from passing but large enough to allow AM fungal hyphae to pass through. The seedlings were grown in a growth chamber equipped with fluorescent light at a photon flux density of 150 μmol m<sup>-2</sup> s<sup>-1</sup> (16-h photoperiod, 25°C) and thinned to 2 plants pot<sup>-1</sup> one week after sowing. The plants (whole pot) received deionized water (DIW) every other day for the first week, then low-P nutrient solution (4 mM NH<sub>4</sub>NO<sub>3</sub>, 1 mM K<sub>2</sub>SO<sub>4</sub>, 75 μM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 50 μM Fe-EDTA and 50 μM KH<sub>2</sub>PO<sub>4</sub>) from the 2nd to 6th week and non-P nutrient solution (KH<sub>2</sub>PO<sub>4</sub> was withheld from the low-P nutrient solution) for the 7th week in sufficient amount until the solution flowed out from the drain. At the beginning of 8th week, an 1 mM Pi (KH<sub>2</sub>PO<sub>4</sub>) solution was applied using a pipette to the HC gently in sufficient amount until the solution flowed out from the drain, and then the Pi solution was washed out by applying DIW with a watering can in sufficient amount one h after Pi application. Mycorrhizal roots and extraradical mycelium in the MC and mycelium in the HC were harvested separately after Pi application at one h intervals as follows. The MC (inner mesh bag) was taken off from the pot and transferred to water, and then mycorrhizal roots and attaching mycelium (roots + mycelium) were collected after removing adhering sand particles by gentle shaking in the water. Detached mycelium in the water was further collected by the wet sieving

and combined with the roots + mycelium fraction. Extraradical mycelium in the HC was collected by the wet sieving after removing the P-diffusion barrier (outer mesh bag). The samples were blotted with a paper towel, frozen in liquid nitrogen immediately and stored at  $-80^{\circ}\text{C}$ .

### Analytical procedures

In the case of mycelium from the HC, 5 to 30 mg (f. wt) material was ground in an ice-cooled mortar and pestle with 10 to 20-fold volume (w/v) of extraction buffer (8 M urea/ 50 mM Tris-HCl, pH 8.0) and transferred to a 1.5 mL tube. In the case of roots + mycelium from the MC, 0.7 to 2.0 g (f. wt) material was ground in a mortar with liquid nitrogen and mixed with 5-fold volume (w/v) of the extraction buffer, and then 500  $\mu\text{L}$  of the slurry was transferred to a 1.5 mL tube.

For determination of total P in the HC samples, 100  $\mu\text{L}$  of the slurry was transferred to a 14.7 mL Teflon vial (Savillex, Minneapolis, USA), mixed with 2.5 mL of 1.8 M sulfuric acid, heated at  $250^{\circ}\text{C}$  for two h to evaporate water (until sulfuric acid was concentrated) and then digested at  $250^{\circ}\text{C}$  for one h by using 0.2 - 0.3 mL hydrogen peroxide as an oxidant. Pi concentrations in the digests were determined by the ascorbic acid method (Watanabe & Olsen, 1965). PolyP concentrations in the slurries prepared from the HC and MC samples were determined by the reverse

reaction of polyphosphate kinase (PPK) as described by Ezawa *et al.* (2004). Free Pi concentrations in the slurries from the HC samples were determined by the ammonium molybdate method (Ohnishi *et al.*, 1975) using 200  $\mu$ L supernatant obtained after centrifuging the slurry at 14,000  $\times g$  for 15 min. Ten  $\mu$ L of the slurry from the HC and MC samples was taken for the determination of protein concentration using the DC Protein Assay Kit (Bio-Rad Laboratories, Tokyo) with bovine serum albumin as standard.

#### Experimental setup and data analysis

The time course analysis (from zero to nine h after Pi application) of total P and that of polyP in the HC were conducted separately using different batches of plant/fungal material. For these analyses, 22 pots were prepared as one batch, and mycelial samples collected from two pots grown in the same batch were combined as one sample (5 – 30 mg f. wt per sample). One set of time course experiment (from zero to nine h after Pi application) was conducted using one batch (without replication) and triplicated using three independent batches. For data analysis, average values were calculated from the data obtained from the three replicated experiments ( $n = 3$ ). For the time course analysis of polyP in roots + mycelium in the MC (from zero to ten h after Pi application), 33 pots were prepared as one batch, and three samples (0.7 – 2.0 g f. wt

per sample) harvested from three pots were analyzed separately ( $n = 3$ ). For the simultaneous analysis of total P, polyP and Pi in the HC (from zero to six h after Pi application), 22 pots were prepared as one batch, and mycelial samples collected from two pots grown in the same batch were combined as one sample. One set of time course experiment (from zero to six h after Pi application) was conducted using one batch (without replication) and replicated five times using five independent batches. For data analysis, average values were calculated from the data obtained from the five replicated experiments ( $n = 5$ ).

Analysis of variance (ANOVA) with the Tukey-Kramer test as a post-hoc test or Student's *t*-test was applied for data analysis using the StatView software (SAS Institute Inc., Cary, USA).

## Results

The total P content of extraradical mycelium in the HC was  $3.6 \mu\text{mol mg}^{-1}$  protein at time zero, which increased to  $8.2 \mu\text{mol mg}^{-1}$  protein 5 h after Pi application and then decreased to  $5.2 \mu\text{mol mg}^{-1}$  protein 9 h after Pi application (Fig. 1). PolyP levels of extraradical mycelium in the HC increased from  $0.5$  to  $7.1 \mu\text{mol mg}^{-1}$  protein from 0 to 6 h after Pi application and decreased to  $2.8 \mu\text{mol mg}^{-1}$  protein by 9 h after Pi application (Fig. 2). The apparent accumulation rates of total P and polyP from 0 to 5 h

after Pi application were 1.03 and 1.14  $\mu\text{mol mg}^{-1} \text{h}^{-1}$  protein, respectively, and were not significantly different (*t*-test,  $P < 0.05$ ). The apparent declining rates of total P and polyP from 5 to 9 h after Pi application were 0.94 and 1.06  $\mu\text{mol mg}^{-1} \text{h}^{-1}$  protein, respectively, and were also not different at  $P < 0.05$ . PolyP levels of mycorrhizal roots + extraradical mycelium in the MC were maintained within a range of 3.8 to 6.6 nmol  $\text{mg}^{-1}$  protein from 0 to 5 h after Pi application, increased to 44.3 nmol  $\text{mg}^{-1}$  protein from 6 to 9 h after Pi application and then decreased to 9.8 nmol  $\text{mg}^{-1}$  protein 10 h after Pi application (Fig. 3). To investigate whether Pi and/or unknown precursors of polyP were accumulated prior to polyP accumulation, free Pi, total P and polyP levels of extraradical mycelium in the HC were measured simultaneously. Total P and polyP levels increased synchronously from 2.9 to 6.7  $\mu\text{mol mg}^{-1}$  protein and from 0.2 to 4.3  $\mu\text{mol mg}^{-1}$  protein, respectively, from 0 to 5 h after Pi application (Fig. 4). In contrast, free Pi content remained constant within a range between 200–500 nmol  $\text{mg}^{-1}$  protein during the experiment. It is noteworthy that the level of polyP reached 64% of total P 5 h after Pi application. Differences between total P and polyP levels were constant from 0 to 6 h after Pi application (Fig. 4).

## Discussion

The present study demonstrated that the AM fungus were capable of accumulating

polyP more than 60% of total cellular P, implying that the potential pool size of polyP in the cell was much larger than previously considered. Rapid and massive accumulation of polyP in microorganisms was first discovered in yeast *Saccharomyces cerevisiae* more than four decades ago and defined as ‘polyP overplus (or overcompensation)’ (reviewed in Harold, 1966), e.g., the level of polyP in *S. cerevisiae* that was grown under P-deficient conditions increased 20-fold 2 h after re-supply of Pi, up to 38 mg g<sup>-1</sup> d. wt (corresponding to 400 μmol g<sup>-1</sup> d. wt) (Trilisenko *et al.*, 2002). In the present study, *Glomus* sp. HR1 was found to respond to Pi similarly: the level of polyP in the fungus grown under P-deficient conditions increased 14-fold 5 h after Pi application, up to 7.1 μmol mg<sup>-1</sup> protein, corresponding to 390 – 500 μmol g<sup>-1</sup> d. wt (the values were estimated based on the following parameters: protein content, 11 – 15 mg g<sup>-1</sup> f. wt; water content, 80%). Although this was achieved by the application of quite high-concentration of Pi that rarely occurs under natural conditions, the level is comparable to that observed in the yeast ‘polyP overplus’ and much higher than those in AM fungi reported previously: *Gl. mosseae* intraradical hyphae, 32 μmol g<sup>-1</sup> d. wt (Capaccio & Callow, 1982); *Gi. margarita* extraradical hyphae, 32 – 48 μmol g<sup>-1</sup> d. wt (Solaiman *et al.*, 1999) (estimated on the assumption that water content was 80%). It has been considered that ‘polyP overplus’ would be the feature evolved in a wide range of microorganisms to prepare P-deficiency, because Pi availability tends to be low in natural environments (Harold, 1966). Glomeromycotan fungi may have acquired the

traits involved in ‘polyP overplus’ during the early evolution and could successfully provide a great competitive advantage in P-acquisition for their host plants by facilitating a large P-pool.

Our observations that total P and polyP increased and decreased synchronously without fluctuation in the level of free Pi suggest that neither Pi nor intermediary P-metabolite such as short-chain polyP or organic P-compound, which could be detected by the total P measurement but not by the PPK/luciferase method, was accumulated prior to polyP accumulation. This supports the ideas that polyP formation in AM fungi is quite rapid (Ezawa *et al.*, 2004) and thus contributes to maintaining cellular Pi at a low level for efficient uptake of Pi (Viereck *et al.*, 2004), confirming that polyP plays a significant role in AM fungal P-metabolism as a temporary but largest P-storage.

PolyP first increased in extraradical mycelium in the HC and later in mycorrhizal roots + mycelium in the MC. These observations were consistent with those reported by Viereck *et al.* (2004) and strongly suggest that polyP mediates long-distance P-translocation through hyphae. In this context, our experimental system could provide further information about parameters for P-translocation in AM symbiosis: the declining rates of polyP in mycelium in the HC from 5 to 9 h after Pi application ( $1.06 \mu\text{mol mg}^{-1} \text{h}^{-1}$ , corresponding to  $58 - 80 \mu\text{mol g}^{-1} \text{d. wt h}^{-1}$ ) can be regarded as the apparent P-translocation rate through hyphae towards the plants. In

addition, the time lag of 5–6 h for the increases in polyP level in roots + mycelium in the MC can be regarded as the time required for P to pass the 10 mm diffusion barrier, i.e. polyP (or Pi) moved towards the plant at 1.7–2.0 mm h<sup>-1</sup>. In the *in vitro* carrot root-organ/*G. intraradices* association, it took 14 h to translocate radio-labeled P from extraradical hyphae to intraradical hyphae (Nielsen *et al.*, 2002), suggesting that the processes of P-accumulation/translocation *in vitro* were slower than those in our open culture system. Differences in experimental systems may affect the energy status of the fungal partner through carbon supply from the plant partner, and this may be reflected in the differences in the rates of P-accumulation/translocation (Olsson *et al.*, 2002; Bücking & Shachar-Hill, 2005). It has been well documented that there are large inter- and intraspecific variations in the efficiency of P uptake by AM fungi (e.g. Jakobsen *et al.*, 1992; Maldonado-Mendoza *et al.*, 2001; Nielsen *et al.*, 2002; Munkvold *et al.*, 2004). In fact, the apparent polyP accumulation rate of *Archaeospora leptoticha* (Ezawa *et al.*, 2004) is more than twice as rapid as that of *Glomus* sp. HR1. Accordingly, the parameters for polyP accumulation/translocation presented in our study could be applicable for the assessment of inter- and intraspecific variations in P-delivery potential among AM fungi.

In the present experimental system, polyP formation (a net increase in polyP) was observed even after the Pi-washing (removal) process conducted one h after Pi application. The washing step shortened the duration of Pi uptake by hyphae and thus

was essential to estimate the declining rate of polyP in mycelia. Two reasons could be proposed to explain the prolonged Pi uptake after the washing process. Firstly, the amount of DIW used for the washing was insufficient, and thus Pi remained in the medium, and secondly, Pi was captured in hydrophilic (viscous) material such as extracellular polysaccharide gels around hyphae, which might be secreted by the fungus and removed by the wet sieving but not by watering. The former is unlikely, because an increase in DIW for the washing process did not shorten the duration of Pi uptake. The fact that adherence of organic material and fine sand particles to mycelia was observed frequently suggests that extracellular polysaccharide is present around hyphae, and thus the latter seems likely.

The present study demonstrated the significance of polyP as a largest P-storage and a mediator of long-distance P-translocation in AM fungi. It remains uncertain, however, whether P is translocated as polyP without turning over or as Pi through dynamic regulation of polyP-synthesis/hydrolysis (Ezawa *et al.*, 2001). Further investigations are required to understand the whole picture of P-delivery system in the symbiotic associations.

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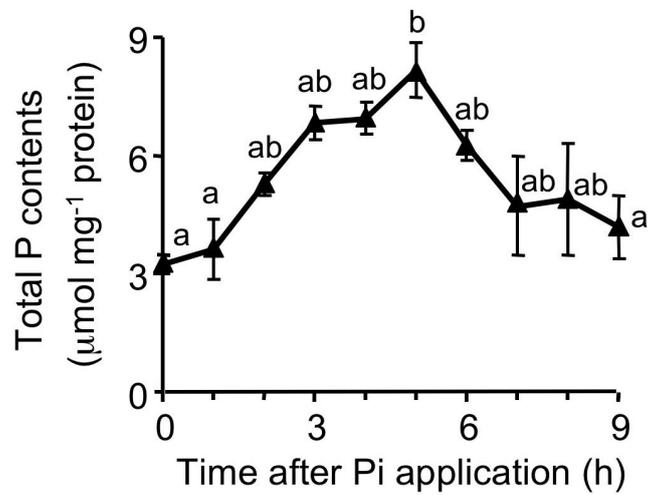
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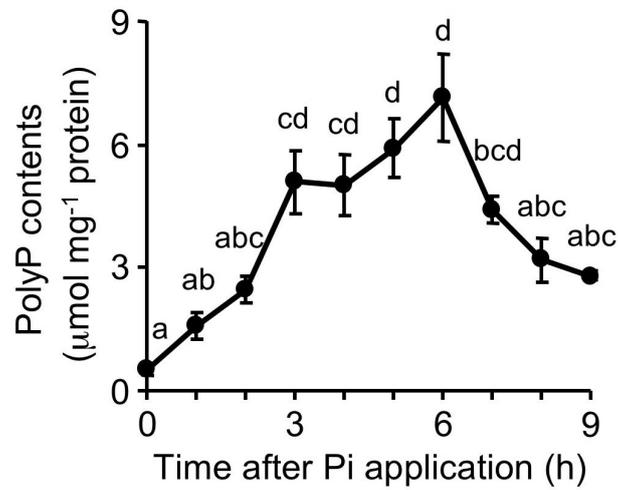
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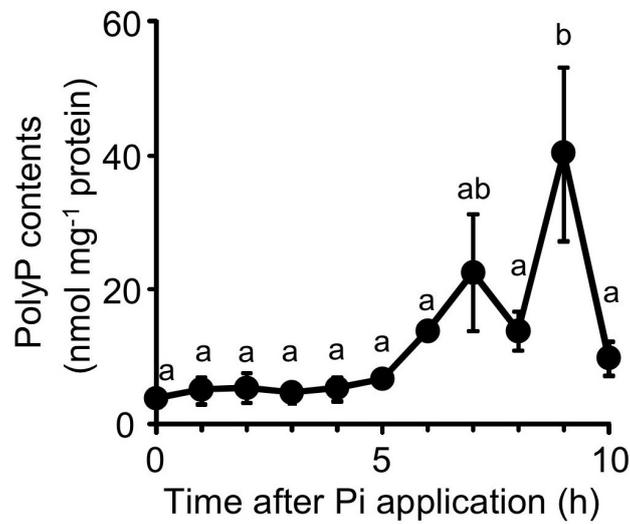
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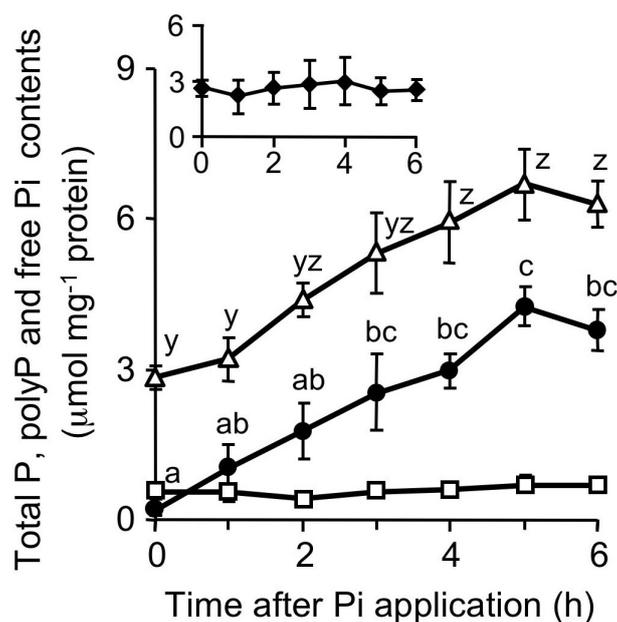
**Fig. 1** Time course assessment of total phosphorus contents in the extraradical mycelium of *Glomus* sp. HR1 in the hyphal compartment. One mM phosphate (Pi) was applied to the hyphal compartment at time zero and washed with deionized water 1 h after Pi application. The average values obtained from three independent experiments ( $n = 3$ ) are shown with SE (vertical bars). Different letters indicate significant difference ( $P < 0.05$ , Tukey-Kramer test).



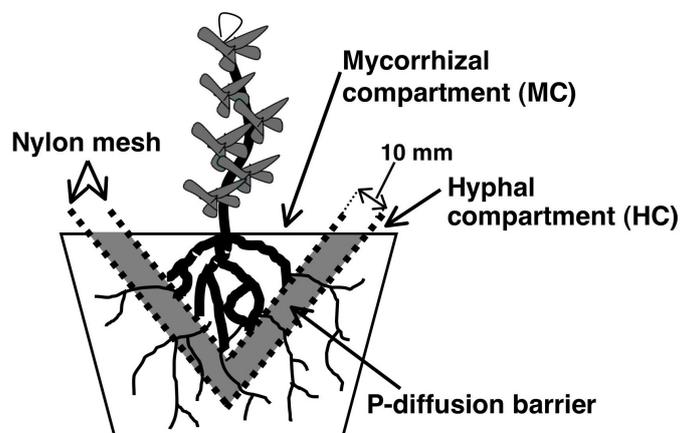
**Fig. 2** Time course assessment of polyphosphate contents in the extraradical mycelium of *Glomus* sp. HR1 in the hyphal compartment. One mM phosphate (Pi) was applied to the hyphal compartment at time zero and washed with deionized water 1 h after Pi application. The average values obtained from three independent experiments ( $n = 3$ ) are shown with SE (vertical bars). Different letters indicate significant difference ( $P < 0.05$ , Tukey-Kramer test).



**Fig. 3** Time course assessment of polyphosphate contents in the *Lotus japonicus*/*Glomus* sp. HR1 mycorrhizal roots and extraradical mycelium in the mycorrhizal compartment. One mM phosphate (Pi) was applied to the hyphal compartment at time zero and washed with deionized water 1 h after Pi application. Average values ( $n = 3$ ) obtained from one representative experiment are shown with SE (vertical bars). Different letters indicate significant difference ( $P < 0.05$ , Tukey-Kramer test).



**Fig. 4** Time course assessment of total phosphorus (open triangles), polyphosphate (black circles) and free phosphate (open squares) contents in the extraradical mycelium of *Glomus* sp. HR1 in the hyphal compartment. One mM phosphate (Pi) was applied to the hyphal compartment at time zero and washed with deionized water 1 h after Pi application. The inserted small graph shows differences between the levels of total phosphorus and polyphosphate during the time course analysis (the units are the same as those in the large graph). The average values obtained from five independent experiments ( $n = 5$ ) are shown with SE (vertical bars). Different letters indicate significant difference ( $P < 0.05$ , Tukey-Kramer test). Differences between the levels of total phosphorus and polyphosphate were not significantly different among all time points (ANOVA,  $P > 0.05$ ).



**Fig. S1** A dual mesh bag culture system. The mycorrhizal (MC) and hyphal (HC) compartments were separated by a cone-shaped dual nylon mesh bag (37  $\mu\text{m}$  pore size). The medium in the MC and HC was autoclaved river sand, and autoclaved subsoil with a high-P absorption coefficient was layered as a P-diffusion barrier in between the inner and outer mesh bags (10 mm in width).