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Rapid report

Rapid accumulation of polyphosphate in extraradical hyphae of an arbuscular mycorrhizal fungus as revealed by histochemistry and a polyphosphate kinase/luciferase system

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Summary

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The rate of polyphosphate accumulation in extraradical hyphae of an arbuscular mycorrhizal fungus was investigated by conventional histochemistry and a new enzymatic method using a bacterial enzyme, polyphosphate kinase. Marigold (*Tagetes patula* cv. Bonanza Orange) was inoculated with *Archaeospora leptoticha* and grown under P-deficient conditions. Extraradical hyphae were harvested at 0, 1, 3 and 24 h after 1 mM P-application. PolyP levels were assessed by both metachromasy of Toluidine blue O and polyphosphate kinase which converted polyP to ATP followed by the ATP-luciferase assay. Percentage of hyphae with metachromatic granules was increased from 25 to 44% from 0 to 1 h, and a maximum of 50% was reached by 3 h. Polyphosphate content was doubled from 1 to 3 h after P-application (4.8–10.0 $\mu\text{mol as Pi mg}^{-1}$ protein) at a rate of $46.4 \pm 15.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$. The rate of polyphosphate accumulation in the hyphae was surprisingly rapid as those of polyphosphate-hyper accumulating microorganisms. The enzymatic method employed in the present study allows highly specific and sensitive assessment of polyphosphate in the mycorrhizal system.

Key words: arbuscular mycorrhizal fungi, luciferase, metachromasy, polyphosphate, polyphosphate kinase, Toluidine Blue O.

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Introduction

Arbuscular mycorrhizal (AM) fungi are ubiquitous and form symbiotic associations with most terrestrial plant species. It is well documented that the fungi promote growth of the host plant through enhanced uptake of phosphate. High-affinity type Pi-transporter genes have been isolated from AM fungi, and the gene products are responsible for uptake of Pi from the soil solution and into the fungal cytosol (Harrison & Van Buuren, 1995; Maldonado-Mendoza *et al.*, 2001). However, there is a paucity of information on the mechanisms of P compartmentation, translocation along hyphae and transfer from fungi to the host.

Inorganic polyphosphate (polyP) is a linear polymer of phosphate linked by high-energy bonds; a wide range of microorganisms store P by accumulation of polyP (Kornberg *et al.*, 1999). It has been suggested that AM fungi accumulate polyP for long distance translocation along hyphae (Callow *et al.*, 1978; Cox *et al.*, 1980; Solaiman *et al.*, 1999). However, detailed information on the dynamics of polyP turnover in AM fungi is lacking, and further research is required to elucidate the role of polyP in the P-delivery system. To this end, establishment of a specific method to quantify polyP is essential. Several colorimetric methods for polyP measurement have been reported (Griffin *et al.*, 1965; Allan & Miller, 1980; Lorenz *et al.*, 1997). Recently, metachromasy of Toluidine

blue O (TBO) by polyP was applied to quantify the polyP pool in intra- and extraradical hyphae of an AM fungus, *Gigaspora margaritata* (Solaiman *et al.*, 1999). TBO (Ezawa *et al.*, 2001) and 4',6-diamidino-2-phenylindole (DAPI) (Boddington & Dodd, 1999a; Boddington & Dodd, 1999b) were used for histochemical assessment of polyP levels in mycorrhizal hyphae. Such methods can be used for both localization and quantification of polyP. However, these stains interact with polyanionic compound such as nucleic acids. Thus, an appropriate control (e.g. nonP treatment) is required to ensure specificity and validity of the methods (Ezawa *et al.*, 2001). *In vivo* ^{31}P NMR is a powerful tool to investigate polyP metabolism in mycorrhizal associations (Martin *et al.*, 1985; Ashford *et al.*, 1994; Gerlitz & Werk, 1994; Martin *et al.*, 1994; Rasmussen *et al.*, 2000); however, this method only allows the detection of mobile (short-chain) polyP (Gerlitz & Werk, 1994; Rasmussen *et al.*, 2000). It is clear that each of the above methods has inherent limitations and use of such methods needs to be considered carefully.

Recently, a novel method for specific measurement of polyP using a bacterial enzyme, polyphosphate kinase (PPK), in conjunction with the ATP-luciferase assay has been developed. The method is based on conversion of polyP to ATP by PPK reverse reaction followed by chemi-luminescence assay of ATP by the luciferase system and allows highly sensitive and specific quantification of polyP (Ault-Riché *et al.*, 1998). In the present study, we investigated polyP accumulation in P-deficient hyphae of an AM fungus by conventional histochemistry and for the first time the PPK/luciferase method. Thus, establishing the PPK/luciferase method for AM fungi and also testing the specificity and validity of the TBO method for polyP quantification and localization.

Materials and Methods

Chemicals and enzyme

Toluidine blue O (TBO) and ADP (highest purity) were purchased from Sigma Chemicals and Roche Diagnostics, respectively. Polyphosphate kinase (PPK) was purified from the PPK-over-expressing *Escherichia coli* as described elsewhere (Ahn & Kornberg, 1990). Specific activity of PPK after purification was 3×10^6 U mg^{-1} protein, in which 1 U of activity was designated to phosphorylate 1 pmol ADP min^{-1} at 37°C using polyphosphate (polyP) as a phosphoryl donor (PPK reverse reaction). Purified PPK was diluted to 3000 U μl^{-1} in 50% glycerol/50 mM Tris/HCl pH 7.5 and stored at -20°C.

Plant and fungal materials

Dwarf marigold (*Tagetes patula* L. cv. Bonanza Orange) was inoculated with *c.* 200 spores of *Archaeospora leptoticha* (Shenck & Smith) Morton & Redecker (strain OK-15, MAFF520072) and grown on a 9 : 1 river sand-soil mixture (soil pH was 4.9, available phosphate in the soil was less than 1.0 mg kg^{-1}) in

100 ml pots (6 cm in diameter) in a growth chamber (16-h photoperiod, 25°C). The plants received liquid fertilizer +P (4 mM NH_4NO_3 , 1 mM K_2SO_4 , 0.75 mM MgSO_4 , 2 mM CaCl_2 , 0.5 mM Fe-EDTA and 50 μM KH_2PO_4) for the first 4 wk and fertilizer -P (as above minus KH_2PO_4) for the next week. At the 6th week, 1 mM KH_2PO_4 solution was applied to the plants, and extraradical hyphae were collected 0 (no P control), 1, 3 and 24 h after P-application by wet sieving followed by manipulation under a dissecting microscope. At each sampling time, hyphae were collected from three individual pots ($n = 3$). Hyphae from the same pot were divided into two subsamples: 2–3 mg were soaked in absolute ethanol for 15 min (for histochemical assessment), and the remaining hyphae were frozen in liquid nitrogen and stored at -80°C (for specific measurement by PPK and polyacrylamide gel-electrophoresis).

Histochemical assessment by Toluidine blue O

The hyphae were removed from the ethanol, blotted on filter paper, stained in 0.05% TBO in 50 mM KCl/HCl pH 1.0 for 15 min at room temperature, rinsed in 50 mM KCl/HCl pH 1.0 and mounted in the same buffer on a glass microscope slide. Percentage of hyphal length showing metachromasy was assessed using a grid line intersect method as described previously (Ezawa *et al.*, 2001).

Specific measurement by polyphosphate kinase

Five to 10 mg hyphae were disrupted in 300 μl ice-cold acetone with *c.* 100 mg of zirconia beads (0.5 mm in diam) by a Mini-Bead Beater (BIOSPEC, OK, USA) at 5000 rpm for 3×10 s with 1 min intervals on dry ice. Acetone was evaporated by vacuum centrifugation after disruption. The pellet was suspended in 400 μl of the extraction buffer (8 M urea in 50 mM Tris/HCl pH 8.0) and centrifuged at $20\,000 \times g$ for 5 min at 4°C, and the supernatant was taken as the hyphal extract. Poly P extraction from hyphae was most efficient using 8 M urea, however, it interfered the PPK reaction. Therefore, following polyP extraction, urea was removed by applying 50–100 μl of the extract to a Bio-Spin 6 gel-filtration column (Bio-Rad Laboratories, Tokyo, Japan). PolyP concentration was quantified using the PPK-reverse reaction (Ahn & Kornberg, 1990) with slight modifications as follows: a PPK reaction mixture consisting of 2 μl of the gel-filtrated extract, 50 mM $(\text{NH}_4)_2\text{SO}_4$, 4 mM MgCl_2 , 40 μM ADP and 600 U PPK, 40 mM HEPES/KOH pH 7.5 in a final volume of 20 μl was incubated at 37°C for 40 min. The reaction was terminated by heating at 95°C for 2 min. The mixture was diluted 1 : 100 in an ATP dilution buffer (4 mM EDTA in 100 mM Tris/HCl pH 7.5), and 20 μl aliquots were mixed with the same volume of the luciferase reaction mixture (ATP Bioluminescence Assay Kit CLS II, Roche Diagnostics, Tokyo, Japan). Luminescence was measured as total luminescent counts in 10 s with a Luminescencer-PSN (ATTO, Tokyo, Japan). Background ATP levels were

determined by the PPK reaction control in which substrate (polyP) was omitted. Concentration of polyP is given in terms of Pi-residues per mg protein. Protein concentration was determined by the BCA Protein Assay Reagent Kit (PIERCE, IL, USA) using bovine serum albumin as a standard.

Qualitative analysis by gel-electrophoresis

Qualitative analysis of polyP was carried out by polyacrylamide gel-electrophoresis. Fifty to 100 μl of the hyphal extract prepared from samples harvested 0 and 3 h after P-application was desalted in a gel-filtration column, mixed with 1 μl of 10 mg ml^{-1} ribonuclease A and 0.5–1.0 μl of 100 mM BeSO_4 (1 mM in final) and incubated at 37°C for 1 h. BeSO_4 was added to inhibit hydrolysis of polyP by polyphosphatase-type enzymes in the hyphal extract (Ezawa *et al.*, 2001). The final solutions were loaded on 10% polyacrylamide gel buffered with Tris/borate/EDTA and run at 10–20 mA constant current. Loading volume was standardized between the samples on the basis of protein concentration of crude extracts. The gel was stained with 0.05% TBO/25% methanol/5% glycerol for 30 min and destained in 25% methanol/5% glycerol. Chain-length of polyP was estimated based on relative mobility (R_m) to bromophenol blue, a tracking dye, according to Clark & Wood (1987)

Results

Addition of P to P-deficient AM hyphae resulted in an exponential increase in TBO stained metachromatic granules over time. At the first harvest time (0 h after P-application) granules were observed in *c.* 25% of extraradical hyphae (Figs 1 and 2). One hour after P-application, the percentage of hyphae containing metachromatic granules increased to 44%. Heavy accumulation of metachromatic granules was observed in 49–50% of hyphae from 3 to 24 h after P-application.

Figure 3 shows polyP content in hyphae measured by the PPK/luciferase method. No increase in polyP was detected from 0 to 1 h after P-application (4.80 ± 0.60 and 4.40 ± 0.45 $\mu\text{mol mg}^{-1}$ protein, respectively). The polyP level increased to 9.97 ± 1.75 $\mu\text{mol mg}^{-1}$ at 3 h and was steady by 24 h after P-application. The net rate of polyP accumulation from 1 to 3 h was calculated as 46.4 ± 15.1 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein ($P < 0.05$).

Qualitative analysis of polyP by electrophoresis clearly showed heavy accumulation of polyP 3 h after the P-application (Fig. 4). Although the range of polyP chain-length was broad, there was a peak of high-molecular weight polyP around R_m 0.12, which was estimated as more than 300 Pi-residues.

Discussion

This study provides the first information about the rate of polyP accumulation in the AM symbiosis. Using two different approaches, the maximum level of polyP was shown to occur in less than 3 h after P-application to hyphae growing in soil.

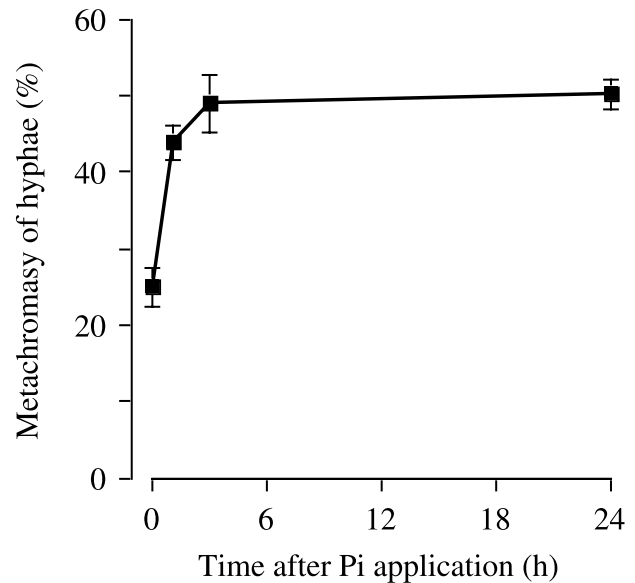


Fig. 1 Time course assessment of polyphosphate in extraradical hyphae of *Archaeospora leptoticha* by histochemistry. Polyphosphate was localized by metachromasy of Toluidine blue O and the percentage of hyphae with metachromatic granules was assessed. Vertical bars show SE ($n = 3$).

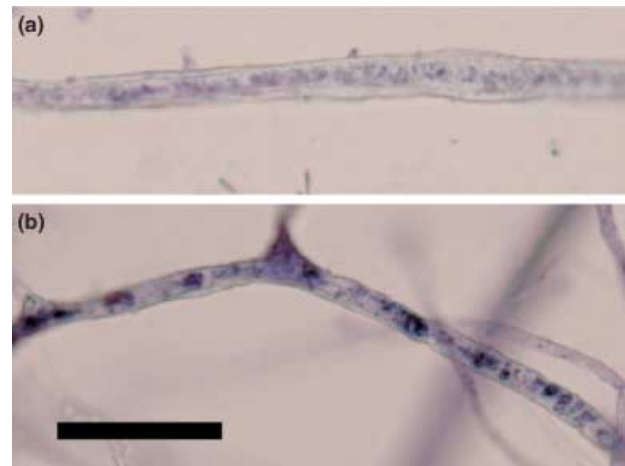


Fig. 2 Accumulation of polyphosphate in extraradical hyphae of *Archaeospora leptoticha* as revealed by metachromasy of Toluidine blue O. (a) Scattered metachromatic granules observed in hyphae harvested at zero time (no P control). (b) Heavy accumulation of metachromatic granules in hyphae harvested 3 h after P-application. Bar, 10 μm .

PolyP accumulation in P-deficient yeast (Schuddemat *et al.*, 1989) and bacterial cells (Castrol *et al.*, 1999) was even more rapid and reached a maximum level within 15–30 min under glucose (energy source)-sufficient conditions. An initial polyP formation rate of Pi-deficient cells of *Acinetobacter johnsonii* 210 A which could accumulate polyP up to 50% of their dry weight was 98 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein (van Niel *et al.*, 1999). The rate of polyP accumulation observed in the present study ($46.4 \text{ nmol min}^{-1} \text{mg}^{-1}$ protein) may be an underestimation,

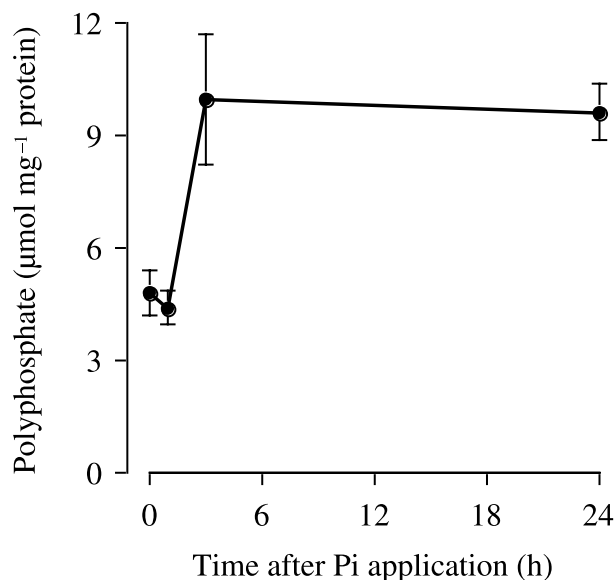


Fig. 3 Time course assessment of polyphosphate in extraradical hyphae of *Archaeospora leptoticha* by the polyphosphate kinase/luciferase system. Polyphosphate was extracted and quantified as detailed in Materials and Methods, and polyphosphate content was expressed as Pi-residues per mg protein. Vertical bars show SE ($n = 3$).

because the TBO staining revealed that only 50% of extraradical hyphae were responsible for polyP biosynthesis, but the rate was calculated on the basis of total protein content of the hyphal material irrespective of the presence of polyP. Therefore, the polyP accumulation rate of the AM fungus could be comparable to that of the polyP-hyper accumulating bacteria, *A. johnsonii* 210 A. It is considered that P-delivery in AM association is regulated through the following four steps: P-uptake, -compartmentation (polyP biosynthesis), -translocation along hyphae and -transfer to the host. The fact that the P-uptake and subsequent polyP biosynthesis were surprisingly rapid suggests that the rate-limiting step of the P-delivery system is the translocation or transfer to the host. This hypothesis requires further confirmation.

It is predicted that external Pi concentration also affects the rate of polyP accumulation. In the present study we applied 1 mM Pi, which was extremely high compared with Pi concentration of normal soil solution. Therefore, the rate of polyP accumulation obtained in the present study can be regarded as a potential rate under optimal conditions. On the other hand, given the heterogenous distribution of nutrients in soil both spatially and temporally, our results may reflect growth of P-starved hyphae exploiting a newly created or discovered patch of nutrient rich soil.

About 50% of extraradical hyphae showed metachromatic granules using TBO in the present study, consistent with observation of *Glomus coronatum* with *T. patula* (Ezawa *et al.*, 2001). Boddington and Dodd (1999b) observed using DAPI that 50–70% of extraradical hyphae of *Gi. rosea* in symbiosis with *Desmodium ovalifolium* accumulated polyP irrespective

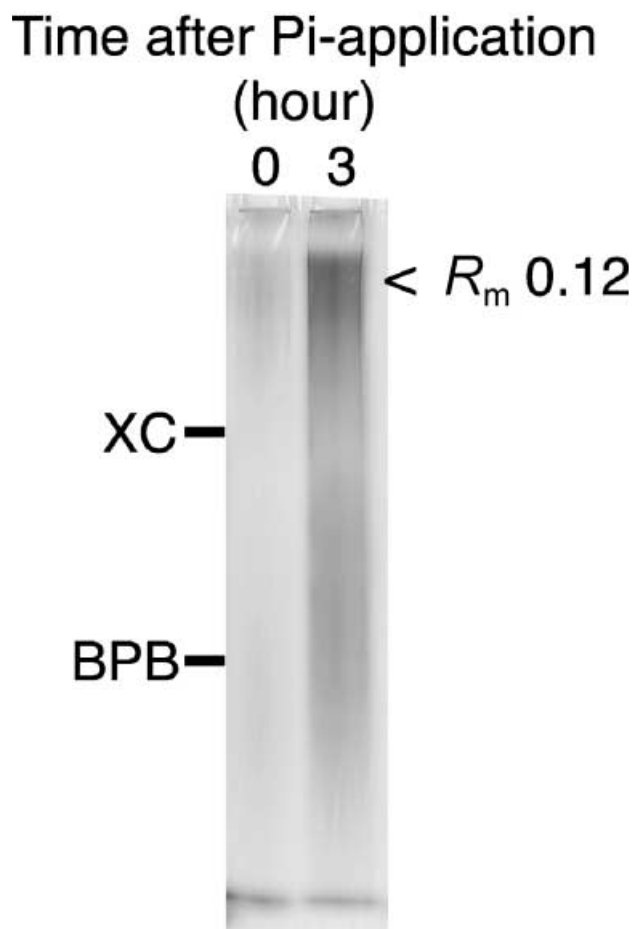


Fig. 4 Electrophoretic analysis of polyphosphate extracted from extraradical hyphae of *Archaeospora leptoticha* 0 and 3 h after P-application. Polyphosphate was extracted, purified as detailed in Materials and Methods and loaded on 10% polyacrylamide gel (buffered with TBE). Loading volume was standardized by protein concentration of the crude extracts (30 μg protein per lane). The position of xylene cyanol (XC) and bromophenol blue (BPB), tracking dyes of electrophoresis, are indicated. R_m , relative mobility to BPB.

of soil P fertility. The percentage of hyphae with polyP may indicate the percentage of hyphae that contribute to P-uptake and translocation in the hyphal network.

The chain-length distribution of polyP in AM fungi has not been determined precisely. An average chain-length of freely mobile polyP in *Gl. intraradices* was 15 as revealed by NMR (Rasmussen *et al.*, 2000). A broad distribution of polyP chain-length was also observed in the extraradical hyphae of *Gl. etunicatum* in which high-molecular weight polyP was estimated as more than 190 residues (Ezawa *et al.*, 1999). Yeast, a model eukaryotic microorganism, is known to accumulate three to several hundred Pi-residues of polyP (Kornberg *et al.*, 1999). PolyP chain-length may depend on the balance between cell energy status (Schuddemat *et al.*, 1989) and probably on P availability in environment and in turn affect solubility of polyP *in situ* (Ezawa *et al.*, 2002).

The PPK/luciferase method is highly specific and sensitive to polyP (Ault-Riché *et al.*, 1998). This is the first case that has employed the method in AM associations. It is worthwhile that the TBO method, a conventional histochemical assessment of polyP, has been validated by the PPK method. Generally, results obtained by the TBO method were in good agreement with those obtained by the PPK method: the values at zero time were doubled at 3–24 h after P-application. The disagreement between the data at 1 h after P-application may be caused by the following two reasons. Firstly, we could not assess the density or size of metachromatic granules which might be less or smaller in hyphae at 1 h than those in hyphae at 3 and 24 h, resulting in an overestimation of the TBO-stained material at 1 h. Secondly, sensitivity of the two methods to short-chain polyP may differ. Reactivity of PPK to polyP shorter than 20 residues is much lower than that to longer polyP, whereas TBO shows relatively constant reactivity to shorter polyP (T. Ezawa and R. Ohtomo, unpublished results). It has been reported that short-chain polyP was accumulated at the initial phase of polyP biosynthesis in yeast (Schuddemat *et al.*, 1989). It is likely that the AM fungus would have accumulated short-chain polyP with which TBO could react, but PPK could not react in the first hour after P-application.

Little is known about mechanisms of polyP biosynthesis and compartmentation in eukaryotic microorganisms. Most of polyP is compartmentated in vacuoles in eukaryotic microorganisms, yeast (Kornberg *et al.*, 1999) and *Neurospora crassa* (Yang *et al.*, 1993). Rasmussen *et al.* (2000) suggested that polyP in AM fungi was also compartmentated in acidic compartment based on the chemical shift of terminal Pi residue of polyP by *in vivo* ³¹P-NMR. At least two types of acidic compartment, neutral red-stained vacuoles (Ezawa *et al.*, 2001) and tubular vacuoles (Uetake *et al.*, 2002), are known in AM fungi, and probably there are more types (K. Saito, pers. comm.). It is expected that the techniques employed in the present study can be applied to reveal subcellular localization of polyP. Furthermore, establishment of the PPK method in conjunction with the histochemical assessment of polyP for AM hyphae extracted from a soil based system could be used as an indicator of 'nutritionally functional' AM fungi under a range of conditions.

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