Title	ATP-Dependent but Proton Gradient-Independent Polyphosphate-Synthesizing Activity in Extraradical Hyphae of an Arbuscular Mycorrhizal Fungus
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Title: ATP-dependent but proton gradient-independent polyphosphate-synthesizing activity in extraradical hyphae of an arbuscular mycorrhizal fungus

Running title: POLYPHOSPHATE-SYNTHESIZING ACTIVITY IN AM FUNGUS

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Abstract

Arbuscular mycorrhizal fungi benefit their host plants by supplying phosphate obtained from the soil. Polyphosphate is thought to act as the key intermediate in this process, however, little is currently understood about how polyphosphate is synthesized or translocated within arbuscular mycorrhizas. *Glomus* sp. HR1 was grown with marigold in a mesh bag compartment system, and extraradical hyphae were harvested and fractionated by density gradient centrifugation. Using this approach, three distinct layers were obtained: Layers 1 and 2 were composed of amorphous and membranous materials, together with mitochondria, lipid bodies and electron-opaque bodies, and Layer 3 was composed mainly of partially broken hyphae and fragmented cell walls. The polyphosphate kinase/luciferase system, a highly sensitive polyphosphate detection method, enabled the detection of polyphosphate-synthesising activity in Layer 2 in the presence of ATP. This activity was inhibited by vanadate but not by bafiromycin A_1 or by a protonophore, suggesting that ATP may not energize the reaction through H^+-ATPase but act as a direct substrate in the reaction. This report represents the first demonstration that AM fungi possess polyphosphate-synthesizing activity that is localized in the organelle fraction and not in the cytosol or at the plasma membrane.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs that form symbiotic associations with most land plants (29). These fungi promote the growth of host plants via enhanced uptake of phosphate (Pi) and thus play important roles in the terrestrial phosphorus cycle. In the symbiotic phase, AM fungi take up Pi from soil through an extensive network of extraradical hyphae and rapidly accumulate inorganic polyphosphate (polyP). This accumulation was as rapid as that for a polyP-hyperaccumulating bacterium found in activated sludge (6). PolyP is a linear polymer of three to hundreds of Pi linked by high energy-phosphoanhydride bonds and has been found across all classes of organisms (19). Although polyP is considered to play a central role in long-distance translocation of Pi in AM fungal associations (4, 10, 30, 31), the translocation mechanism, metabolism and dynamics in the fungi have not been elucidated due to the difficulty in obtaining sufficient fungal material for analysis.

Many enzymes/genes involved in polyP synthesis/metabolism have been identified and characterized in prokaryotes (19). For instance, exopolyphosphatase (PPX) hydrolyzes the terminal high-energy bonds of polyP and polyphosphate glucokinase (PPGK) transfers the terminal Pi residue to glucose. Polyphosphate kinase 1 (PPK1) is responsible both for polyP synthesis using ATP as a phosphoryl donor and for the reverse ATP-generating reaction. This enzyme is bound to plasma membrane (18) and has been found from a wide range of bacteria

(17). Unlike prokaryotes, knowledge of polyP synthesis/metabolism in eukaryotes remains limited. The first eukaryotic PPK genes, *DdPPK1* (32) and *DdPPK2* (14), were identified from the social slime mold *Dictyostelium discoiderm*. The products of these genes are, as known for bacterial PPK1s, responsible both for polyP synthesis and for ATP-generating reaction and have been suggested to be associated with vacuoles or small vesicles (14, 32). Although several homologues of bacterial PPK1 genes have now been found in the genomes of eukaryotic microorganisms (17), yeast *Candida humicola* is the only organism apart from *D. discoiderm* for which PPK-like activity has been confirmed (22). The model organism *Saccharomyces cerevisiae* is known to accumulate polyP up to 10% of its dry weight (19). A unique polyP synthetic pathway different from those of PPK1 has been proposed for *S. cerevisiae* based on the observation that the vacuolar-type H^+-ATPase (V-ATPase)-defective mutants could not accumulate polyP (23). In this hypothetical pathway, Pi would be polymerized by an analogous system (enzyme) of mitochondrial F_1-ATPase on the vacuolar membrane using the proton motive force created by V-ATPase (23). On the other hand, Hothorn *et al*. (16) demonstrated very recently that vacuolar transporter chaperone 4 (VTC4), a small transmembrane protein associated with membrane, polymerizes Pi using γ-Pi residue of ATP as a phosphoryl donor in *S. cerevisiae*.

More than two decades ago, Cappacio and Callow (3) reported the presence of polyP-hydrolyzing, -metabolizing (PPGK) and -synthesizing (PPK-like) activities in the soluble (cytosolic) fractions of the hyphae of AM fungus *Glomus mosseae*. Recently, polyP-hydrolyzing activity has been found both in the cytosolic and insoluble (membrane) fractions and characterized (8). PPGK activity has also been confirmed in the cytosolic fraction, although the activity was quite low and hexokinase (ATP-hexose phosphotransferase) activity appeared to dominate in the glucose phosphorylation process (9). PPK-like activity, however, could not be detect in the same fraction (10), and this seems likely because all other prokaryotic (reviewed in 17) and eukaryotic (14, 16, 22, 32) polyP-synthesizing enzymes, so far, are associated with membranes. These observations suggest that AM fungi possess a polyP-synthesizing enzyme that is probably associated with membranes and that ATP may be essential in the synthesis as a phosphoryl donor or via H^+-ATPase as suggested by Ogawa et al. (23). In this study, a cell fractionation technique was applied to demonstrate polyP-synthesizing activity in an AM fungus, and then the role of ATP in the synthesis was investigated.

MATERIAL AND METHODS

Fungal material. *Glomus* sp. HR1 (MAFF520076) was isolated from the rhizosphere soil of *Lespedeza* sp. grown in acidic soil and deposited in the NIAS Genebank (http://www.gene.affrc.go.jp/about_en.php). The small subunit ribosomal RNA gene sequence (GenBank accession number is AB220171) showed high-similarity to those of *G.*

manihotis and *G. clarum* that belong to *Glomus* Group A, Glomeromycota (28). Dwarf marigold (*Tagetes patula* L. cv. Bonanza Orange, Murakami Seed, Ibaraki, Japan) was inoculated with 1,000 spores of *Glomus* sp. HR1 and grown in a mesh bag compartment system in which the root/hyphal (R + H) compartment and the hyphal (H) compartment was separated by a mesh bag (37 μm nylon mesh, 40 ml in vol) in a plastic pot (5.5 cm in diam, 90 ml in vol) (8). The R + H compartment was filled with 1 : 2 autoclaved washed pumice (4 - 10 mm in diam)-river sand mixture, while the H compartment was filled with autoclaved river sand. The plants (one batch consisted of 60 to 70 pots) were grown in growth chambers (16 h photoperiod, 25°C, relative humidity 60%) and received distilled water for the first week, followed by Peters Professional 25-5-20 (N-P_2O_5-K_2O) liquid fertilizer (Scotts-Sierra Horticultural Products, OH, USA) at 50 mg N l^{-1} for the second week and then low-P liquid fertilizer (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 third to sixth week every other day in sufficient amount until the solutions flowed out from the drain holes. At the end of sixth week, 1 mM KH_2PO_4 solution was applied to the plants in sufficient amount 4-9 h prior to harvest, and extraradical hyphae were collected from the H compartment from all pots of the batch by wet sieving, combined, cleaned under a dissecting microscope as quickly as possible and placed on ice.

Cell fractionation. All of the following experiments were done under ice-cooled conditions. Approximately 0.5 - 1.5 g hyphal samples were homogenized immediately after the harvest on mortar and pestle with 5-fold volume (w/v) of buffer A (1 mM Na_2ATP and 1.2 M sorbitol in 10 mM HEPES/KOH pH 7.4) with the Protease Inhibitor Cocktail for use with fungal and yeast extract (Sigma-Aldrich, Tokyo). The slurry was transferred to a 15 ml plastic tube, and the mortar and pestle were washed with the same volume of buffer A, and then the solutions were combined. After centrifugation at 160 ×*g* for 5 min at 4˚C, the upper layer was transferred to a new tube, and the pellet was resuspended in 5-fold volume (w/v) of buffer A and centrifuged under the same conditions. The upper layers were combined, layered on a continuous density gradient that was prepared by centrifugation of 50% (v/v) Percoll (GE Healthcare, Tokyo) in buffer A at 20,000 ×*g* for 5 h at 4˚C and centrifuged at 2,500 ×*g* for 2 h at 4˚C. The resultant fractions with densities of 1.02 - 1.05, 1.06 - 1.09 and > 1.15 g ml^{-1} were designated as Layers 1, 2 and 3, respectively (Fig. 1). These fractions were collected separately, mixed with 20-fold volume of buffer A and centrifuged at 18,000 ×*g* for 15 min at 4˚C. The pellets were washed twice with the same buffer, resuspended in a minimum volume of the buffer and stored on ice.

For transmission electron microscope (TEM) observation of the fractions, the pellets of Layers 1-3 were fixed with 4% (w/v) paraformaldehyde-1% (w/v) glutaraldehyde in buffer A for 16 h at 4˚C. After the fixation, the pellets were centrifuged and rinsed three times with 10 mM HEPES/KOH at pH 7.4 for 10 min, embedded in a drop of 1% (w/v) low-temperature melting agarose (Sigma-Aldrich) and processed with standard procedure for TEM. Briefly, the materials were dehydrated with ethanol series, substituted with a propylenoxide and infiltrated in Supper's resin (Nisshin EM, Tokyo) that was polymerized at 70°C overnight. Ultra-thin sections were cut with glass-knives or a diamond knife, put on copper grids, stained with tannic acid followed by lead citrate and observed using a JEM-1200EX TEM (JEOL, Tokyo).

Quantification of polyphosphate. Each fraction was mixed with 9-fold volume of polyP extraction buffer (8 M urea in 50 mM Tris/HCl pH 8.0), vortexed for 30 s and centrifuged at 18 000 × *g* for 15 min at 4°C. The supernatant was collected, and urea was eliminated using a Micro Bio-Spin P-6 gel filtration column (Bio-Rad Laboratories, Tokyo) pretreated with TE buffer (1 mM EDTA in 10 mM Tris/HCl pH 8.0) according to the manufacturers' instructions. PolyP content was determined by the *E. coli* PPK/luciferase method (2) with some modifications (6). Total protein in the fractions was precipitated with trichloroacetic acid and reextracted with NaOH (7), and the concentrations were determined by the modified Lowry method using a *DC* Protein Assay Kit (Bio-Rad Laboratories, Tokyo) according to the manufacturers' instructions.

Enzyme assay conditions. PolyP-synthesizing activity was assessed based on an increase in polyP content after incubation in the presence of ATP. Fifteen microliters of the fractions were mixed with an equal volume of buffer A on ice, and two 10 μl aliquots of the mixture were transferred to a new tube. One was mixed with 90 μl of polyP extraction buffer and left on ice as the 0-time control, while the other was incubated for 30 min at 30°C. After incubation, solutions were mixed with 90 μl of polyP extraction buffer. PolyP concentrations in the mixtures before (0-time control) and after the incubation were determined by the PPK/luciferase method. To examine the involvement of proton motive force in polyP synthesis, the effects of 500 μM vanadate [Plasma membrane-type H^+-ATPase (P-ATPase) inhibitor], 100 nM bafilomycin A_1 (V-ATPase inhibitor) and 50 μM carbonylcyanide-*m*-chlorophenylhydrazone (CCCP, protonophore) on the synthesizing activity were assessed. In this assessment, 0.5% (v/v) dimethylsulfoxide (DMSO) was added to all reaction mixtures as the stock solutions of bafilomycin A_1 (20 μM) and CCCP (10 mM) were prepared with DMSO. PolyP-synthesizing activity was defined based on an increase in Pi-residues of polyP per min per unit protein under the specified conditions.

P- and V-ATPases and cytochrome *c* oxidase (COX) were employed as marker enzymes for the plasma membrane, tonoplast and mitochondria, respectively, to characterize the fractions. The fractions used for the assessment of P- and V-ATPase activities were prepared from the hyphae to which 1 mM Pi solution was not applied prior to harvest, as fractions prepared from the Pi-applied hyphae contained a large amount of polyP that was hydrolyzed during incubation and interfered with the assay. P- and V-ATPase activities were determined as the 'specific inhibitor-sensitive activities' as follows:

*Inhibitor-**sensitive** activity = Total ATPase activity* –Inhibitor-**insensitive** activity*

**Total ATPase activity = ATP hydrolyzing activity – non-specific phosphatase activity*†

†Non-specific phosphatase activity was determined using ADP as a substrate.

Twenty five microliters of the fractions were mixed with an equal volume of reaction mixture consisting of 100 mM KCl, 20 mM $MgCl_2$, 2.4 M sorbitol, 0.2% DMSO, 2 mM substrate (Na_2ATP or Na_2ADP) and 80 mM HEPES/KOH at pH 7.5 in the presence or absence of inhibitors on ice, and two 20 µl aliquots of the mixture were transferred to new tubes. One was mixed with an equal volume of 10% (w/v) sodium dodecylsulfate (SDS) and left on ice as a 0-time control, and the other was incubated for 30 min at 30°C, and then an equal volume of 10% SDS was mixed with the solution. The levels of released Pi in the solutions before (0-time control) and after the incubation were determined as previously described (24). The activity was expressed as the amount of Pi released per min per unit protein under the specified conditions. COX activity was measured as previously described (15). Prior to the assessment, 1 ml of 2 mg ml^{-1} horse heart cytochrome *c* (Wako Pure Chemicals, Osaka) in 100 mM potassium phosphate buffer (pH 7.5) was reduced by mixing with 2-3 mg sodium dithionite and passed through a PD-10 Sephadex G-25M column (GE Healthcare, Tokyo) equilibrated with the phosphate buffer to remove excess sodium dithionite. Ten microliters of the fractions were mixed with an equal volume of 1% (v/v) TritonX-100 in the phosphate buffer and 180 µl of 1 mg ml^{-1} reduced cytochrome *c* in the phosphate buffer, and decreases in absorbance at 550 nm were monitored for 3 min at room temperature. The concentration of oxidized cytochrome *c* was calculated based on an extinction coefficient of 18.5 mM^{-1} cm^{-1}, and the activity was expressed as an increase in oxidized cytochrome *c* per min per unit protein under the specified conditions. Polyphosphate hydrolyzing activity was measured based on liberation of Pi at pH 7.5 using 1 mM polyP type 75+ (average chain-length 79, Sigma-Aldrich) as a substrate (8).

Each enzyme assay was triplicated using the same batch of material ($n = 3$). The treatments that showed zero or negative values were excluded from subsequent statistical analysis and expressed as 'not detected'. ANOVA followed by Fisher's protected least significant difference test or Student's *t*-test was performed for tests of significance by the StatView software (SAS Institute Inc., NC).

RESULTS

Cell fractionation and polyphosphate-synthesizing activity. Cell fractionation was carried out several times using different batches of fungal material (Table 1 and Table S1), and representative results are shown in Table 1. After centrifugation of the hyphal homogenate at 160 ×*g*, 20-45% of polyP was recovered in the upper layer. Layers 1, 2 and 3 that were obtained from the 160 ×*g* upper layer by the Percoll density gradient centrifugation retained only 0.1-0.5% of total polyP. Whereas 30-70% of polyP of the 160 ×*g* upper layer was recovered from the supernatant of the density gradient centrifugation. PolyP concentrations per unit protein in Layers 1-3 were lower than that of the homogenate.

Ultrastructural observations of the fractions revealed that Layers 1 and 2 were composed of membranous and amorphous materials, together with organelles that were identified as mitochondria, lipid bodies and electron-opaque bodies (Fig. 2a-c). A few bacteria were observed in these layers (data not shown). No conspicuous difference in composition between Layer 1 and 2 was found. Layer 3 was composed of mainly partially broken hyphae and fragmented cell walls (Fig. 2d and e). Amorphous material associating with the cell wall fragments was also observed.

PolyP-synthesizing activity was consistently detected in Layer 2 of all batches in the presence of ATP (Table 2 and Table S2). Although Layer 1 showed polyP-synthesizing activity in some batches, the detection of the activity in Layer 1 was poorly reproducible (data not shown). Layer 2 was thus used for subsequent characterization of the polyP-synthesizing activity. P-ATPase activity was enriched in Layer 3, while V-ATPase activity could not be detected in any of the layers after the density gradient centrifugation (Table 3). COX activity was enriched in Layers 1 and 2. PolyP-hydrolyzing activity was diluted in all layers after the density gradient centrifugation.

Substrate for polyphosphate synthesis. To examine whether ATP was used as a direct substrate or as an energy source to create the proton gradients for polyP synthesis (Pi would be substrate in this case), Layer 2 was incubated with ATP, Pi or both using the fraction prepared in the presence (original method) or absence (ATP was withheld from buffer A used for the washing step after density gradient centrifugation) of ATP. The activity of the fraction prepared with ATP and incubated with ATP was regarded as a positive control. No polyP-synthesizing activity was detected in the fraction prepared without ATP and incubated with no substrate or with 1 mM Pi (Fig. 3). The fraction prepared without ATP and incubated in the presence of ATP showed polyP-synthesizing activity, although the activity was lower than that of the positive control. No activity was observed in the fraction prepared in the presence of ATP and incubated with both ATP and Pi. The effects of P- and V-ATPase inhibitors and protonophore were examined for further characterization. Vanadate showed an inhibitory effect on polyP-synthesizing activity, whereas bafilomycin A_1 did not (Fig. 4a). CCCP had no inhibitory effect on the activity (Fig. 4b).

DISCUSSION

Given the fact that AM fungal associations play a key role in phosphorus acquisition of the majority of land plants, it is of significance to clarify the polyP synthetic pathway, the first step of polyP metabolism and translocation, in the biotrophic fungi. PolyP-synthesizing activity in an AM fungus *Glomus* sp. HR1 was successfully demonstrated in combination with cell fractionation. PolyP-synthesizing activity could not be detected in the soluble (cytosolic) fractions but was associated with the insoluble cellular components (membranous and amorphous material and organelles) of which the density was within the range of 1.06-1.09 g ml^{-1}. It has been shown that bacterial PPKs bounds peripherally to the inner plasma membrane (19). The association of the polyP-synthesizing activity with plasma membrane, however, could be excluded in the AM fungus as activity of the P-ATPase, a plasma membrane marker, was not concurrent with the polyP-synthesizing activity. The association of the activity with mitochondria may also be unlikely, because polyP-synthesizing activity was consistently detected only in Layer 2 but the mitochondrial marker enzyme, COX, was enriched both in Layer 1 and 2 after fractionation to the same extent. The amorphous material was not specific to Layer 2 and observed in all fractions, and thus the association of polyP-synthesizing activity with the material is unlikely. The membranous material observed in Layer 2 is likely to be vacuolar membrane due to the fact that AM fungal cells contain numerous vacuoles, although the vacuolar marker enzyme, V-ATPase, could not be detected in any fraction. Possibly, V-ATPase of the fungus is susceptible to physical disruption/fractionation or that the activity is intrinsically very low in the fungus. It seems likely that polyP-synthesizing activity is associated with vacuolar membrane due to the following two reasons. Firstly, all other eukaryotic polyP-synthesizing enzymes, DdPPK1 (32), DdPPK2 (14) in the slime mold and VTC4 in yeast (16), are associated with vacuoles or small vesicles. Secondly, AM fungi accumulate polyP in vacuoles as observed in the germ tubes of *Gigaspora margarita* (20) and in the extra- and intraradical hyphae in *Glomus* sp. HR1 (Kuga & Ezawa, unpublished observations). Further characterization is required to localize the activity.

Our results are not consistent with those reported by Capaccio and Callow (3) in which Poly-synthesizing activity was detected in the extract (soluble fraction) of an AM fungus. They incubated radioactive ATP with the extract and identified polyP as trichloroacetic acid-precipitated radioactive compound. This approach was the most reliable for detection of polyP at that time but may not be specific to polyP. One possibility, therefore, is that the polyP-synthesizing activity reported previously represented an activity of another phosphotransferase-type enzyme that was localized in the cytosol and used ATP as a phosphoryl donor. It is also likely that the extract was contaminated with the membrane fraction that retained polyP-synthesizing activity. The radioactive compound-based method is highly sensitive such that contamination by trace amounts of membrane may also result in the detection of the activity.

The following three technical breakthroughs were indispensable for the detection of polyP-synthesizing activity in the fungus. i) Small-scale and high-sensitive polyP assay system: the PPK/luciferase-polyP assay system (2) has been applied to AM fungi recently (6, 25). Although the method is relatively less sensitive to polyP shorter than twenty Pi-residues (26), it was essential for the measurement of picomole levels of polyP in the present study. ii) Selection of fungal species that produces few spores and a large amount of extraradical hyphae: *Glomus* sp. HR1 produced fewer spores and greater hyphal mass over 6- to 8-week-culture than other species examined (data not shown), although *Glomus* sp. HR1 produced a large amount of spores after 4-month-culture. This is a quite important characteristic for cell fractionation, because AM fungal spores are, in general, filled with lipids that aggregate with organelles and interferes with cell fractionation. iii) Enrichment of polyP-synthesizing activity by cell fractionation: the activities of PPX (3, 8), acid (8, 12) and alkaline (1, 7, 11, 13) phosphatases have been detected in AM fungi, and these enzymes are likely to be involved in polyP hydrolysis. In fact, polyP-hydrolyzing activity in the hyphal homogenate was quite high in the present study. Therefore, cell fractionation that could enrich polyP-synthesizing activity and dilute polyP-hydrolyzing activity was necessary for the detection. It should be noted, in addition, that the polyP-synthesizing activity estimated in our study might have been underestimated due to the concurrence of polyP-hydrolyzing activity in the fraction.

ATP was essential for the polyP synthesis in the fungus, and the results strongly suggest that ATP did not energize the reaction via P- or V-ATPase but acted as a direct substrate in the reaction due to the following reasons: firstly, the V-ATPase inhibitor and protonophore did not inhibit the activity. Secondly, P-ATPase activity was enriched in Layer 3 that showed no polyP-synthesizing activity. Although vanadate, the P-ATPase inhibitor, inhibited the polyP-synthesizing activity, the involvement of P-ATPase, i.e. the proton gradient across plasma membrane, could be validated only if the activity was inhibited by both vanadate and the protonophoric reagent, because vanadate has been known to inhibit many phosphoryl transfer enzymes as a structural and chemical mimic of phosphate (5). The facts that all polyP-synthesizing enzymes found from prokaryotes (19) and eukaryotes

(14, 16, 22, 32) used ATP as a direct phosphoryl donor also support our observations. Catalysis of the reverse ATP-regenerating reaction is a typical feature of PPK-type enzymes in both prokaryotes (19) and eukaryotes (14, 32) but is unlikely in the VTC4 found in yeast (16). In the present study, it was difficult to examine whether Layer 2 catalyzed the reverse reaction due to the high-background of ATP that was essential for the protection/stabilization of the polyP-synthesizing activity during fractionation. The polyP-synthesizing activity was inhibited by the coexistence of ATP and Pi. It is postulated that Pi interfered with the activity through binding to the catalytic center or that Pi might act as an allosteric effector that regulates the balance between ATP consumption and polyP synthesis in the cell. However, given the fact that cytoplasmic Pi homeostasis is strictly maintained at millimolar levels, e.g. 5-10 mM in the case of plants (27), it is unlikely that cytoplasmic Pi is directly involved in the regulation of polyP synthesis in AM fungi. The underlying mechanism will be clarified if the enzyme is purified and localized at the subcellular level.

It is of importance to identify enzymes/genes involved in polyP metabolism for a clear understanding of the role of polyP in Pi translocation in AM fungi. The present study suggests that purification/localization of the enzyme will be possible using polyP-synthesizing activity as a marker. Application of forthcoming genomic information (21) in conjunction with biochemical analysis will be one promising approach.

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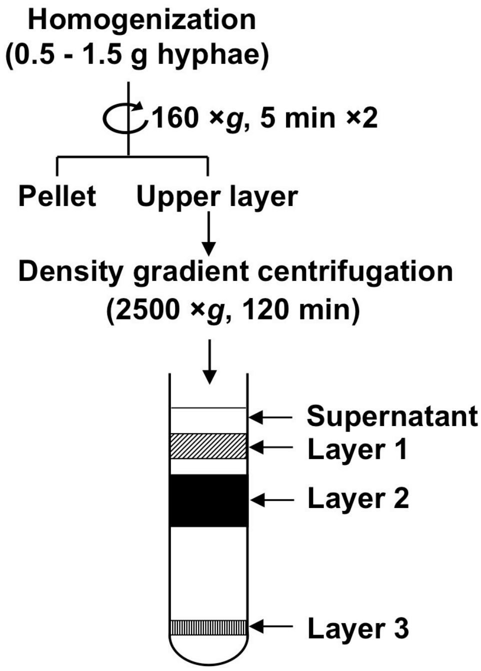

FIG. 1. Schematic diagram for the fractionation of the extraradical hyphae of *Glomus* sp. HR1. The densities of the fractions are as follows: Layer 1, 1.02-1.05 g ml^{-1}; Layer 2, 1.06-1.09 g ml^{-1}; Layer 3, > 1.15 g ml^{-1}.

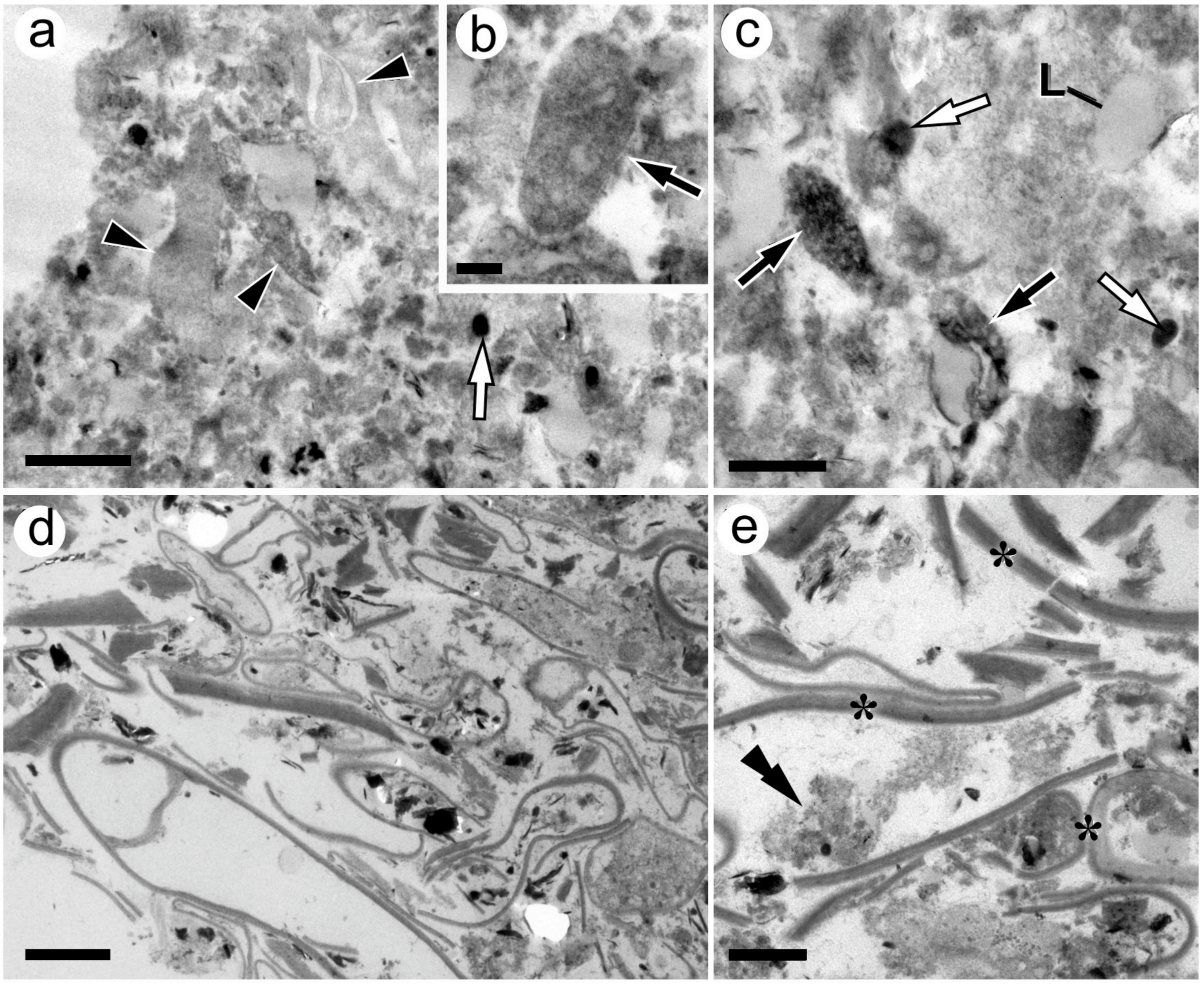

FIG. 2a-e. Ultrastructure of Layers 2 and 3 fractions of *Glomus* sp. HR1 extraradical hyphae. a-c. Layer 2 composed of amorphous and membranous (black arrowheads) materials with mitochondria (black arrows), lipid body (L) and electron opaque body (white arrows). d and e. Layer 3 composed of partially broken hyphae and fragmented hyphal cell wall (asterisks) with which amorphous material was associated (double arrowhead). Bars: a, 1 µm; b, 0.2 µm; c, 0.5 µm; d, 2 µm; e, 1 µm.

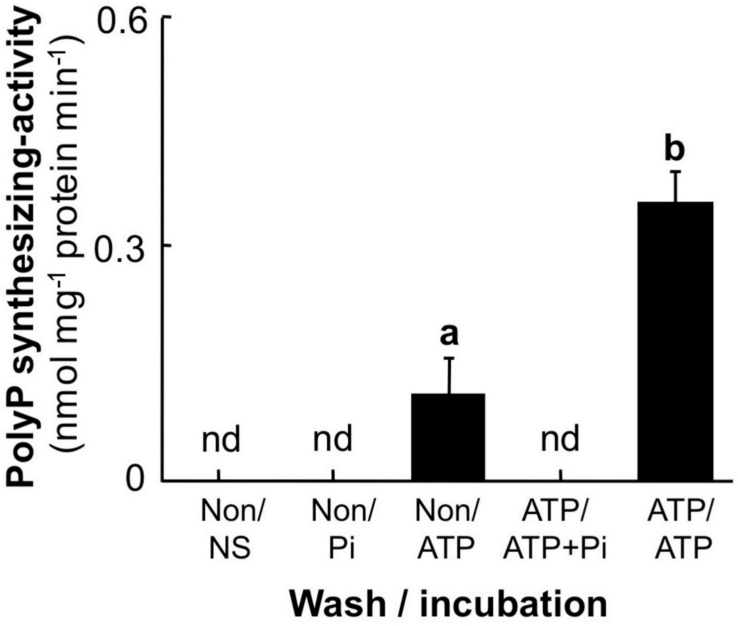

FIG. 3. Effect of ATP and phosphate (Pi) on polyphosphate (polyP)-synthesizing activity in Layer 2 prepared from the extraradical hyphae of *Glomus* sp. HR1 in symbiosis with *T. patula*. The fraction was washed with (ATP) or without (Non) 1 mM ATP and incubated in the absence (NS, no substrate) or presence of 1 mM ATP, Pi or both. Vertical bars indicate ± SE (n = 3). nd, not detected. Different letters indicate significant difference ($P < 0.01$, Student's t-test between the two treatments).

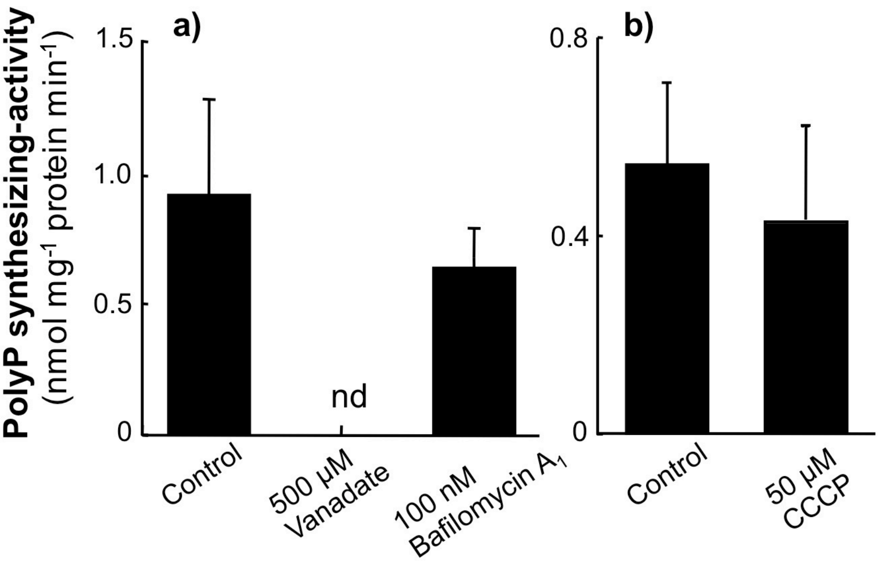

FIG. 4. Effects of H^+-ATPase inhibitors (a) and protonophore (b) on polyphosphate (polyP) synthesizing-activity in Layer 2 prepared from extraradical hyphae of *Glomus* sp. HR1 in symbiosis with *T. patula*. a, The layer 2 was incubated with 1 mM ATP in the presence or absence of 500 µM vanadate (plasma membrane-type H^+-ATPase inhibitor) or 100 nM bafilomycin A_1 (vacuolar-type H^+-ATPase inhibitor). nd, not detected. No significant difference in the activity between the control and bafilomycin A_1 treatments was observed ($P > 0.05$, Student's *t*-test). b, The layer 2 was incubated with 1 mM ATP in the presence or absence of 50 µM carbonylcyanide-*m*-chlorophenylhydrazone (CCCP). No significant difference in the activity between the control and CCCP treatments was observed ($P > 0.05$, Student's *t*-test). Vertical bars indicate ± SE ($n = 3$).

TABLE 1. Fractionation of extraradical hyphae of *Glomus* sp. HR1

	Polyphosphate			Protein	
Fraction[a]	Total (nmol)	Concentration (nmol mg^{-1} protein)	Recovery (%)	Total (mg)	Concentration (mg ml^{-1})
Homogenate	14 448	504.6	100.0	28.6	3.58
160 ×*g* centrifugation					
Upper layer	4 464	244.8	30.9	15.2	1.69
Pellet	6 382	386.1	44.2	16.5	2.07
Density gradient centrifugation					
Supernatant	1 409	123.4	9.8	11.4	1.27
Layer 1 (1.02-1.05 g ml^{-1})	75	124.7	0.5	0.6	3.34
Layer 2 (1.06-1.09 g ml^{-1})	74	45.7	0.5	0.6	2.46
Layer 3 (>1.15 g ml^{-1})	42	59.2	0.3	0.7	2.36

[a] Detailed procedure for the fractionation is described in Materials and methods. The experiment was conducted several times using independent batches of fungal material, and one set of the results is shown.

TABLE 2. Polyphosphate synthesizing-activity in the fractions prepared from extraradical hyphae of *Glomus* sp. HR1

Fractions[a]	Activity (nmol Pi mg^{-1} protein min^{-1})[b]
160 × *g* Upper layer	nd
Density gradient centrifugation	
Supernatant	nd
Layer 1	nd
Layer 2	1.01 ± 0.31
Layer 3	nd

[a] The detailed procedure for the fractionation is described in Materials and methods. The experiment was conducted several times using independent batches of fungal material, and one set of the results is shown.

[b] The rates of increase in polyP during 30 min incubation at 30˚C in the presence of ATP. The amount of polyP is expressed as Pi-residues. The values are indicated as mean ± SE ($n = 3$). nd, not detected.

TABLE 3. Characterization of the layers that obtained by the cell fractionation of *Glomus* sp. HR1 extraradical hyphae based on the activities[a] of plasma membrane-type H^+-ATPase, vacuolar-type H^+-ATPase, cytochrome *c* oxidase and polyphosphatase

Fractions[b]	P-ATPase[c]	V-ATPase[c]	COX[d]	PolyPase[e]
		(nmol mg^{-1} protein min^{-1})		
160 × *g* Upper layer	5.44	0.95	2.55 ± 0.07	45.6 ± 3.8
Density gradient centrifugation				
Supernatant	nd	nd	nd	30.5 ± 3.0
Layer 1	nd	nd	5.04 ± 0.16	15.2 ± 0.5
Layer 2	nd	nd	6.55 ± 0.04	13.1 ± 0.4
Layer 3	7.68	nd	1.99 ± 0.03	23.0 ± 2.8

[a] Each enzyme activity was measured using different batches of fungal material.

[b] Detailed procedure for the fractionation is described in Materials and methods. The fractions for the assessment of P- and V-ATPase activities were prepared from the hyphae to which 1 mM Pi solution was not applied prior to harvest.

[c] The rates of specific inhibitor-sensitive and ATP-specific hydrolysis during 30 min incubation at 30˚C. Vanadate and bafilomycin A_1 were used as specific inhibitors for P- and V-ATPases, respectively. Only the mean values (n = 3) are indicated for P- and V-ATPase activities, because these activities were determined by subtracting the inhibitor-sensitive activity from total ATP-specific activity. nd, not detected.

[d] The rates of cytochrome *c* oxidation during 3 min incubation at room temperature. The values are mean ± SE (n = 3).

[e] The rates of polyphosphate hydrolysis during 30 min incubation at 30°C. The values are mean ± SE ($n = 3$).

TABLE S1. Polyphosphate concentrations and recoveries during cell fractionation

	Batch numbers[a]			
Fractions	1	2	3	4
	Polyphosphate concentration (nmol Pi mg^{-1} protein)			
Homogenate	459.5 (100)[b]	306.1 (100)	1248.4 (100)	915.7 (100)
160 ×*g* centrifugation				
Upper layer	155.4 (25.8)	189.3 (38.0)	422.5 (21.3)	481.7 (46.4)
Pellet	602.5 (65.6)	427.6 (75.8)	944.8 (63.6)	929.1 (61.4)
Density gradient centrifugation				
Supernatant	488.9 (16.8)	635.3 (13.8)	626.2 (15.1)	477.2 (12.7)
Layer 1	268.5 (0.2)	56.7 (0.1)	105.7 (0.1)	201.3 (0.2)
Layer 2	151.7 (0.2)	49.5 (0.1)	111.4 (0.3)	93.3 (0.4)
Layer 3	198.7 (0.2)	42.8 (0.1)	89.7 (0.2)	158.9 (0.2)

[a] The experiments were conducted several times by using different batches of fungal material. One batch consisted of 60-70 pots from which 0.5-1.5 g (fw) of hyphal material was collected.

[b] Values in parentheses are the percentages of recovered polyphosphate.

TABLE S2. Polyphosphate synthesizing-activity in the layer 2 prepared by cell fractionation

	Batch numbers[a]			
Fraction	1	2	3	4
	(nmol Pi mg^{-1} protein min^{-1})			
Layer 2	0.37 ± 0.07 [b]	0.43 ± 0.28	0.80 ± 0.60	0.83 ± 0.16

[a] The experiments were conducted several times by using different batches of fungal material. One batch consisted of 60-70 pots from which 0.5-1.5 g (fw) of hyphal material was collected.

[b] Mean value ± SD ($n = 3$).