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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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1 **Abstract**

2

3 Arbuscular mycorrhizal fungi benefit their host plants by supplying phosphate obtained
4 from the soil. Polyphosphate is thought to act as the key intermediate in this process,
5 however, little is currently understood about how polyphosphate is synthesized or
6 translocated within arbuscular mycorrhizas. *Glomus* sp. HR1 was grown with marigold in a
7 mesh bag compartment system, and extraradical hyphae were harvested and fractionated by
8 density gradient centrifugation. Using this approach, three distinct layers were obtained:
9 Layers 1 and 2 were composed of amorphous and membranous materials, together with
10 mitochondria, lipid bodies and electron-opaque bodies, and Layer 3 was composed mainly
11 of partially broken hyphae and fragmented cell walls. The polyphosphate kinase/luciferase
12 system, a highly sensitive polyphosphate detection method, enabled the detection of
13 polyphosphate-synthesizing activity in Layer 2 in the presence of ATP. This activity was
14 inhibited by vanadate but not by bafiromycin A₁ or by a protonophore, suggesting that ATP
15 may not energize the reaction through H⁺-ATPase but act as a direct substrate in the reaction.
16 This report represents the first demonstration that AM fungi possess
17 polyphosphate-synthesizing activity that is localized in the organelle fraction and not in the
18 cytosol or at the plasma membrane.

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INTRODUCTION

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

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

39 (17). Unlike prokaryotes, knowledge of polyP synthesis/metabolism in eukaryotes remains
40 limited. The first eukaryotic PPK genes, *DdPPK1* (32) and *DdPPK2* (14), were identified
41 from the social slime mold *Dictyostelium discoiderm*. The products of these genes are, as
42 known for bacterial PPK1s, responsible both for polyP synthesis and for ATP-generating
43 reaction and have been suggested to be associated with vacuoles or small vesicles (14, 32).
44 Although several homologues of bacterial PPK1 genes have now been found in the genomes
45 of eukaryotic microorganisms (17), yeast *Candida humicola* is the only organism apart from
46 *D. discoiderm* for which PPK-like activity has been confirmed (22). The model organism
47 *Saccharomyces cerevisiae* is known to accumulate polyP up to 10% of its dry weight (19). A
48 unique polyP synthetic pathway different from those of PPK1 has been proposed for *S.*
49 *cerevisiae* based on the observation that the vacuolar-type H⁺-ATPase (V-ATPase)-defective
50 mutants could not accumulate polyP (23). In this hypothetical pathway, Pi would be
51 polymerized by an analogous system (enzyme) of mitochondrial F₁-ATPase on the vacuolar
52 membrane using the proton motive force created by V-ATPase (23). On the other hand,
53 Hothorn *et al.* (16) demonstrated very recently that vacuolar transporter chaperone 4 (VTC4),
54 a small transmembrane protein associated with membrane, polymerizes Pi using γ -Pi residue
55 of ATP as a phosphoryl donor in *S. cerevisiae*.

56 More than two decades ago, Cappacio and Callow (3) reported the presence of
57 polyP-hydrolyzing, -metabolizing (PPGK) and -synthesizing (PPK-like) activities in the
58 soluble (cytosolic) fractions of the hyphae of AM fungus *Glomus mosseae*. Recently,

59 polyP-hydrolyzing activity has been found both in the cytosolic and insoluble (membrane)
60 fractions and characterized (8). PPGK activity has also been confirmed in the cytosolic
61 fraction, although the activity was quite low and hexokinase (ATP-hexose
62 phosphotransferase) activity appeared to dominate in the glucose phosphorylation process (9).
63 PPK-like activity, however, could not be detect in the same fraction (10), and this seems likely
64 because all other prokaryotic (reviewed in 17) and eukaryotic (14, 16, 22, 32)
65 polyP-synthesizing enzymes, so far, are associated with membranes. These observations
66 suggest that AM fungi possess a polyP-synthesizing enzyme that is probably associated with
67 membranes and that ATP may be essential in the synthesis as a phosphoryl donor or via
68 H⁺-ATPase as suggested by Ogawa et al. (23). In this study, a cell fractionation technique was
69 applied to demonstrate polyP-synthesizing activity in an AM fungus, and then the role of ATP
70 in the synthesis was investigated.

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MATERIAL AND METHODS

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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.*

79 *manihotis* and *G. clarum* that belong to *Glomus* Group A, Glomeromycota (28). Dwarf
80 marigold (*Tagetes patula* L. cv. Bonanza Orange, Murakami Seed, Ibaraki, Japan) was
81 inoculated with 1,000 spores of *Glomus* sp. HR1 and grown in a mesh bag compartment
82 system in which the root/hyphal (R + H) compartment and the hyphal (H) compartment was
83 separated by a mesh bag (37 μ m nylon mesh, 40 ml in vol) in a plastic pot (5.5 cm in diam,
84 90 ml in vol) (8). The R + H compartment was filled with 1 : 2 autoclaved washed pumice
85 (4 - 10 mm in diam)-river sand mixture, while the H compartment was filled with
86 autoclaved river sand. The plants (one batch consisted of 60 to 70 pots) were grown in
87 growth chambers (16 h photoperiod, 25°C, relative humidity 60%) and received distilled
88 water for the first week, followed by Peters Professional 25-5-20 (N-P₂O₅-K₂O) liquid
89 fertilizer (Scotts-Sierra Horticultural Products, OH, USA) at 50 mg N l⁻¹ for the second
90 week and then low-P liquid fertilizer (4 mM NH₄NO₃, 1 mM K₂SO₄, 0.75 mM MgSO₄, 2
91 mM CaCl₂, 0.5 mM Fe-EDTA and 50 μ M KH₂PO₄) for the third to sixth week every other
92 day in sufficient amount until the solutions flowed out from the drain holes. At the end of
93 sixth week, 1 mM KH₂PO₄ solution was applied to the plants in sufficient amount 4-9 h
94 prior to harvest, and extraradical hyphae were collected from the H compartment from all
95 pots of the batch by wet sieving, combined, cleaned under a dissecting microscope as
96 quickly as possible and placed on ice.

97 **Cell fractionation.** All of the following experiments were done under ice-cooled
98 conditions. Approximately 0.5 - 1.5 g hyphal samples were homogenized immediately after

99 the harvest on mortar and pestle with 5-fold volume (w/v) of buffer A (1 mM Na₂ATP and
100 1.2 M sorbitol in 10 mM HEPES/KOH pH 7.4) with the Protease Inhibitor Cocktail for use
101 with fungal and yeast extract (Sigma-Aldrich, Tokyo). The slurry was transferred to a 15 ml
102 plastic tube, and the mortar and pestle were washed with the same volume of buffer A, and
103 then the solutions were combined. After centrifugation at 160 ×g for 5 min at 4°C, the upper
104 layer was transferred to a new tube, and the pellet was resuspended in 5-fold volume (w/v)
105 of buffer A and centrifuged under the same conditions. The upper layers were combined,
106 layered on a continuous density gradient that was prepared by centrifugation of 50% (v/v)
107 Percoll (GE Healthcare, Tokyo) in buffer A at 20,000 ×g for 5 h at 4°C and centrifuged at
108 2,500 ×g for 2 h at 4°C. The resultant fractions with densities of 1.02 - 1.05, 1.06 - 1.09 and
109 > 1.15 g ml⁻¹ were designated as Layers 1, 2 and 3, respectively (Fig. 1). These fractions
110 were collected separately, mixed with 20-fold volume of buffer A and centrifuged at 18,000
111 ×g for 15 min at 4°C. The pellets were washed twice with the same buffer, resuspended in a
112 minimum volume of the buffer and stored on ice.

113 For transmission electron microscope (TEM) observation of the fractions, the
114 pellets of Layers 1-3 were fixed with 4% (w/v) paraformaldehyde-1% (w/v) glutaraldehyde
115 in buffer A for 16 h at 4°C. After the fixation, the pellets were centrifuged and rinsed three
116 times with 10 mM HEPES/KOH at pH 7.4 for 10 min, embedded in a drop of 1% (w/v)
117 low-temperature melting agarose (Sigma-Aldrich) and processed with standard procedure
118 for TEM. Briefly, the materials were dehydrated with ethanol series, substituted with a

119 propylenoxide and infiltrated in Supper's resin (Nisshin EM, Tokyo) that was polymerized
120 at 70°C overnight. Ultra-thin sections were cut with glass-knives or a diamond knife, put on
121 copper grids, stained with tannic acid followed by lead citrate and observed using a
122 JEM-1200EX TEM (JEOL, Tokyo).

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

133 **Enzyme assay conditions.** PolyP-synthesizing activity was assessed based on an
134 increase in polyP content after incubation in the presence of ATP. Fifteen microliters of the
135 fractions were mixed with an equal volume of buffer A on ice, and two 10 µl aliquots of the
136 mixture were transferred to a new tube. One was mixed with 90 µl of polyP extraction
137 buffer and left on ice as the 0-time control, while the other was incubated for 30 min at 30°C.
138 After incubation, solutions were mixed with 90 µl of polyP extraction buffer. PolyP

139 concentrations in the mixtures before (0-time control) and after the incubation were
 140 determined by the PPK/luciferase method. To examine the involvement of proton motive
 141 force in polyP synthesis, the effects of 500 μ M vanadate [Plasma membrane-type
 142 H^+ -ATPase (P-ATPase) inhibitor], 100 nM bafilomycin A_1 (V-ATPase inhibitor) and 50 μ M
 143 carbonylcyanide-*m*-chlorophenylhydrazone (CCCP, protonophore) on the synthesizing
 144 activity were assessed. In this assessment, 0.5% (v/v) dimethylsulfoxide (DMSO) was
 145 added to all reaction mixtures as the stock solutions of bafilomycin A_1 (20 μ M) and CCCP
 146 (10 mM) were prepared with DMSO. PolyP-synthesizing activity was defined based on an
 147 increase in Pi-residues of polyP per min per unit protein under the specified conditions.

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

155

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

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

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

159

160 Twenty five microliters of the fractions were mixed with an equal volume of reaction
161 mixture consisting of 100 mM KCl, 20 mM MgCl₂, 2.4 M sorbitol, 0.2% DMSO, 2 mM
162 substrate (Na₂ATP or Na₂ADP) and 80 mM HEPES/KOH at pH 7.5 in the presence or
163 absence of inhibitors on ice, and two 20 µl aliquots of the mixture were transferred to new
164 tubes. One was mixed with an equal volume of 10% (w/v) sodium dodecylsulfate (SDS)
165 and left on ice as a 0-time control, and the other was incubated for 30 min at 30°C, and then
166 an equal volume of 10% SDS was mixed with the solution. The levels of released Pi in the
167 solutions before (0-time control) and after the incubation were determined as previously
168 described (24). The activity was expressed as the amount of Pi released per min per unit
169 protein under the specified conditions. COX activity was measured as previously described
170 (15). Prior to the assessment, 1 ml of 2 mg ml⁻¹ horse heart cytochrome *c* (Wako Pure
171 Chemicals, Osaka) in 100 mM potassium phosphate buffer (pH 7.5) was reduced by mixing
172 with 2-3 mg sodium dithionite and passed through a PD-10 Sephadex G-25M column (GE
173 Healthcare, Tokyo) equilibrated with the phosphate buffer to remove excess sodium
174 dithionite. Ten microliters of the fractions were mixed with an equal volume of 1% (v/v)
175 TritonX-100 in the phosphate buffer and 180 µl of 1 mg ml⁻¹ reduced cytochrome *c* in the
176 phosphate buffer, and decreases in absorbance at 550 nm were monitored for 3 min at room
177 temperature. The concentration of oxidized cytochrome *c* was calculated based on an
178 extinction coefficient of 18.5 mM⁻¹ cm⁻¹, and the activity was expressed as an increase in

179 oxidized cytochrome *c* per min per unit protein under the specified conditions.
180 Polyphosphate hydrolyzing activity was measured based on liberation of Pi at pH 7.5 using
181 1 mM polyP type 75+ (average chain-length 79, Sigma-Aldrich) as a substrate (8).

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

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RESULTS

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

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

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

214 **Substrate for polyphosphate synthesis.** To examine whether ATP was used as a
215 direct substrate or as an energy source to create the proton gradients for polyP synthesis (Pi
216 would be substrate in this case), Layer 2 was incubated with ATP, Pi or both using the
217 fraction prepared in the presence (original method) or absence (ATP was withheld from
218 buffer A used for the washing step after density gradient centrifugation) of ATP. The activity

219 of the fraction prepared with ATP and incubated with ATP was regarded as a positive control.
220 No polyP-synthesizing activity was detected in the fraction prepared without ATP and
221 incubated with no substrate or with 1 mM Pi (Fig. 3). The fraction prepared without ATP
222 and incubated in the presence of ATP showed polyP-synthesizing activity, although the
223 activity was lower than that of the positive control. No activity was observed in the fraction
224 prepared in the presence of ATP and incubated with both ATP and Pi. The effects of P- and
225 V-ATPase inhibitors and protonophore were examined for further characterization. Vanadate
226 showed an inhibitory effect on polyP-synthesizing activity, whereas bafilomycin A₁ did not
227 (Fig. 4a). CCCP had no inhibitory effect on the activity (Fig. 4b).

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DISCUSSION

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232 Given the fact that AM fungal associations play a key role in phosphorus
233 acquisition of the majority of land plants, it is of significance to clarify the polyP synthetic
234 pathway, the first step of polyP metabolism and translocation, in the biotrophic fungi.
235 PolyP-synthesizing activity in an AM fungus *Glomus* sp. HR1 was successfully
236 demonstrated in combination with cell fractionation. PolyP-synthesizing activity could not
237 be detected in the soluble (cytosolic) fractions but was associated with the insoluble cellular
238 components (membranous and amorphous material and organelles) of which the density

239 was within the range of 1.06-1.09 g ml⁻¹. It has been shown that bacterial PPKs bounds
240 peripherally to the inner plasma membrane (19). The association of the polyP-synthesizing
241 activity with plasma membrane, however, could be excluded in the AM fungus as activity of
242 the P-ATPase, a plasma membrane marker, was not concurrent with the polyP-synthesizing
243 activity. The association of the activity with mitochondria may also be unlikely, because
244 polyP-synthesizing activity was consistently detected only in Layer 2 but the mitochondrial
245 marker enzyme, COX, was enriched both in Layer 1 and 2 after fractionation to the same
246 extent. The amorphous material was not specific to Layer 2 and observed in all fractions,
247 and thus the association of polyP-synthesizing activity with the material is unlikely. The
248 membranous material observed in Layer 2 is likely to be vacuolar membrane due to the fact
249 that AM fungal cells contain numerous vacuoles, although the vacuolar marker enzyme,
250 V-ATPase, could not be detected in any fraction. Possibly, V-ATPase of the fungus is
251 susceptible to physical disruption/fractionation or that the activity is intrinsically very low
252 in the fungus. It seems likely that polyP-synthesizing activity is associated with vacuolar
253 membrane due to the following two reasons. Firstly, all other eukaryotic polyP-synthesizing
254 enzymes, DdPPK1 (32), DdPPK2 (14) in the slime mold and VTC4 in yeast (16), are
255 associated with vacuoles or small vesicles. Secondly, AM fungi accumulate polyP in
256 vacuoles as observed in the germ tubes of *Gigaspora margarita* (20) and in the extra- and
257 intraradical hyphae in *Glomus* sp. HR1 (Kuga & Ezawa, unpublished observations). Further
258 characterization is required to localize the activity.

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

270 The following three technical breakthroughs were indispensable for the detection
271 of polyP-synthesizing activity in the fungus. i) Small-scale and high-sensitive polyP assay
272 system: the PPK/luciferase-polyP assay system (2) has been applied to AM fungi recently (6,
273 25). Although the method is relatively less sensitive to polyP shorter than twenty Pi-residues
274 (26), it was essential for the measurement of picomole levels of polyP in the present study.
275 ii) Selection of fungal species that produces few spores and a large amount of extraradical
276 hyphae: *Glomus* sp. HR1 produced fewer spores and greater hyphal mass over 6- to
277 8-week-culture than other species examined (data not shown), although *Glomus* sp. HR1
278 produced a large amount of spores after 4-month-culture. This is a quite important

279 characteristic for cell fractionation, because AM fungal spores are, in general, filled with
280 lipids that aggregate with organelles and interferes with cell fractionation. iii) Enrichment of
281 polyP-synthesizing activity by cell fractionation: the activities of PPX (3, 8), acid (8, 12)
282 and alkaline (1, 7, 11, 13) phosphatases have been detected in AM fungi, and these enzymes
283 are likely to be involved in polyP hydrolysis. In fact, polyP-hydrolyzing activity in the
284 hyphal homogenate was quite high in the present study. Therefore, cell fractionation that
285 could enrich polyP-synthesizing activity and dilute polyP-hydrolyzing activity was
286 necessary for the detection. It should be noted, in addition, that the polyP-synthesizing
287 activity estimated in our study might have been underestimated due to the concurrence of
288 polyP-hydrolyzing activity in the fraction.

289 ATP was essential for the polyP synthesis in the fungus, and the results strongly
290 suggest that ATP did not energize the reaction via P- or V-ATPase but acted as a direct
291 substrate in the reaction due to the following reasons: firstly, the V-ATPase inhibitor and
292 protonophore did not inhibit the activity. Secondly, P-ATPase activity was enriched in Layer
293 3 that showed no polyP-synthesizing activity. Although vanadate, the P-ATPase inhibitor,
294 inhibited the polyP-synthesizing activity, the involvement of P-ATPase, i.e. the proton
295 gradient across plasma membrane, could be validated only if the activity was inhibited by
296 both vanadate and the protonophoric reagent, because vanadate has been known to inhibit
297 many phosphoryl transfer enzymes as a structural and chemical mimic of phosphate (5).
298 The facts that all polyP-synthesizing enzymes found from prokaryotes (19) and eukaryotes

299 (14, 16, 22, 32) used ATP as a direct phosphoryl donor also support our observations.
300 Catalysis of the reverse ATP-regenerating reaction is a typical feature of PPK-type enzymes
301 in both prokaryotes (19) and eukaryotes (14, 32) but is unlikely in the VTC4 found in yeast
302 (16). In the present study, it was difficult to examine whether Layer 2 catalyzed the reverse
303 reaction due to the high-background of ATP that was essential for the
304 protection/stabilization of the polyP-synthesizing activity during fractionation. The
305 polyP-synthesizing activity was inhibited by the coexistence of ATP and Pi. It is postulated
306 that Pi interfered with the activity through binding to the catalytic center or that Pi might act
307 as an allosteric effector that regulates the balance between ATP consumption and polyP
308 synthesis in the cell. However, given the fact that cytoplasmic Pi homeostasis is strictly
309 maintained at millimolar levels, e.g. 5-10 mM in the case of plants (27), it is unlikely that
310 cytoplasmic Pi is directly involved in the regulation of polyP synthesis in AM fungi. The
311 underlying mechanism will be clarified if the enzyme is purified and localized at the
312 subcellular level.

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

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REFERENCES

329

- 330 1. **Aono, T., I. E. Maldonado-Mendoza, G. R. Dewbre, M. J. Harrison, and M.**
331 **Saito.** 2004. Expression of alkaline phosphatase genes in arbuscular mycorrhizas.
332 *New Phytol.* **162**:525-534.
- 333 2. **Ault-Riché, D., C. D. Fraley, C. M. Tzeng, and A. Kornberg.** 1998. Novel assay
334 reveals multiple pathways regulating stress-induced accumulations of inorganic
335 polyphosphate in *Escherichia coli*. *J. Bacteriol.* **180**:1841-1847.
- 336 3. **Capaccio, L. C. M., and J. A. Callow.** 1982. The enzymes of polyphosphate
337 metabolism in vesicular-arbuscular mycorrhizas. *New Phytol.* **91**:81--91.
- 338 4. **Cox, G., K. J. Moran, F. Sanders, C. Nockolds, and P. B. Tinker.** 1980.

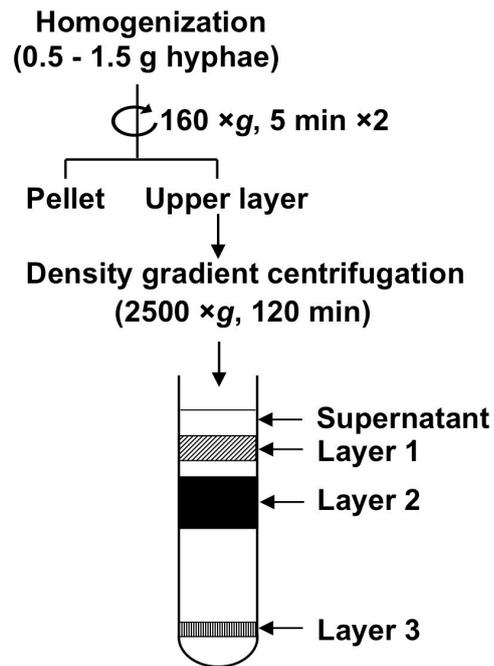
- 339 Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. III.
340 Polyphosphate granules and phosphorus translocation. *New Phytol.* **84**:649-659.
- 341 5. **Davies, D. R., and W. G. J. Hol.** 2004. The power of vanadate in crystallographic
342 investigations of phosphoryl transfer enzymes. *FEBS Lett.* **577**:315-321.
- 343 6. **Ezawa, T., T. R. Cavagnaro, S. E. Smith, F. A. Smith, and R. Ohtomo.** 2004.
344 Rapid accumulation of polyphosphate in extraradical hyphae of an arbuscular
345 mycorrhizal fungus as revealed by histochemistry and a polyphosphate
346 kinase/luciferase system. *New Phytol.* **161**:387-392.
- 347 7. **Ezawa, T., S. Kuwahara, K. Sakamoto, T. Yoshida, and M. Saito.** 1999. Specific
348 inhibitor and substrate specificity of alkaline phosphatase expressed in the symbiotic
349 phase of the arbuscular mycorrhizal fungus, *Glomus etunicatum*. *Mycologia*
350 **91**:636-641.
- 351 8. **Ezawa, T., S. E. Smith, and F. A. Smith.** 2001. Differentiation of polyphosphate
352 metabolism between the extra- and intraradical hyphae of arbuscular mycorrhizal
353 fungi. *New Phytol.* **149**:555-563.
- 354 9. **Ezawa, T., S. E. Smith, and F. A. Smith.** 2001. Enzyme activity involved in
355 glucose phosphorylation in two arbuscular mycorrhizal fungi: indication that polyP
356 is not the main phosphagen. *Soil Biol. Biochem.* **33**:1279-1281.
- 357 10. **Ezawa, T., S. E. Smith, and F. A. Smith.** 2002. P metabolism and transport in AM
358 fungi. *Plant Soil* **244**:221-230.

- 359 11. **Funamoto, R., K. Saito, H. Oyaizu, M. Saito, and T. Aono.** 2007. Simultaneous in
360 situ detection of alkaline phosphatase activity and polyphosphate in arbuscules
361 within arbuscular mycorrhizal roots. *Func. Plant Biol.* **34**:803-810.
- 362 12. **Gianinazzi, S., V. Gianinazzi-Pearson, and J. Dexheimer.** 1979. Enzymatic
363 studies on the metabolism of vesicular-arbuscular mycorrhiza. III. Ultrastructural
364 localization of acid and alkaline phosphatase in onion roots infected by *Glomus*
365 *mosseae* (Nicol. & Gerd.). *New Phytol.* **82**:127-132.
- 366 13. **Gianinazzi-Pearson, V., and S. Gianinazzi.** 1978. Enzymatic studies on the
367 metabolism of vesicular-arbuscular mycorrhiza. II. Soluble alkaline phosphatase
368 specific to mycorrhizal infection in onion roots. *Physiol. Plant Pathol.* **12**:45-53.
- 369 14. **Gomez-Garcia, M. R., and A. Kornberg.** 2004. Formation of an actin-like filament
370 concurrent with the enzymatic synthesis of inorganic polyphosphate. *Proc. Natl.*
371 *Acad. Sci. USA* **101**:15876-15880.
- 372 15. **Horie, S., M. Morrison, and B. With the technical assistance of John.** 1963.
373 Cytochrome c Oxidase Components. I. Purification and properties. *J. Biol. Chem.*
374 **238**:1855-1860.
- 375 16. **Hothorn, M., H. Neumann, E. D. Lenherr, M. Wehner, V. Rybin, P. O. Hassa, A.**
376 **Uttenweiler, M. Reinhardt, A. Schmidt, J. Seiler, A. G. Ladurner, C. Herrmann,**
377 **K. Scheffzek, and A. Mayer.** 2009. Catalytic core of a membrane-associated
378 eukaryotic polyphosphate polymerase. *Science* **324**:513-516.

- 379 17. **Kornberg, A.** 2008. Abundant microbial inorganic polyphosphate, poly P kinase are
380 underappreciated. *Microbe* **3**:119-123.
- 381 18. **Kornberg, A.** 1995. Inorganic polyphosphate: toward making a forgotten polymer
382 unforgettable. *J. Bacteriol.* **177**:491-496.
- 383 19. **Kornberg, A., N. N. Rao, and D. Ault-Riche.** 1999. Inorganic polyphosphate: A
384 Molecule of Many Functions. *Ann. Rev. Biochem.* **68**:89-125.
- 385 20. **Kuga, Y., K. Saito, K. Nayuki, R. L. Peterson, and M. Saito.** 2008. Ultrastructure
386 of rapidly frozen and freeze-substituted germ tubes of an arbuscular mycorrhizal
387 fungus and localization of polyphosphate. *New Phytol.* **178**:189-200.
- 388 21. **Martin, F., V. Gianinazzi-Pearson, M. Hijri, P. Lammers, N. Requena, I. R.**
389 **Sanders, Y. Shachar-Hill, H. Shapiro, G. A. Tuskan, and J. P. W. Young.** 2008.
390 The long hard road to a completed *Glomus intraradices* genome. *New Phytol.*
391 **180**:747-750.
- 392 22. **McGrath, J. W., A. N. Kulakova, L. A. Kulakov, and J. P. Quinn.** 2005. In vitro
393 detection and characterisation of a polyphosphate synthesising activity in the yeast
394 *Candida humicola* G-1. *Res. Microbiol.* **156**:485-491.
- 395 23. **Ogawa, N., J. DeRisi, and P. O. Brown.** 2000. New components of a system for
396 phosphate accumulation and polyphosphate metabolism in *Saccharomyces*
397 *cerevisiae* revealed by genomic expression analysis. *Mol. Biol. Cell* **11**:4309-21.
- 398 24. **Ohnishi, T., R. S. Gall, and M. L. Mayer.** 1975. An improved assay of inorganic

- 399 phosphate in the presence of extralabile phosphate compounds: Application to the
400 ATPase assay in the presence of phosphocreatine. *Anal. Biochem.* **69**:261-267.
- 401 25. **Ohtomo, R., and M. Saito.** 2005. Polyphosphate dynamics in mycorrhizal roots
402 during colonization of an arbuscular mycorrhizal fungus. *New Phytol.* **167**:571-578.
- 403 26. **Ohtomo, R., Y. Sekiguchi, T. Mimura, M. Saito, and T. Ezawa.** 2004.
404 Quantification of polyphosphate: different sensitivities to short-chain polyphosphate
405 using enzymatic and colorimetric methods as revealed by ion chromatography. *Anal.*
406 *Biochem.* **328**:139-146.
- 407 27. **Schachtman, D. P., R. J. Reid, and S. M. Ayling.** 1998. Phosphorus uptake by
408 plants: From soil to cell. *Plant Physiol.* **116**:447-453.
- 409 28. **Schwarzott, D., C. Walker, and A. Schüssler.** 2001. *Glomus*, the largest genus of
410 the arbuscular mycorrhizal fungi (Glomales), is nonmonophyletic. *Mol. Phylogenet.*
411 *Evol.* **21**:190-197.
- 412 29. **Smith, S. E., and D. J. Read.** 2008. *Mycorrhizal symbiosis*, 3rd ed. Academic
413 Press.
- 414 30. **Solaiman, M., T. Ezawa, T. Kojima, and M. Saito.** 1999. Polyphosphates in
415 intraradical and extraradical hyphae of an arbuscular mycorrhizal fungus, *Gigaspora*
416 *margarita*. *Appl. Environ. Microbiol.* **65**:5604-5606.
- 417 31. **Viereck, N., P. E. Hansen, and I. Jakobsen.** 2004. Phosphate pool dynamics in the
418 arbuscular mycorrhizal fungus *Glomus intraradices* studied by *in vivo* ³¹P NMR

- 419 spectroscopy. *New Phytol.* **162**:783-794.
- 420 32. **Zhang, H., M. R. Gomez-Garcia, X. Shi, N. N. Rao, and A. Kornberg.** 2007.
- 421 Polyphosphate kinase 1, a conserved bacterial enzyme, in a eukaryote, *Dictyostelium*
- 422 *discoideum*, with a role in cytokinesis. *Proc. Natl. Acad. Sci. USA*
- 423 **104**:16486-16491.
- 424
- 425



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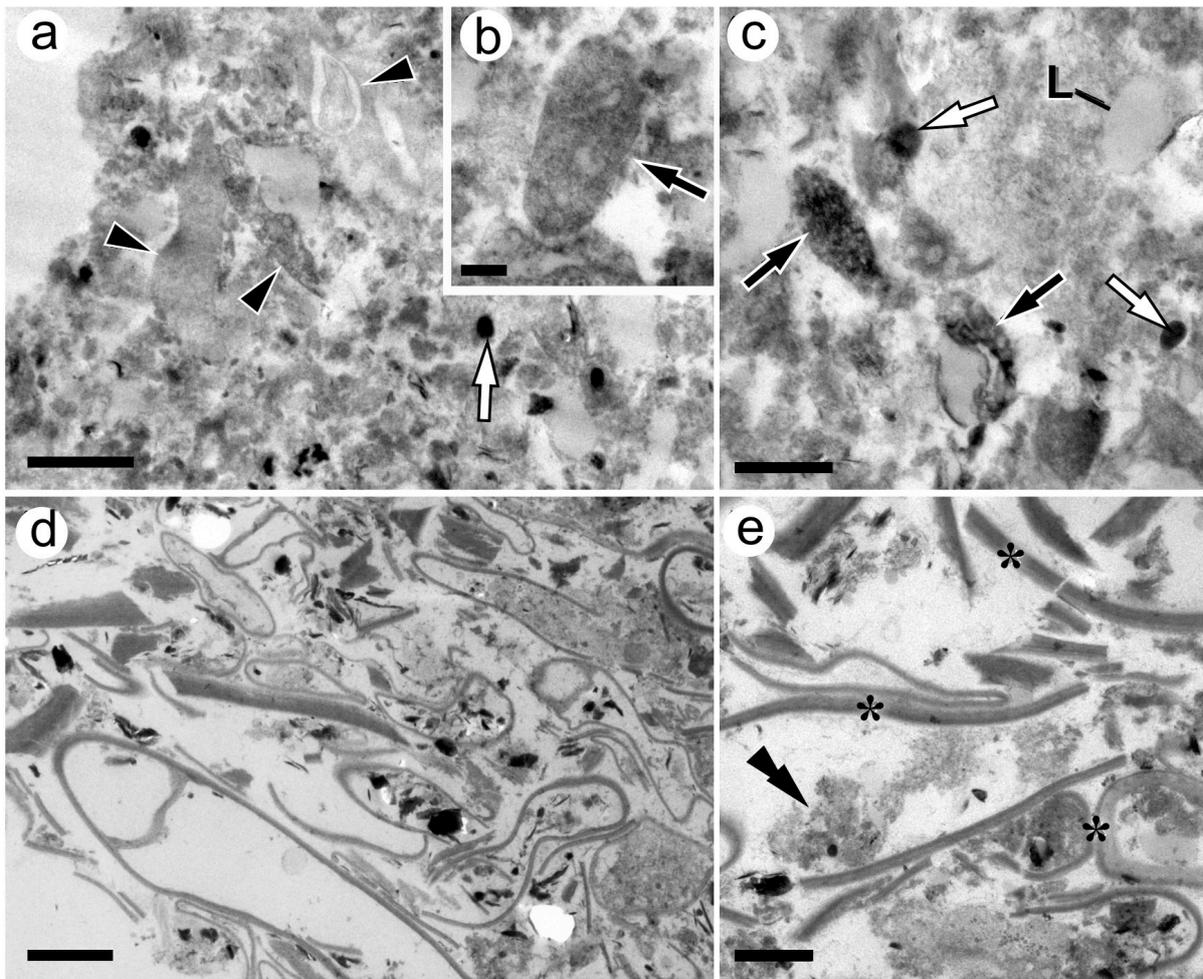
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427 FIG. 1. Schematic diagram for the fractionation of the extraradical hyphae of *Glomus* sp. HR1.

428 The densities of the fractions are as follows: Layer 1, 1.02-1.05 g ml⁻¹; Layer 2, 1.06-1.09 g

429 ml⁻¹; Layer 3, > 1.15 g ml⁻¹.

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432 FIG. 2a-e. Ultrastructure of Layers 2 and 3 fractions of *Glomus* sp. HR1 extraradical hyphae.

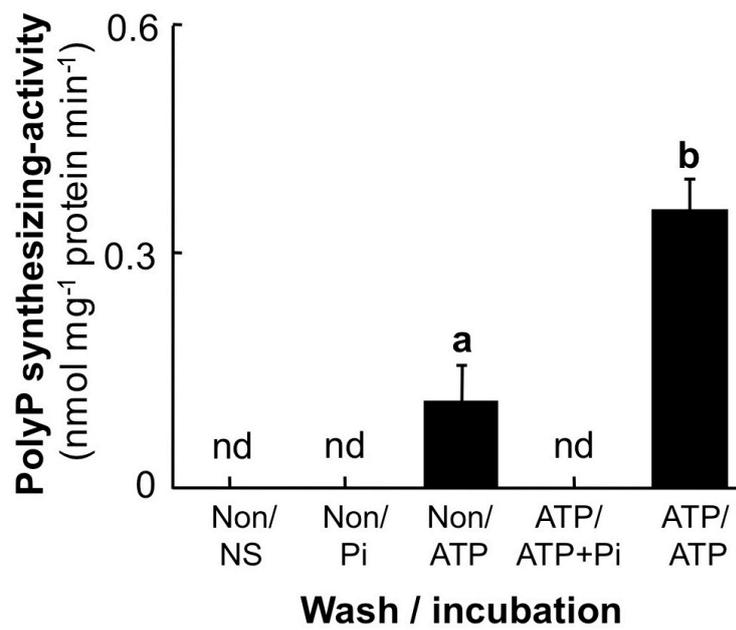
433 a-c. Layer 2 composed of amorphous and membranous (black arrowheads) materials with

434 mitochondria (black arrows), lipid body (L) and electron opaque body (white arrows). d and

435 e. Layer 3 composed of partially broken hyphae and fragmented hyphal cell wall (asterisks)

436 with which amorphous material was associated (double arrowhead). Bars: a, 1 μm ; b, 0.2437 μm ; c, 0.5 μm ; d, 2 μm ; e, 1 μm .

438

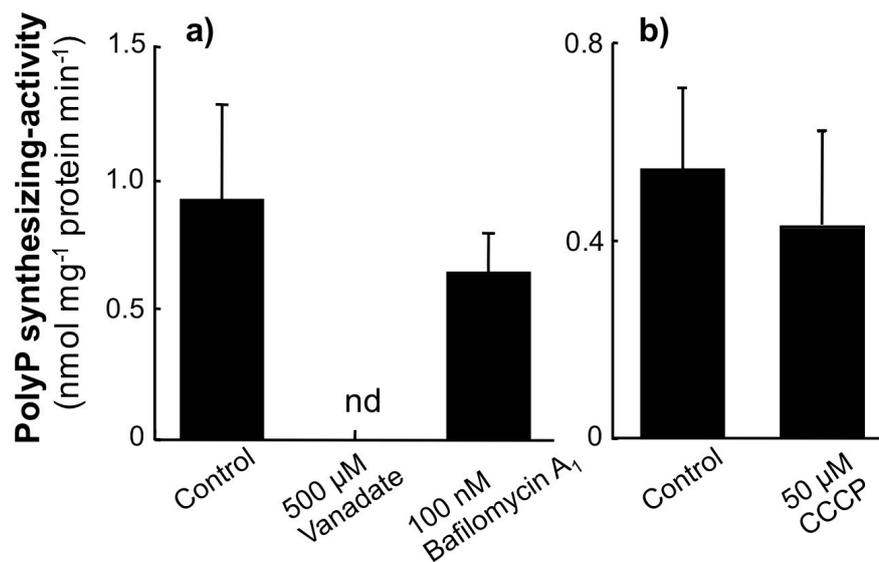


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439

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

446



446

447

448 FIG. 4. Effects of H⁺-ATPase inhibitors (a) and protonophore (b) on polyphosphate (polyP)

449 synthesizing-activity in Layer 2 prepared from extraradical hyphae of *Glomus* sp. HR1 in

450 symbiosis with *T. patula*. a, The layer 2 was incubated with 1 mM ATP in the presence or

451 absence of 500 μM vanadate (plasma membrane-type H⁺-ATPase inhibitor) or 100 nM

452 bafilomycin A₁ (vacuolar-type H⁺-ATPase inhibitor). nd, not detected. No significant

453 difference in the activity between the control and bafilomycin A₁ treatments was observed

454 ($P > 0.05$, Student's *t*-test). b, The layer 2 was incubated with 1 mM ATP in the presence or

455 absence of 50 μM carbonylcyanide-*m*-chlorophenylhydrazone (CCCP). No significant

456 difference in the activity between the control and CCCP treatments was observed ($P > 0.05$,

457 Student's *t*-test). Vertical bars indicate ± SE ($n = 3$).

458

458

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

Fraction ^a	Polyphosphate			Protein	
	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

459

460

^aDetailed procedure for the fractionation is described in Materials and

461

methods. The experiment was conducted several times using independent

462

batches of fungal material, and one set of the results is shown.

463

463

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

464

465 ^aThe detailed procedure for the fractionation is described in Materials and methods.

466 The experiment was conducted several times using independent batches of fungal material,

467 and one set of the results is shown.

468 ^bThe rates of increase in polyP during 30 min incubation at 30°C in the presence of

469 ATP. The amount of polyP is expressed as Pi-residues. The values are indicated as mean ± SE

470 ($n = 3$). nd, not detected.

471

471

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

472

473 ^aEach enzyme activity was measured using different batches of fungal material.

474 ^bDetailed procedure for the fractionation is described in Materials and methods. The
 475 fractions for the assessment of P- and V-ATPase activities were prepared from the hyphae to
 476 which 1 mM Pi solution was not applied prior to harvest.

477 ^cThe rates of specific inhibitor-sensitive and ATP-specific hydrolysis during 30 min
 478 incubation at 30°C. Vanadate and bafilomycin A₁ were used as specific inhibitors for P- and
 479 V-ATPases, respectively. Only the mean values (*n* = 3) are indicated for P- and V-ATPase
 480 activities, because these activities were determined by subtracting the inhibitor-sensitive
 481 activity from total ATP-specific activity. nd, not detected.

482 ^dThe rates of cytochrome *c* oxidation during 3 min incubation at room temperature.

483 The values are mean ± SE (*n* = 3).

484 °The rates of polyphosphate hydrolysis during 30 min incubation at 30°C. The
485 values are mean \pm SE ($n = 3$).
486

TABLE S1. Polyphosphate concentrations and recoveries during cell fractionation

Fractions	Batch numbers ^a			
	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)

487 ^aThe experiments were conducted several times by using different batches of fungal
488 material. One batch consisted of 60-70 pots from which 0.5-1.5 g (fw) of hyphal
489 material was collected.

490 ^bValues in parentheses are the percentages of recovered polyphosphate.

491

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

Fraction	Batch numbers ^a			
	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

492 ^aThe experiments were conducted several times by using different batches of fungal
 493 material. One batch consisted of 60-70 pots from which 0.5-1.5 g (fw) of hyphal
 494 material was collected.

495 ^bMean value ± SD ($n = 3$).