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A New Hypothesis on the Strategy for Acquisition of Phosphorus in Arbuscular Mycorrhiza: Up-Regulation of Secreted Acid Phosphatase Gene in the Host Plant

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The mycorrhiza-responsive phosphatase of *Tagetes patula* in symbiosis with *Glomus etunicatum* was detected by electrophoresis, was purified by column chromatography, and was characterized as acid phosphatase that was secreted into rhizosphere. The N-terminal amino acid sequence was determined by a gas-phase sequencer, and a cDNA fragment of the phosphatase gene (*TpPAP1*) was amplified by degenerate primers designed based on the N-terminal amino acid sequence. The full-length cDNA was obtained by the rapid amplification of cDNA ends technique. The *TpPAP1* was of host origin, and the cDNA was 1,843 bp long with a predicted open reading frame of polypeptide of 466 amino acids. Phylogenetic analysis revealed that the gene fell into the cluster of plant high-molecular-weight purple acid phosphatase. Expression analysis of the *TpPAP1* in *T. patula* in symbiosis with *Archaeospora leptoticha* showed that the levels of transcripts increased eightfold by mycorrhizal colonization. Western blot analysis revealed that the 57-kDa protein corresponding to the mycorrhiza-responsive phosphatase increased by mycorrhizal colonization. The present study proposes a new strategy for acquisition of P in arbuscular mycorrhizal associations in which the fungal partner activates a part of the low-P adaptation system of the plant partner, phosphatase secretion, and improves the overall efficiency of P uptake.

Availability of phosphorus (P) in soil is relatively low in nature, i.e., P concentration in soil rarely exceeds 10 μ M (Bielecki 1973); thus, plant growth is likely limited by P. Plants have two strategies to adapt to a low-P environment: i) the enhancement of P uptake capability and ii) mobilization of inaccessible P such as sparingly soluble inorganic P and organic P. The formation of symbiotic associations with arbuscular mycorrhizal (AM) fungi improves P uptake capability (Schachtman et al. 1998). In the association, the host plant supplies a carbon source to the fungal partner and the fungi construct an extraradical hyphal network that provides a huge

contact area between soil solution and host plant and enables the host plant to take up P far beyond the P-depletion zone around the roots. Expression of the high-affinity phosphate (Pi) transporter gene (Smith 2002) and a change in root architecture (Watt and Evans 1999; Williamson et al. 2001) are also typical responses of plants to enhance P uptake capability. It is known, however, that AM formation reduces expression levels of the high-affinity Pi-transporter gene of the host plant and the fungal Pi transporter on extraradical hyphae plays an alternative role in P uptake (Liu et al. 1998). To mobilize sparingly soluble Pi such as Al- and Fe-P, plants secrete low-molecular-weight organic acid (Ryan et al. 2001). Secretion of acid phosphatase into the rhizosphere has been observed in many plants grown under P-deficient conditions and is considered to be involved in mineralization of organic P (Duff et al. 1994). Secretion of acid phosphatase by AM fungi, on the other hand, is controversial. Tarafdar and Marschner (1994) observed an increase in phosphatase activity in the hyphosphere (hyphae-soil interface). Joner and Johansen (2000) also found extracellular phosphatase activity, but the enzyme was associated with the hyphal wall and was not secreted into the hyphosphere.

Phosphatase specific to mycorrhizal colonization was first found in the *Allium cepa*–*Glomus mosseae* association by an electrophoretic analysis and was characterized as alkaline phosphatase localized in the fungal vacuoles (Gianinazzi-Pearson and Gianinazzi 1976; Gianinazzi et al. 1979). The fungal alkaline phosphatase activity visualized by histochemical staining was employed as a biochemical index of active hyphae in terms of P nutrition (Boddington and Dodd 1999; Tisserant et al. 1993). However, subsequent detailed characterization of the enzyme suggested that the alkaline phosphatase was involved in fungal carbon metabolism (Ezawa et al. 1999). Furthermore, the gene expression analyses of *GmALP* and *GiALP*, alkaline phosphatase genes of *Gigaspora margarita* and *Glomus intraradices*, respectively, revealed that the expression levels of these genes were constitutive irrespective of the growth stages of both of the symbionts and external Pi concentration (Aono et al. 2004). Phosphatase specific to mycorrhizal colonization was also found in the *Tagetes patula*–AM fungal associations, and the activity correlated with the growth response of the plant partner to mycorrhizal formation under P-deficient conditions (Ezawa and Yoshida 1994a). This enzyme was purified and characterized as nonspecific acid phosphatase (EC 3.1.3.2) and was suggested to be of host origin (Ezawa and Yoshida 1994b). In this study, a gene encoding the mycorrhiza-responsive acid phosphatase was cloned, and the possible role of the enzyme in the P-acquisition strategy of the plant-fungal symbiotic system is discussed.

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The nucleotide sequence data for *TpPAP1* cDNA is available at the GenBank database under accession number AB029086.

RESULTS

Mycorrhiza-responsive acid phosphatase in *Tagetes patula* is secreted into rhizosphere.

Dwarf marigold (*Tagetes patula*) was inoculated with *Glomus etunicatum* and was grown for 6 weeks in a growth chamber. The percentage of fungal colonization reached 40 to 45% at the time of harvest, while the control plants were uncolonized. Soluble enzyme was extracted from mycorrhizal and nonmycorrhizal roots and was subjected to electrophoresis, and acid phosphatase activity (pH 5.0) was visualized on the gel. Activity of phosphatase corresponding to a relative mobility (R_m) of 0.12 increased in response to mycorrhizal colonization (Fig. 1A) that had been characterized as nonspecific acid phosphatase (E.C.3.1.3.2) (Ezawa and Yoshida 1994b). Low activity of phosphatase at the equivalent R_m was also observed in the nonmycorrhizal roots. The phosphatase showed a typical feature of secreted enzyme that could be extracted by 1 M NaCl without disruption of tissue (Fig. 1B). Secretion of the enzyme was inhibited by application of 1 mM

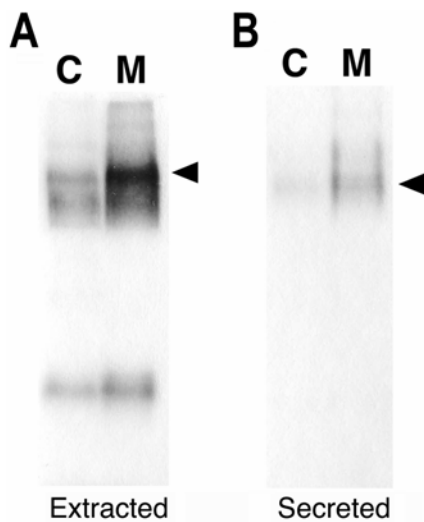


Fig. 1. Electrophoretic analysis of phosphatase in the roots of nonmycorrhizal *Tagetes patula* (C) and *Tagetes patula*-*Glomus etunicatum* association (M). Phosphatase activity was visualized at pH 5.0 by the azo-dye method. **A**, Crude extract was prepared from mycorrhizal and nonmycorrhizal roots, and 12.5 μ g protein was loaded on the gel. **B**, Intact mycorrhizal and nonmycorrhizal roots were immersed in a 1:50 volume (wt/vol) of 1 M NaCl-50 mM Tris/HCl, pH 7.5, at 5°C for 4 h, and 150 ng protein was loaded on the gel after concentration by an ODS cartridge (Tosoh Corp.). Arrowheads indicate the mycorrhiza-responsive phosphatase ($R_m = 0.12$).

phosphate 1 week before harvest (data not shown). Denatured molecular weight of the phosphatase was estimated as 57 kDa, whereas the native molecular weight had been estimated as 102 kDa (Ezawa and Yoshida 1994b).

Isolation of a gene encoding the mycorrhiza-responsive phosphatase.

The mycorrhiza-responsive phosphatase was purified as described at Ezawa and Yoshida (1994b), and the N-terminal amino acid sequence was determined by a gas-phase sequencer as D-I-S-A-D-M-P-L-N-S-D-V-F-A-L. Degenerate primers for reverse-transcription-polymerase chain reaction (RT-PCR) were designed based on the sequence. The nucleotide sequence and relative location of the PCR primers used in this study are shown in Table 1 and Figure 2, respectively. The first-strand cDNA was synthesized with the oligo (dT)-T3-anchor primer, using mRNA prepared from the mycorrhizal roots. First and second (nested) PCR were performed with the degenerate primers and T3-anchor primer, and a 1.5-kbp cDNA fragment (*TpPAP1*) was obtained, cloned, and sequenced. The 5' end of the *TpPAP1* was amplified by 5' RACE (rapid amplification of cDNA end) technique, and the RACE fragment was cloned and sequenced. Subsequently, a full-length coding region of the *TpPAP1* cDNA was amplified by nested PCR with primer sets designed to the 5' and 3' end untranslated regions to confirm that the RACE fragment was from *TpPAP1* mRNA. The full-length *TpPAP1* cDNA was 1,843 bp long with a predicted open reading frame of polypeptide of 466 amino acids (Fig. 3). The N-terminal polypeptide of 35 amino acids was rich in hydrophobic amino acids and a putative signal peptide. The sequence of 15 amino acids following the signal peptide was identical to

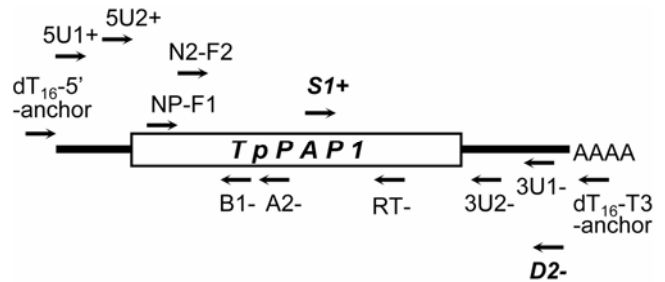


Fig. 2. Relative location of polymerase chain reaction primers used for amplification of *TpPAP1* cDNA. An open box indicates the open reading frame. The italicized primers (S1+ and D2-) were employed for the assessment of *TpPAP1* mRNA levels.

Table 1. Primers used for reverse transcription-polymerase chain reaction

Name	Sequence (5' to 3')	Position in <i>TpPAP1</i>	Notes
Oligo(dT)-T3-anchor	ATT AAC CCT CAC TAA AGG GA(T) ₁₆	–	First strand cDNA synthesis for cloning
T3-anchor	ATT AAC CCT CAC TAA AGG GA	–	First and 2nd primer for cloning
NP-F1	GAY ATH TCN GCN GAY ATG CC	287 to 306	First degenerate primer for cloning
N2-F2	CCN YTI AAY AGY GAY GTI TTY GC	305 to 327	Second degenerate primer for cloning
RT-	CAT AAG CCG AGT AG	1,027 to 1,040	First-strand cDNA synthesis for 5' RACE
Oligo(dT)-5'-anchor	GAC CAC GCG TAT CGA TGT CGA C(T) ₁₆ V	–	First sense primer for 5' RACE
A2-	CTC CTC TCC CTT CAT GAT CCC C	374 to 395	First antisense primer for 5' RACE
5'-Anchor	GAC CAC GCG TAT CGA TGT CGA C	–	Second sense primer for 5' RACE
B1-	GGA GCA TTA AAG CCA TGA GG	332 to 351	Second antisense primer for 5' RACE
5U1+	TCC AAA CAC GCA AAC CAA GG	100 to 119	Sense primer for full-length cDNA
5U2+	GAT CGC AAT AAC ATC TCC CC	125 to 144	Sense primer for full-length cDNA
3U1-	GCC CAT AGT TTA CAT CCA AC	1,689 to 1,708	Antisense primer for full-length cDNA
3U2-	CTA TTC GTC CGT GGT ATT CG	1,606 to 1,625	Antisense primer for full-length cDNA
S1+	GGT GGG ATT CAT GGG CAA GG	804 to 823	Sense primer for <i>TpPAP1</i> quantification
D2-	CTC ATG CCC ATA GTT TAC ATC C	1,692 to 1,713	Antisense primer for <i>TpPAP1</i> quantification
NS1	GTA GTC ATA TGC TTG TCT C	–	Sense primer for rDNA quantification
NS21	AAT ATA CGC TAT TGG AGC TGG	–	Antisense primer for rDNA quantification

that of N-terminal of the mature peptide determined by a gas-phase sequencer. The predicted molecular mass of the mature peptide was 49.9 kDa.

***TpPAP1* is classified in the super family of purple acid phosphatase.**

The deduced amino acid sequence of *TpPAP1* showed high similarity to plant purple acid phosphatases (PAP). The highest identity was observed with those of *Ipomea batatas* PAP2 (73%), *Glycine max* PAP (69%), and *Nicotiana tabacum* PAP19 (68%). The phylogenetic analysis of the amino acid sequences of *TpPAP1* with other PAP from plants, fungi, and animals was carried out (Fig. 4). The *TpPAP1* fell into the cluster of plant high-molecular-weight PAP (Schenk et al. 2000). Plant PAP are homodimeric Fe(III)-Zn(II) metalloenzymes (Durmus et al. 1999; Klabunde et al. 1996; Schenk et al. 2000; Sträter et al. 1995). Two motifs are involved in the Fe(III)-Zn(II) binuclear metal center: motif A, DX[G/H]-(X)₋₂₅-GDXX[Y/X]₋₂₅-GNH[E/D] and motif B, GH-(X)₋₅₀-GHXH. These motifs are conserved in *TpPAP1* (Fig. 5). The cystein residue that was reported to form disulfide bond between monomers in red kidney bean (*Phaseolus vulgaris*) PAP existed in the equivalent position of *TpPAP1*.

Expression of *TpPAP1* is up-regulated by mycorrhizal colonization and down-regulated by P fertilization.

The expression of *TpPAP1* in response to AM formation was investigated by relative quantitative RT-PCR (Burleigh 2001). Marigold was inoculated with *Archaeospora leptoticha* and was grown for five weeks. The percentage of fungal coloniza-

tion reached 35 to 40% at the time of harvest, while the control plants were uncolonized. Total RNA was extracted from the mycorrhizal and nonmycorrhizal roots, and first-strand cDNA was synthesized with both the oligo(dT) and NS21 primers, which targeted mRNA and 18S ribosomal RNA, respectively (Table 1). The cDNA of *TpPAP1* and 18S ribosomal RNA were amplified with the primer sets of S1+/D2- and NS1/NS21, respectively, were Southern blotted, and were detected by digoxigenin (DIG)-labeled probes. At this stage, the *TpPAP1* was confirmed to be of host origin, because the 0.9-kbp fragment of *TpPAP1* could be amplified not only from mRNA extracted from the mycorrhizal roots but also from those of the nonmycorrhizal roots. Levels of *TpPAP1* transcripts were estimated by PCR kinetics and were standardized based on 18S ribosomal RNA content, which was also determined by PCR kinetics (Fig. 6). The *TpPAP1* transcripts increased in the mycorrhizal roots at an average of 8.3 ± 3.7 fold, compared with those in the nonmycorrhizal controls (Table 2). The *TpPAP1* transcripts were not detectable in the mycorrhizal plants nor nonmycorrhizal plants fertilized with 1 mM phosphate 24 h prior to harvest (data not shown).

Western blot analysis of *TpPAP1* product.

To raise an antiserum against the *TpPAP1* product, synthetic peptide (YP-337) corresponding to the first 20 residues of the N terminal of the mature peptide was employed as an immunogen. Two rabbits were immunized with YP-337 and antiserum showing higher titer was used for the subsequent Western blotting analysis. *T. patula* was inoculated with *Archaeospora leptoticha* and was grown for six weeks, and crude extracts were prepared from mycorrhizal and nonmycorrhizal roots. Protein

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1 caacataaa ctatcttatt attattaaga gcaaatcacag gtctctcacc ccaatccacc taatctaadc cagccaataa tcacaatcat aacactttat
M A M G L G L
101 ccaaacacgc aaaccaaggt cagcgcagc aataaacatc ccctgccta ctgcaattac agttcagata aacatccatc tatggccatg ggattgggat
G S V Y L L L L C F V L T S C C N G G I S S S Y S R T N D I S A D
201 tgggatcagt ttatctgttg ttactgtgtt tcgttctcac atcctgctgc aatggagggga tcagtagcag ttatcdaa accaatgaca tcagtctgta
M P L N S D V F A L F N A P Q V H I T Q G D H E G R G V I
301 tatgccatta aacagcgatg tttttgcact tcctcatggc ttttaagtctc ctcaacagggt tcacataaca caaggggatc atgaagggag aggagtaatt
V S W V T P N E P G S S K V I Y W A E N S N V K Q H A V G S F V T Y
401 gtttcttggg tcacaccaaa tgaacctggt tcaagtaag taatttactg gcccgagaat agcaacgtca aacaacacgc agtgggatct tttgtcacct
K Y Y N Y S S P Y I H H C T I K N L E Y N T K Y F Y E L G T G N V
501 acaagtacta caactacagc tcaccgtaca tccatcattg caccatcaag aatcttgaat ataatacaca gtatttttac gagcttqga caggcaacgt
T R Q F W F T T P P E V G P D V P Y T F G L I G D L G Q T F D S N
601 cacacgacag ttttggttta ccacacctcc tgaagittggc cctgatgttc cttatacttt tggctctatc ggggatcttg gtcaaacatt tgactccaac
R T L T H Y E S N P A K G Q A V L F V G D L S Y A D A Y P L H D N N
701 cgaactttga ctcaatata gtcnaaccca gcaaaagggc aggcagtgct ttttggttgt gaccttctgt atgcagatgc gtacccttg catgataata
R W D S W A R F V E R S V A Y Q P W I W S A G N H E I D Y L P E Y
801 atagttggga ttcatgggca aggtttgtag aaagaagcgt ggcttaccaa ccatggattt ggagtgtctg gaatcatgaa attgattacc ttctgtaata
G E G E P F K P Y T H R Y Y V P Y E A P G V H L R F G Y S I K R A
901 tggtagaagt gaaccattta agccttatac acatcggtat tatgtgccat atgaagcacc gggagtacat ctccgcttgg ggtactcgat taaagagct
S A Y I I V M S S Y S A Y G M Y T P Q Y K W L M N E L P K V N R S E
1001 tctgcttaca taatcgtgat gtcacccac tcggcttatg gaatgtacac gcctcaatat aatggctca tgaacgagct accaaaggtt aaccgaagcg
T P W L I V V M H C P L Y S T Y L H H Y M E G E T M R V M Y E Q Y
1101 agacaccatg gtaattgtg gttatgcat gtccattata tagtacctat ttgatcatt atatggaagg tgaacaatg agagtgatgt atgaacaata
F V K Y K V D V V F S G H V H A Y E R T E R I S N V A Y N I E N G
1201 ttttgttaag tataaagttg atgtcgtgtt ttctggacat gttcatgcct atgagcgaac ggaaccgata tcaaatgittg cttacaatat cgaaaacgga
L C T P R N D E Y A P V Y I T I G D G G N Q E G L L Y E M V D P Q P
1301 ttgtgcaact ctagaatga cgaatatgcc ccgcttaca ttaccattgg tgatgggtga aaccaagaag gattgctata tgagatgta gatccacagc
K Y S A F R E P S Y G H A T F E I K N R T T A Y Y A W H R N Q D G
1401 ctaagtattc agcatttagg gaaccgagct acggcagtc tacatttagg atcaagaaca gaactactgc ttactatgct tggcatcgta atcaagatgg
Y S V E A D S V W F H N L Y W K S L S D S S A A S L *
1501 gtattctgtt gaagccgatt ccgtatggtt tcacaatttg tactggaat cattgtcaga ttcatctgct gcactcttgg gagtcgaat tcaagattat
1601 acgtccgaat accacggagc aataggttcc tcagctgcat cacaagattc atgatgtga cttgataac tttttattct tagatcatgt tggatgtaa
1701 ctatgggcat gagattgata tctacagatg ttttttcaa tcagtaaat gttgtagaat atactgtttg gatgtgttat ctgtatttga tcttaataat
1801 aataagatag ttaaatgittg aagtgtttaa aaaaaaaaaaaa aaa

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Fig. 3. Nucleotide and deduced amino acid sequences of *TpPAP1*. The first 35 amino acids (italicized) are a putative signal peptide, followed by an identical sequence of the mature peptide of mycorrhiza-responsive phosphatase determined by a gas-phase protein sequencer (underlined).

(30 µg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was electroblotted onto a nitrocellulose membrane. Immunodetection was carried out with the ECL Western blotting detection system according to the manufacturer's instructions. The antiserum reacted with the 57-kDa protein that corresponded to the mycorrhiza-responsive phosphatase (Fig. 7). More intensive signal was observed in the mycorrhizal roots than in the nonmycorrhizal roots.

DISCUSSION

The following evidence confirmed that the *TpPAP1* encodes the mycorrhiza-responsive acid phosphatase in *T. patula*: i) the N-terminal amino acid sequence following the putative signal peptide in the predicted open reading frame of *TpPAP1* was identical to that of the mycorrhiza-responsive phosphatase determined by a gas-phase sequencer, ii) deduced amino acid sequence of the *TpPAP1* showed highest similarity to those of

other plant acid phosphatases, iii) the transcript levels of *TpPAP1* increased in response to AM formation, and iv) the antiserum against the synthetic peptide, designed based on the N-terminal amino acid sequence of *TpPAP1* mature polypeptide, reacted with the 57-kDa protein that corresponded to the mycorrhiza-responsive acid phosphatase. The *TpPAP1* was further classified in the super family of PAP in which the metallo-phosphoesterase motifs were conserved. The native and denatured molecular mass of the mycorrhiza-responsive phosphatase suggests that the enzyme is functioning as a homodimer, which would be linked by a disulfide bond between the cystein residue conserved in the *TpPAP1*, as those found in other PAP (Schenk et al. 2000).

The gene expression of plant PAP is regulated by P nutrition. The transcription levels of PAP increased in response to P deficiency and decreased under P-sufficient conditions (Haran et al. 2000; Li et al. 2002; Miller et al. 2001; Nishikoori et al. 2001). Generally, AM formation improves P nutrition, and this may result in decrease in PAP gene expression in theory. In

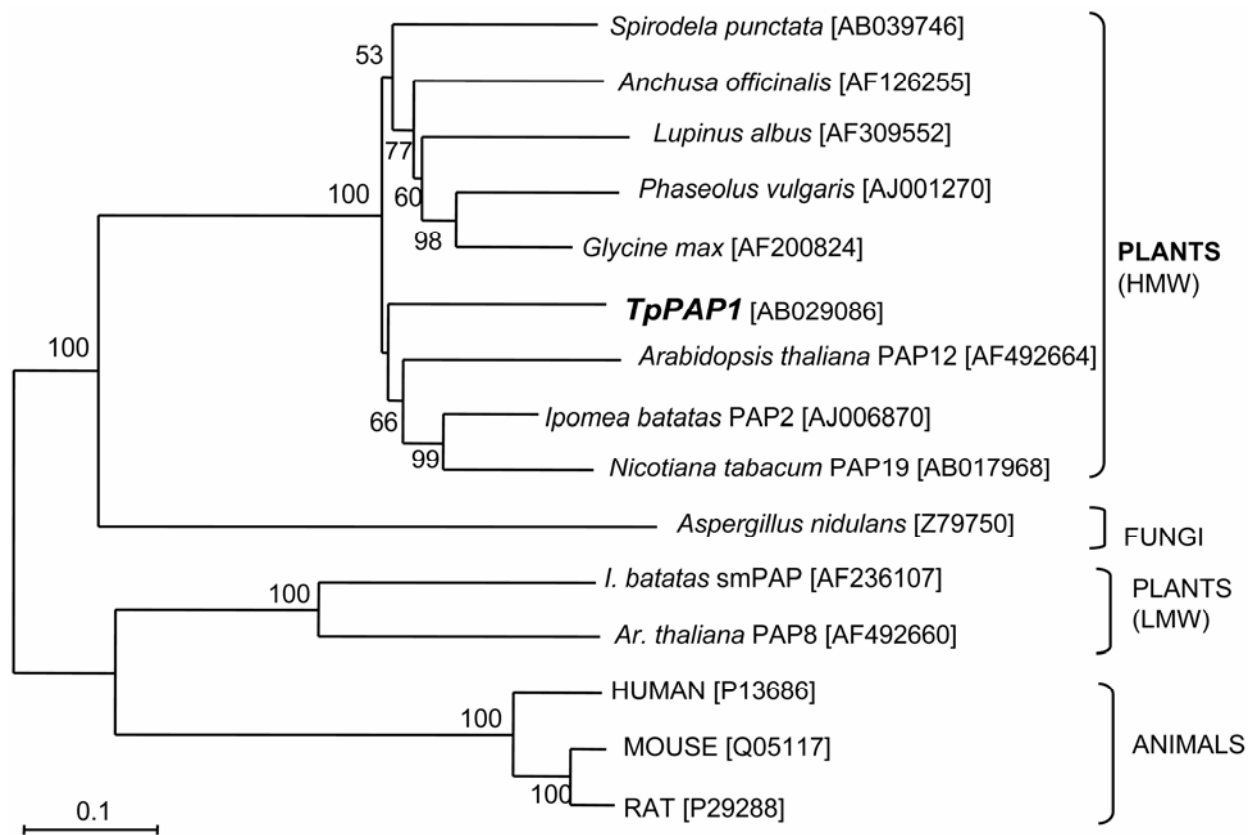


Fig. 4. Phylogenetic analysis of purple acid phosphatases (PAP) of plants, fungi, and animals. The deduced amino acid sequences were aligned using ClustalW, and the phylogenetic tree was drawn by the neighbor-joining method. Bootstrap values are shown at the nodes as a percentage of 1,000 replications. GenBank accession numbers are indicated. HMW = plant high molecular weight PAP, LMW = plant low molecular weight PAP.

		Motif A				Motif B			S-S bridge				
<i>P. vulgaris</i>	157	GLIGDLGQ	21	LFVGDLSYADR	26	WTAGNHEIEF	87	NHHFM	20	WFAGHVHAYE	14	GLCTP	85
<i>N. tabacum</i> (NtPAP19)	163	GLIGDLGQ	21	LFVGDLSYADN	26	WTAGNHELDF	87	VHHYM	20	VFAGHVHAYE	14	RKCSP	88
<i>I. batatas</i> (IbPAP2)	121	GLIGDLGQ	21	LFVGDLSYADR	26	WTAGNHEIDF	87	VHHYM	20	VFAGHVHASYE	14	GLCSP	89
<i>G. max</i>	157	GLIGDLGQ	21	LFVGDLSYADN	26	WTAGNHNHF	87	NYHYM	20	VFAGHVHAYE	14	GLCAP	90
<i>T. patula</i> (TpPAP1)	160	GLIGDLGQ	21	LFVGDLSYADA	26	WSAGNHEIDY	87	LHHYM	20	VFSGHVHAYE	14	GLCTP	89

Fig. 5. Partial alignment of the deduced amino acid sequences of plant purple acid phosphatases (PAP). The GenBank accession numbers are: *Nicotiana tabacum* PAP19, AB017968; *Ipomea batatas* PAP2, AJ006870; *Glycine max* PAP, AF200824; *Tagetes patula* TpPAP1 (this study), AB029086. Motifs A and B are the metallo-phosphoesterase domains, and the S-S bridge is the cystein residue forming a disulfide bond between the monomers. The numbers indicate the number of amino acid residues before, between, and following the regions. The conserved residues are indicated with shading.

fact, the expression of the *Medicago truncatula* PAP gene probed by one of the *Arabidopsis* PAP cDNAs decreased by AM formation (Liu et al. 1998). However, it has been known that several plants, *Triticum aestivum*, *Allium cepa* (Dodd et al. 1987), *Zea mays* (Fries et al. 1998), and *Tagetes patula* (Ezawa and Yoshida 1994a), increased acid phosphatase activity in roots in response to AM formation. The present study revealed that the increase in acid phosphatase activity in the *T. patula*-AM association was regulated at the gene expression levels. Plants possess several PAP genes in genome (Durmus et al. 1999; Kaida et al. 2003; Li et al. 2002), and the expressions of these genes are differently regulated (Kaida et al. 2003; Li et al. 2002). It is postulated that plants, including *M. truncatula*, have an isoform of PAP that would respond to AM formation. Further investigation is required to confirm this hypothesis.

The role of secretory acid phosphatase has not yet been well defined. It is considered, however, that the most probable function is hydrolyzation of organic P present in rhizosphere to release Pi, which could be taken up by the roots (Duff et al. 1994). A wide range of plant species secretes acid phosphatase into rhizosphere under P-deficient conditions irrespective of mycotrophic status. *Lupinus albus* secretes substantial amounts of acid phosphatase from proteoid (cluster) roots formed under P stress (Miller et al. 2001; Wasaki et al. 1999). Since *L. albus* does not form mycorrhiza, the secretion of acid phosphatase is regarded as an important part of P acquisition strategies of nonmycorrhizal plants. Secretion of acid phosphatase could be beneficial also for mycotrophic plants, especially for those associated with the AM fungi developing a hyphal network relatively close to the roots, such as *Scutellospora calospora* (Smith et al. 2000). The promotion of PAP gene expression

and the subsequent secretion of PAP by AM formation would accelerate mineralization of organic P in the rhizosphere, resulting in the enhancement of P uptake through the extraradical hyphae as well as through the roots.

Another function of extracellular phosphatase, proposed by Barrett-Lennard and associates (1993), is salvaging P esters leaked from the root cell. Plant roots exude various organic compounds such as sugar and organic acid, especially under nutrient-deficient conditions (Dakora and Phillips 2002). In addition, AM formation altered membrane permeability of the root cell to provide a carbon source for the fungal partner, e.g., the up-regulation of sugar transporter gene (Harrison 1996). It is likely that P esters also leak from the root cell and that the PAP hydrolyzes those compounds for P recycling.

The P acquisition mechanism of mycorrhizal plants has been well documented. The establishment of mycorrhizal association enables P uptake through the hyphal network extended beyond the P-depletion zone around the roots (Schachtman et al. 1998). The present study, however, proposes a new mechanism that the fungal partner activates a part of the low-P adaptation system of the plant partner, PAP secretion, and improves the overall efficiency of P uptake. The molecular mechanism involved in this interaction is of interest and remains to be clarified.

Table 2. Influence of mycorrhizal formation (*Archaeospora leptoticha*) on *Tagetes patula* on *TpPAP1* transcription levels^a

Tissue	Levels of <i>TpPAP1</i> transcripts ^b		
	Replication 1	Replication 2	Replication 3
Mycorrhizal (M)	0.0294	0.0541	0.1450
Control (C)	0.0049	0.0158	0.0094
M/C (fold)	6.0	3.4	15.5
Average (fold ± SE)	8.3 ± 3.7		

^a As revealed by quantitative reverse transcription-polymerase chain reaction. Measurement in arbitrary units.

^b The transcription levels of *TpPAP1* were standardized by the amount of 18S rRNA.

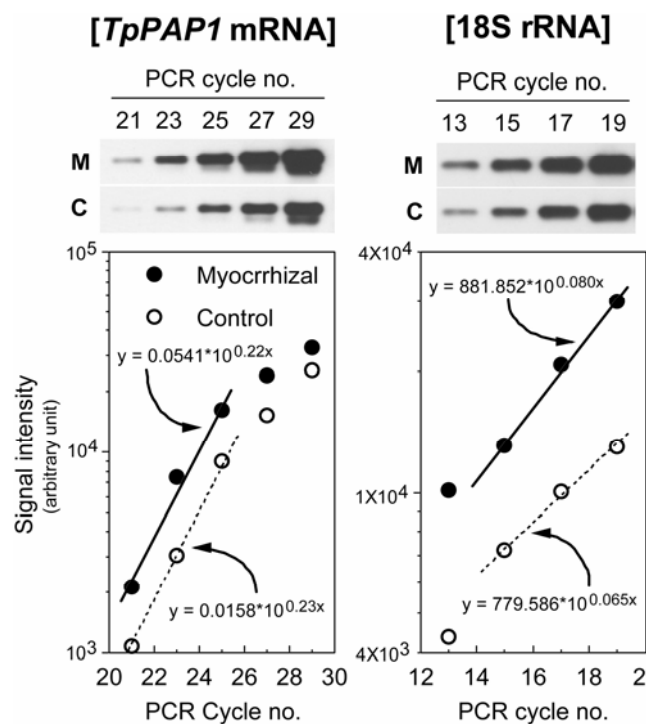


Fig. 6. Quantification of *TpPAP1* mRNA in the roots of nonmycorrhizal *Tagetes patula* (Control) and *T. patula*-*Archaeospora leptoticha* association (Mycorrhizal) by reverse transcription-polymerase chain reaction (PCR). The *TpPAP1* mRNA and 18S rRNA (constitutive control) were reverse-transcribed, amplified, electrophoresed, transferred onto a nylon membrane, and detected by the digoxigenin-labeled probes. Signal intensity of the Southern hybridization was quantified by image analysis, and the intensity versus cycle number of the PCR was plotted. The levels of the cDNA template in the PCR were estimated by the regression equations determined by the linear portions of the PCR kinetics.

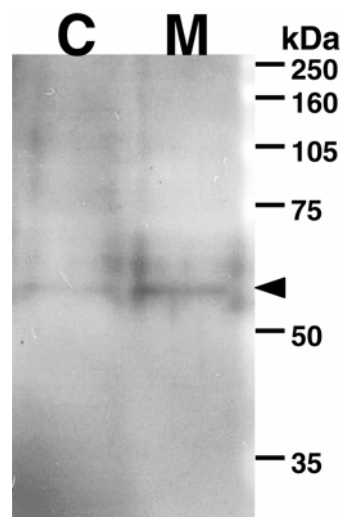


Fig. 7. Western blot analysis of *TpPAP1* product in the roots of nonmycorrhizal *Tagetes patula* (C) and *Tagetes patula*-*Archaeospora leptoticha* association (M). Crude extract was prepared from the mycorrhizal and nonmycorrhizal roots, and 30 µg protein was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and was electroblotted onto a nitrocellulose membrane. The membrane was reacted with a rabbit antiserum against the synthetic peptide corresponding to the first 20 residues of the N-terminal of mature *TpPAP1* protein and was detected by secondary antibody with the chemiluminescent detection system.

MATERIALS AND METHODS

Plant and fungal materials.

Dwarf marigold (*T. patula* cv. Bonanza Orange) was inoculated with 1 ml of a spore/water suspension (200 spores ml⁻¹) of *Glomus etunicatum* (isolated by T. Wood, NPI, Salt Lake City, UT, U.S.A., for protein purification and gene isolation) or with *Archaeospora leptoticha* (strain OK-15, MAFF 520072, for gene expression analysis and Western blot) and was grown on river sand (for *Glomus etunicatum*) or a 9:1 sand/soil (pH 4.9, available phosphate was less than 1.0 mg kg⁻¹) mixture (for *Archaeospora leptoticha*) in a growth chamber (16-h photoperiod, 25°C). The spore/water suspension was passed through a 53- μ m sieve and was used as the mock inoculum for the nonmycorrhizal controls. The plants received liquid fertilizer (4 mM NH₄NO₃, 1 mM K₂SO₄, 0.75 mM MgSO₄, 2 mM CaCl₂, 0.5 mM Fe-EDTA, 50 μ M KH₂PO₄) two to three times per week in sufficient amounts, until the solution flowed out from the drain holes. After five weeks, the plants were harvested and the roots were washed on a 500- μ m mesh, were wiped with a paper towel, were weighed and frozen in liquid nitrogen, and were stored at -80°C.

Electrophoretic analysis of phosphatase.

Roots (500 to 700 mg fresh weight) were ground on a mortar at 0°C with an equal volume (wt/vol) of 5 mM ascorbate, 2% (wt/vol) polyvinylpyrrolidone in 100 mM borate buffer (pH 8.8) and an equal weight of sea sand. The slurry was transferred to a 1.5-ml tube and was centrifuged at 15,000 \times g for 5 min at 4°C, and the supernatant was transferred to a new tube and was subjected to electrophoresis.

Secreted enzyme was prepared from intact roots. The mycorrhizal and nonmycorrhizal roots were harvested and gently washed in water, were blotted on a paper towel, were weighed, and were immersed in a 1:50 volume (wt/vol) of 1 M NaCl in 50 mM Tris/HCl buffer (pH 7.5) at 5°C with slow stirring. After 4 h, the roots were removed, and the solution was centrifuged at 1,500 \times g for 5 min. Solid ammonium sulfate was added to the supernatant to 80% saturation, and then, the solution was introduced onto the Toyopak ODS-S cartridge (Tosoh Corp., Tokyo). Adsorbed protein was eluted with 1.5 ml of 100 mM Tris/citrate buffer (pH 7.5) and was subjected to electrophoresis.

Protein concentration in the enzyme solutions was determined by the DC protein assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.), using bovine serum albumin as a standard. The enzyme solutions were loaded on a 10% SDS-polyacrylamide gel without denaturing (Ezawa and Yoshida 1994a). Phosphatase activity on the gel was visualized by the azo dye method (Scandalios 1969). Molecular weight of the denatured protein was estimated by relative mobility to the protein standards on a 10% SDS-polyacrylamide gel.

Cloning of the *TpPAP1*.

Acid phosphatase specific to arbuscular mycorrhizal colonization in marigold was purified as described by Ezawa and Yoshida (1994b), was electrophoresed on a 10% SDS-polyacrylamide gel, and was blotted on a polyvinylidene difluoride membrane. The N-terminal amino acid sequence of the protein was determined by a gas-phase protein sequencer (ABI 476A, Applied Biosystems, Foster City, CA, U.S.A.). The degenerate primers for RT-PCR were designed based on the sequence.

Roots (500 to 700 mg) were ground in a mortar with liquid nitrogen, and total RNA was extracted and purified with the RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.). Poly(A)⁺ RNA was prepared with the mRNA isolation kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. First-strand cDNA was synthe-

sized with the First-strand cDNA synthesis kit for RT-PCR (Roche Diagnostics), using the oligo (dT)-T3-anchor primer (Table 1). The High-fidelity expand PCR system (Roche Diagnostics) was employed for PCR amplification. The first PCR was performed with the sense-degenerate primer NP-F1 (5 μ M) and the T3-anchor primer (0.3 μ M), using the 1- μ l cDNA template in a total volume of 50 μ l. The amplification cycles were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 52°C for 1 min, 72°C for 1 min, plus cycle elongation of 15 s for each cycle, with a final extension at 72°C for 10 min. The second (nested) PCR was done with the sense-degenerate primer N2-F2 (5 μ M) and the 0.3- μ M T3-anchor primer, using the 0.5- μ l PCR product of the first PCR in a total volume of 50 μ l. The amplification cycles of the second PCR were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 49°C for 1 min, 72°C for 1 min, plus cycle elongation of 15 s for each cycle, with a final extension at 72°C for 10 min. The 1.5-kbp cDNA fragment of the second PCR was cloned into the pUC119 plasmid vector (Takara Bio Inc., Shiga, Japan) and was sequenced with the ABI Prism 373A sequencer (Applied Biosystems).

The 5' end of the cDNA was amplified with the 5'/3' RACE kit (Roche Diagnostics). Poly(A)⁺ RNA was prepared from total RNA as described above, and first-strand cDNA was synthesized using the antisense primer RT-. After homopolymeric dA-tailing to the 3' end of the cDNA, PCR amplification was performed with the oligo(dT)-5'-anchor primer (0.75 μ M) and the antisense primer A2- (0.25 μ M), using 2.5 μ l of cDNA template in a total volume of 25 μ l. The amplification cycles were as follows: initial denaturation at 94°C for 2 min, followed by 10 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 40 s, 25 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 40 s, plus cycle elongation of 20 s for each cycle, with a final extension at 72°C for 7 min. The second (nested) PCR was done with the 5'-anchor primer (0.75 μ M) and the antisense primer B1- (0.25 μ M), using 0.05 μ l of the first PCR product in a total volume of 25 μ l. The amplification cycles of the second PCR were as follows: initial denaturation at 94°C for 2 min, followed by 10 cycles of 94°C for 15 s, 57°C for 30 s, 72°C for 40 s, 25 cycles of 94°C for 15 s, 57°C for 30 s, 72°C for 40 s, plus cycle elongation of 20 s for each cycle, with a final extension at 72°C for 7 min. The product of the second PCR was cloned into the pT7Blue-2 plasmid vector (Novagen, Madison, WI, U.S.A.) and was sequenced with the ABI Prism 3100 sequencer (Applied Biosystems).

To assure that the RACE fragment was originated from the *TpPAP1* cDNA, full-length of the coding region of *TpPAP1* cDNA was amplified by using the 5U1+, 5U2+, 3U1-, and 3U2- primers, which were located in the 5' and 3' untranslated regions of the *TpPAP1* cDNA. Poly(A)⁺ RNA was prepared as described above, and the first-strand cDNA was synthesized using the oligo(dT)₁₅ primer. The first PCR amplification was performed with the sense primer 5U1+ (0.2 μ M) and the antisense primer 3U1- (0.2 μ M), using 2.5 μ l of cDNA template in a total volume of 25 μ l. The amplification cycles were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 53°C for 50 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. The second (nested) PCR was done with either the primer set of 5U1+ and 3U2- (0.2 μ M, respectively) or that of 5U2+ and 3U1- (0.2 μ M, respectively), using the 0.025 μ l of the first PCR product in a total volume of 25 μ l. The amplification cycles of the second PCR were the same as those of first PCR. The products of the second PCR were cloned into the pT7Blue-2 plasmid vector (Novagen) and were sequenced.

Quantitative RT-PCR for expression analysis.

The quantitative RT-PCR technique was employed to compare the amount of *TpPAP1* mRNA between mycorrhizal and nonmycorrhizal roots, based on the amount of 18S rRNA as an internal standard (Burleigh 2001). Total RNA was extracted and purified as described above, and 1 µg of RNA was reverse-transcribed with both the oligo(dT)₁₅ primer (3.75 µM) for mRNA and the NS21 primer (White et al. 1990) (1 µM) for the plant (and fungal) 18S rRNA. The cDNA of 18S rRNA was amplified with the sense primer NS1 and antisense primer NS21 (0.2 µM, respectively), using the 1:20 diluted RT product as a template. The amplification conditions were as follows: initial denaturation at 94°C for 2 min, followed by 9 to 21 cycles of 9°C for 30 s, 50°C for 50 s, and 72°C for 1 min. The PCR product (10 µl) from each cycle were electrophoresed on an agarose gel and were blotted on a nylon membrane with a vacuum blotter (Bio-Rad model 785). The *TpPAP1* cDNA was amplified with the sense primer S1+ and antisense primer D2- (0.2 µM, respectively), using the 1:20 diluted RT product as a template. The amplification conditions were as follows: initial denaturation at 94°C for 2 min, followed by 19 to 29 cycles of 94°C for 30 s, 45°C for 50 s, and 72°C for 1 min. The PCR products (10 µl) from each cycle were electrophoresed on an agarose gel, were transferred onto a nylon membrane by a vacuum blotter (Bio-Rad Laboratories). The cDNA fragments of *TpPAP1* transcripts and 18S rRNA were detected by DIG-labeled probes that were amplified with the primer sets of S1+ and D2- (for *TpPAP1*) and the NS1 and NS21 (for 18S rRNA) and were labeled using the DIG high prime DNA labeling and detection kit (Roche Diagnostics) according to the manufacturer's instructions.

Signal intensity of the Southern hybridization was quantified by the NIH Image (National Institutes of Health, Bethesda, MD, U.S.A.). Cycle numbers that corresponded to the linear phase of the PCR reaction were identified on a semilogarithmic plot of signal intensity of the Southern blot versus cycle number of the PCR. The initial amount of the *TpPAP1* cDNA and rDNA in each RT product was estimated according to Iwai and associates (1995), and the amount of *TpPAP1* cDNA was standardized by that of 18S rRNA.

Preparation of antiserum and Western blot analysis.

To raise an antiserum against the *TpPAP1* product, the first 20 residues of the N-terminus of the mature protein, D-I-S-A-D-M-P-L-N-S-D-V-F-A-L-P-H-G-F-N, were chosen as an immunogen. Bio-Research, Co., Ltd. (Tokyo) synthesized the peptide (YP-337), immunized two rabbits by injecting the immunogen every two weeks over three months, and prepared antisera by standard protocols. Antibody titer of the sera against YP-337 was evaluated by enzyme-linked immunosorbent assay, and a serum showing higher titer was used thereafter.

Crude enzyme solution (soluble protein) was extracted as described at "Electrophoretic analysis of phosphatase." The samples were mixed with the SDS-PAGE sample buffer and were denatured at 95°C for 5 min. Protein (30 µg) was loaded on a 10% SDS-polyacrylamide gel and was run at 10 to 20 mV constant current. The proteins were electroblotted onto a nitrocellulose membrane in the transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) at 80 V and 800 to 1,000 mA for 4 h under ice-cooled conditions. The blot was stored in the transfer buffer at -20°C until use. After transfer, the gel was stained with Coomassie brilliant blue R-250 to evaluate the efficiency of the transfer. Immunodetection was carried out with the ECL Western blotting detection system (Amersham Bioscience, Buckinghamshire, U.K.), according to the manufacturer's instructions.

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