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Phytase activity in tobacco (*Nicotiana tabacum*) root exudates is exhibited by a purple acid phosphatase

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Abstract

Phytases are enzymes that catalyze liberation of inorganic phosphates from phytate, the major organic phosphorus in soil. Tobacco (*Nicotiana tabacum*) responds to phosphorus starvation with an increase in extracellular phytase activity. By a three-step purification scheme, a phosphatase with phytase activity was purified 486-fold from tobacco root exudates to a specific activity of 6,028 nkat mg^{-1} and an overall yield of 3%. SDS–PAGE revealed a single polypeptide of 64 kDa, thus indicating apparent homogeneity of the final enzyme preparation. Gel filtration chromatography suggested that the enzyme was a ca. 56 kDa monomeric protein. *De novo* sequencing by tandem mass spectrometry resulted in a tryptic peptide sequence that shares high homology with several plant purple acid phosphatases. The identity of the enzyme was further confirmed by molybdate-inhibition assay and cDNA cloning. The purified enzyme exhibited pH and temperature optima at 5.0–5.5 and 45 °C, respectively, and were found to have high affinities for both *p*-nitrophenyl phosphate (*p*NPP; $K_m = 13.9$ μM) and phytate ($K_m = 14.7$ μM), but a higher kcat for *p*NPP (2,056 s^{-1}) than phytate (908 s^{-1}). Although a broad specificity of the enzyme was observed for a range of physiological substrates in soil, maximum activity was achieved using mononucleotides as substrates. We conclude that the phytase activity in tobacco root exudates is exhibited by a purple acid phosphatase and its catalytic properties are pertinent to its role in mobilizing organic P in soil.

Keywords: *Nicotiana tabacum*; *Solanaceae*; Phytase; Purple acid phosphatase; Phytate; Phosphorus; Root exudates; Tobacco

1. Introduction

Phytate (*myo*-inositolhexakisphosphate; $InsP_6$ Fig. 1) (**1**) is a major storage form of phosphorus (P) in seeds and pollens. A considerable amount of $InsP_6$ (**1**) is introduced into soil by natural decomposition of terrestrial plant materials and animal manure, and it was frequently detected as the most abundant organic phosphorus (P_o) compound in soil in a number of studies (Cade-Menun, 2005; Nziguheba and Bunemann, 2005; Turner et al., 2002). P_o can constitute up to 90% of total P in soil (Harrison, 1987). The assimilation of these organic compounds in soil is crucial to the growth of terrestrial plants because P is a limiting but essential mineral for the synthesis of vital biomolecules such as nucleic acids, phospholipids, and sugar phosphates. Although the majority of soil P (50–80% of the total) exists as organic compounds (Turner et al., 2002), it cannot be assimilated by plants unless it is hydrolyzed into inorganic phosphate (P_i; Raghothama, 1999). In this context, higher plants possess the innate ability to mineralize organic P compounds by the exudation of acid phosphatases, which is a broad classification of hydrolytic enzymes that catalyze the breakdown of P-monoesters with acid pH optima (Vincent et al., 1992).

Both intracellular and secreted acid phosphatases are induced in higher plants in response to P starvation (Duff et al., 1994). Intracellular acid phosphatases are likely to

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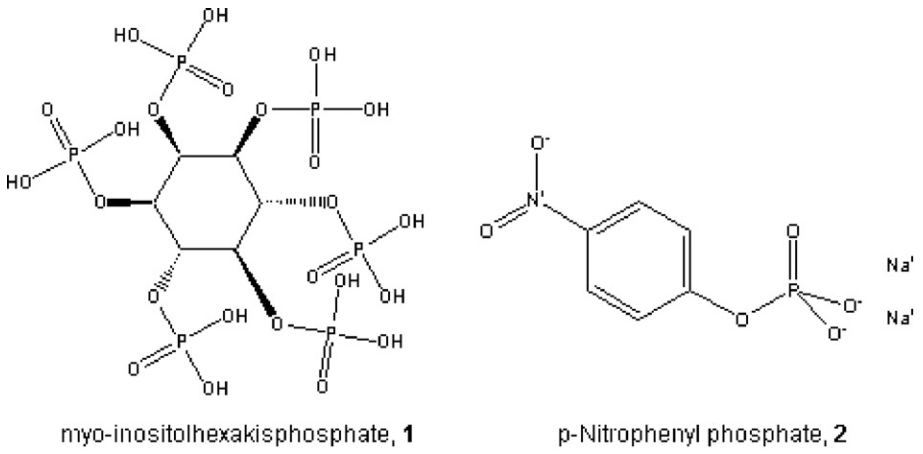

Fig. 1. Chemical structure of substrates.

play a role in internal P homeostasis, whereas secreted acid phosphatases may be pertinent to external P acquisition (Duff et al., 1994). Over the past decade, secreted acid phosphatases have been purified and characterized from a number of P-starved plants, including lupins (Li et al., 2002; Ozawa et al., 1995), tomato (Bozzo et al., 2002; Li and Tadano, 1996) and Arabidopsis (Coello, 2002). These enzymes generally display a broad substrate specificity for various phosphorylated compounds, but their ability to hydrolyze $InsP_6$ (**1**) has either not been reported (Li and Tadano, 1996; Ozawa et al., 1995) or found to be negligible (Bozzo et al., 2002; Coello, 2002). Li et al. (1997a) reported that a purified acid phosphatase from root extracts of P-starved tomato displayed relatively high substrate specificity for $InsP_6$ (**1**). However, its identity and whether it is exuded into the extracellular medium are yet to be investigated.

Hydrolytic enzymes that catalyze the breakdown of phosphomonoester bonds in $InsP_6$ (**1**) are collectively known as phytases. A number of phytase-encoding genes have been identified in the plant kingdom (Hegeman and Grabau, 2001; Maugenest et al., 1997; Rasmussen et al., 2003; Xiao et al., 2005). By sequence homology, currently known plant phytases are classified into two families, histidine acid phosphatases (HAPs) and purple acid phosphatases (PAPs), which were first discovered in maize (Maugenest et al., 1997) and soybean (Hegeman and Grabau, 2001), respectively. Both maize and soybean phytases were temporally expressed at the early stage of germination, which suggests their role in the mobilization of stored $InsP_6$-P to nourish seedling growth. However, the physiological role of plant phytases in external P acquisition is still poorly understood. Although P_i deficiency elicits phytase activity in plant roots (Hayes et al., 1999; Li et al., 1997a,b), and extracellular phytase activities have been detected in several plant species (Asmar, 1997; Richardson et al., 2000), little is known about the identity of these phytases.

Our previous study revealed that tobacco (*Nicotiana tabacum*) has low-P-inducible extracellular phytase activities, which enabled the plants to assimilate soluble $InsP_6$ (**1**) acid but not insoluble $InsP_6$ salts in sand culture (Lung and Lim, 2006). The study reported herein aimed to purify and characterize the phytase activity in tobacco root exudates, and to verify its identity by *de novo* peptide sequencing and cDNA cloning.

2. Results and discussion

2.1. Purification of the root-secreted phytase

Our previous study indicated that P starvation of tobacco seedlings triggered a substantial increase in the specific phytase activity in root exudates (Lung and Lim, 2006). Accordingly, the seedlings were cultivated in liquid MS medium with a 10-fold reduction in P_i concentration. Growth media that were collected on day 28 were pooled and concentrated by lyophilization, which did not significantly change the total and specific activities of the enzyme (data not shown). Phytase activity was detected at acidic pH (5.0) but undetectable at alkaline pH (8.0). A summary of the purification is outlined in Table 1. The target protein was first separated from the root exudates by Mono S

Table 1
Summary of the purification of the secreted NtPAP with phytase activity

Step	Volume (mL)	Total activity (nkat)	Total protein (μg)	Specific activity (nkat mg^{-1})	Yield (%)	Purification (fold)
Root exudates	1,300	33.2	2,675	12.4	100	1
Mono-S HR 5/5	8	8.18	49.8	164	25	13
Superose 12 HR 10/30	0.5	1.09	0.18	6,028	3	486

cation-exchange chromatography into a single activity peak that was eluted at 200–240 mM NaCl. This purification step effectively eliminated most of the contaminating proteins and resulted in several major polypeptides in the active fractions as indicated by silver-stained SDS–PAGE (Fig. 2). The target protein was purified 13-fold to a specific activity of 164 nkat mg^{-1} and a yield of 25% (Table 1). Subsequently, Superose 12 HR 10/30 gel filtration was used to purify the target protein to a specific activity of 6,028 nkat mg^{-1} and an overall yield of ca. 3% (Table 1). The homogeneity of the final preparation was confirmed by SDS–PAGE, in which a single silver-stained polypeptide of ca. 64 kDa was detected (Fig. 2). The apparent molecular weight (M_r) of the native enzyme was estimated to be ca. 56 kDa using preparative grade Superose 12 gel filtration (data not shown), confirming that the native enzyme is a monomeric protein. As only a single activity peak was detected in both Mono S cation-exchange and gel filtration chromatographies (data not shown), the target protein is likely to be the sole enzyme with phytase activity in tobacco root exudates.

2.2. Catalytic properties

A pH-activity profile of the purified enzyme is depicted in Fig. 3a. The enzyme exhibited phytase activity over a typical pH-activity range (3.5–6) of acid phosphatases, with complete abolishment of enzyme activity at or above pH 6.5. The optimum pH was 5–5.5 (Fig. 3a). A temperature-activity profile is depicted in Fig. 3b. The enzyme displayed a maximum activity at 45 °C and was completely denatured at 60 °C or above. The thermostability of the enzyme was studied at various temperatures from 25 to 80 °C (Fig. 3b). The enzyme was stable at or below 55 °C, exhibiting at least 80% of residual activity. The

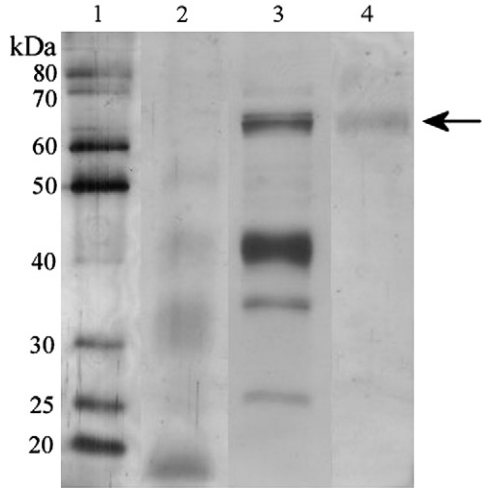

Fig. 2. SDS–PAGE analysis of proteins at different purification stages. Lane 1, molecular weight standards; lane 2, root exudates; lane 3, activity fraction obtained from Mono S HR 5/5 chromatography; lane 4, activity fraction purified by Superose 12 HR 10/30 gel filtration. The proteins were detected by silver staining.

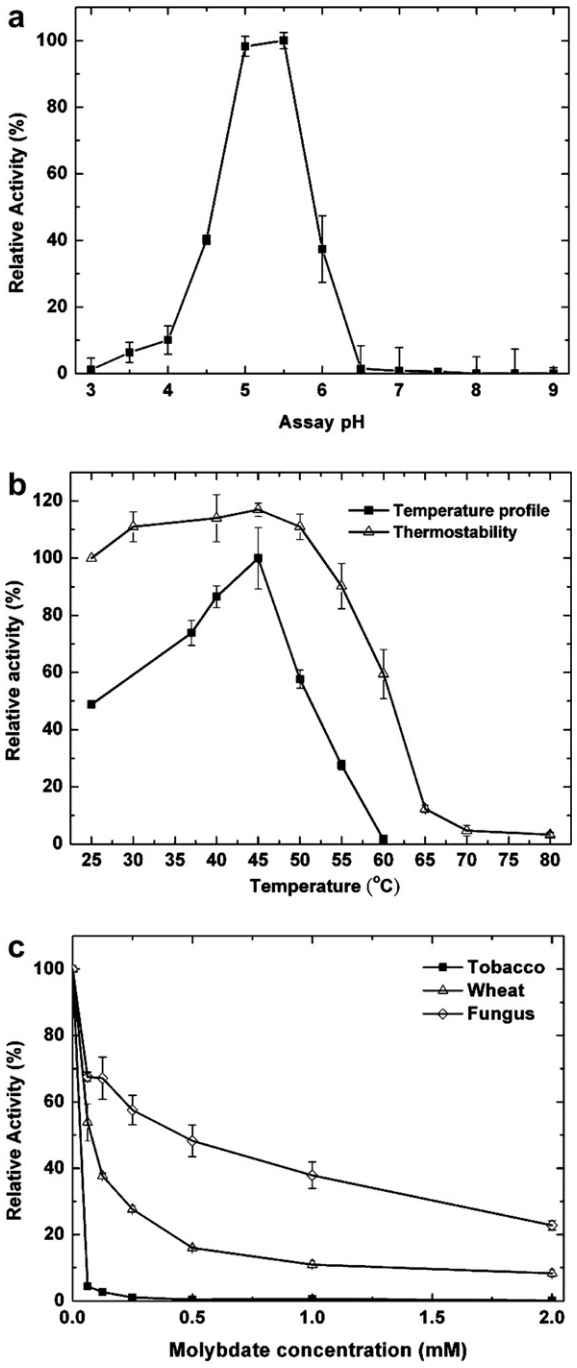

Fig. 3. (a) The pH profile. Phytase activity was assayed in various buffers (100 mM) that contained 1 mM Na-$InsP_6$. The buffers included Glycine/HCl (pH 3–4), NaOAc (pH 4.5–5.5), Tris/maleate (pH 6–7.5) and Tris/HCl (pH 8–9). The reactions were carried out at 37 °C for 30 min. (b) The temperature and thermostability profiles. The temperature profile was conducted in 100 mM NaOAc buffer (pH 5.5) containing 1 mM Na-$InsP_6$ at various temperatures for 30 min. The thermostability profile was constructed by incubating the enzyme preparations in 50 mM NaOAc buffer (pH 5.0) at various temperatures for 10 min, followed by phytase activity assays in the same buffer containing 1 mM Na-$InsP_6$ at 37 °C for 30 min. (c) Effect of molybdate on the activities of the secreted NtPAP, wheat bran phytase, and *Aspergillus ficuum* phytase. Enzyme preparations were assayed in 50 mM MES/NaOH buffer (pH 6.0) that contained 1 mM Na-$InsP_6$ and various concentrations of molybdate. The reactions were carried out at 37 °C for 30 min. Each point represents the mean of three-experiments.

residual activity decreased dramatically after treatment of the enzyme at temperatures above 55 °C (Fig. 3b).

2.3. Substrate specificity

The ability of the purified enzyme to hydrolyze various phosphorylated compounds was investigated. The rates of hydrolysis relative to Na-InsP_6 (**1**) are summarized in Table 2. In the assay mixtures, P_i was liberated to a detectable level from each of the 21 phosphate esters tested, which suggested that the enzyme exhibited broad substrate specificity. It displayed the highest rates of hydrolysis for various deoxyribonucleotide triphosphates (dNTPs; 189–389%) and ribonucleoside triphosphates (NTPs; 271–365%), while its activity toward adenosine monophosphate (AMP; 30%) was significantly lower than that of diphosphate (ADP; 206%) and triphosphate (ATP; 365%). *p*-Nitrophenyl phosphate (*p*NPP; Fig. 1) (**2**), which is a synthetic substrate utilized in routine phosphatase assays, was also hydrolyzed by the enzyme at a relatively high rate (311%), whereas sugar phosphates were broken down at comparatively lower rates (5–75%) than Na-InsP_6.

2.4. Kinetic parameters

The kinetic properties of the purified enzyme were analyzed using Na-InsP_6 (**1**) and *p*NPP (**2**) as two representative substrates. As outlined in Table 3, the purified enzyme exhibited relatively high affinities for both Na-InsP_6 (**1**) ($K_m = 14.7$ μM) and *p*NPP (**2**) ($K_m = 13.9$ μM), while the turnover number for *p*NPP (kcat = 2,056 s^{-1}) was higher than that for Na-InsP_6 (**1**) (kcat = 908 s^{-1}), leading to a 2.4-fold higher catalytic efficiency for *p*NPP (**2**) (kcat/K_m = 148 s^{-1} μM^{-1}) than that for Na-InsP_6 (**1**) (kcat/K_m = 62 s^{-1} μM^{-1}). The V_{max} values for Na-InsP_6 (**1**) and *p*NPP (**2**) were 7,200 and 16,267 nkat mg^{-1} (Table 3), respectively.

Table 2
Substrate specificity of the secreted NtPAP with phytase activity

Substrate	Relative activity (%)
dTTP	389 ± 40
dCTP	367 ± 60
ATP	365 ± 37
CTP	352 ± 47
UTP	350 ± 47
dATP	338 ± 29
Phosphoenolpyruvate	328 ± 7
*p*NPP	311 ± 17
GTP	271 ± 19
ADP	206 ± 15
dGTP	189 ± 7
Phenyl phosphate	188 ± 11
3-Phosphoglycerate	168 ± 37
Tyrosine phosphate	142 ± 8
Na-InsP6	100
Glucose-3-phosphate	75 ± 8
Glucose-6-phosphate	57 ± 15
Ribose-5-phosphate	49 ± 8
Fructose-6-phosphate	46 ± 9
AMP	30 ± 5
Glucose-1-phosphate	5 ± 3

Enzyme activities were assayed at 37 °C for 1 h in 100 mM NaOAc buffer (pH 5.5) that contained various substrates (1 mM). Each value represents the mean of three-experiments ±1 standard deviation and is expressed as a percentage relative to the measurement using Na-InsP_6 as substrate.

Table 3
Catalytic properties of the secreted NtPAP

Substrates	V_{max} (nkat mg^{-1})	K_m (μM)	kcat (s^{-1})	kcat/K_m (s^{-1} μM^{-1})
Na-InsP_6	7,200	14.7	908	61.8
*p*NPP	16,267	13.9	2,056	147.9

2.5. Effect of molybdate on phytase activity

To verify the PAP identity of the target protein, phytase activity of the purified enzyme was assayed in the presence of molybdate, which has been shown to be an inhibitor of PAP activity (Vogel et al., 2002). Our previous study indicated that the phytase activity of wheat PAP was severely inhibited by molybdate at less than 1 mM concentration and could be clearly distinguished from that of a fungal HAP, which was moderately inhibited within the same range of molybdate concentration (Tang et al., 2006). The molybdate-activity curves were reproduced in the present study (Fig. 3c). The inclusion of 1 mM molybdate in the assay resulted in ca. 90% inhibition of wheat PAP activity, which was comparable to that of red kidney bean PAP (Vogel et al., 2002), whereas fungal HAP activity was moderately suppressed by 60% (Fig. 3c). The purified enzyme from tobacco root exudates was even more sensitive toward inhibition by molybdate, of which 62.5 μM was sufficient to suppress 95% activity (Fig. 3c).

2.6. The secreted enzyme is a PAP

To further confirm the PAP identity of the purified enzyme (64 kDa; Fig. 2), it was in-gel digested with trypsin, and the resulting trypsin digests were analyzed with matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). Peaks with signal intensities above 10,000 were further subjected to MS/MS analysis using a QSTAR tandem mass spectrometer. For each peptide, *de novo* sequencing was also performed manually to confirm the sequence information. Using this strategy, an amino acid sequence, GHAVDLPDTDPR, was found in the liberated peptides. When compared with protein sequences in the NCBI and MSDB databases using the MASCOT search engine, the tryptic peptide sequence matched completely and exclusively with a putative phytase from *Oryza sativa* (Genbank accession no. XP_470408). It also exhibits significant sequence similarity to several plant PAP-phytases (Fig. 4), including *Arabidopsis thaliana* AtPAP15 (Li et al., 2002), *Glycine max* GmPhy (Hegeman and Grabau, 2001), and *Medicago truncatula*

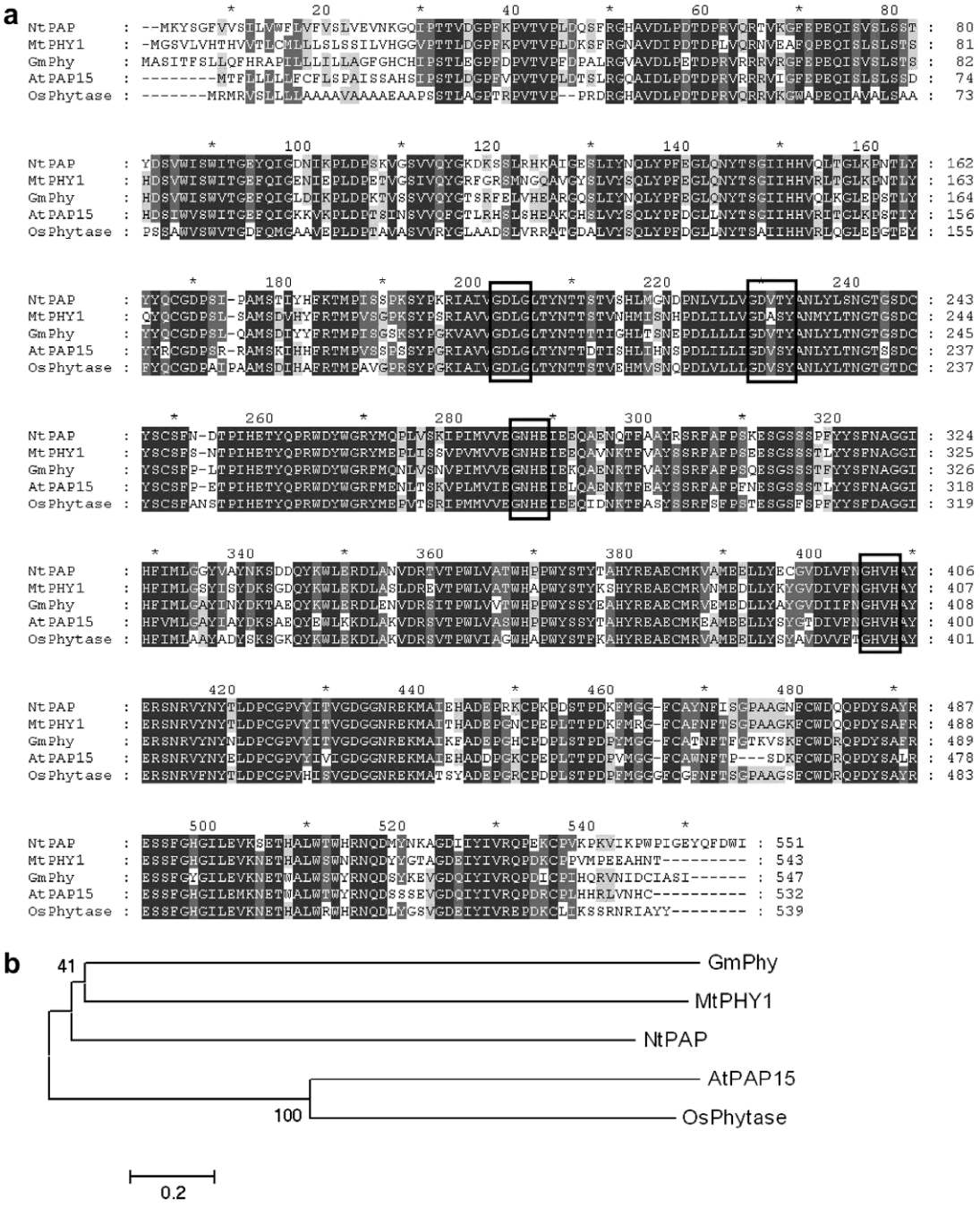

Fig. 4. Deduced amino acid sequence comparison of the secreted NtPAP with different plant PAP-phytases. (a) Amino acid alignment. Alignment was performed using ClustalX v1.83 formatted in Genedoc (Multiple Sequence Alignment Editor and Shading Utility v.2.6). The residues blocked with a white foreground on a dark grey background are 100% conserved residues, residues with a white foreground on a grey background are 75% or more conserved residues, and residues aligned with a black foreground on a light grey background are 50–74% conserved residues. Conserved amino acid sequences of PAPs for metal coordination are boxed. GenBank accession numbers are shown in parentheses: *NtPAP*, *Nicotiana tabacum* (EF397753); *MtPHY1*, *Medicago truncatula* (AAX71115); *GmPhy*, *Glycine max* (AAK49438); *AtPAP15*, *A. thaliana* (AF448726); *OsPhytase*, *Oryza sativa* (XP_470408). (b) The phylogenetic tree was constructed by the neighbor joining method using the MEGA 3 program (Kumar et al., 2004) with 1,000 bootstrap repetitions. The bootstrap values (%) are given at the respective nodes.

MtPHY1 (Xiao et al., 2005). Subsequently, cDNA sequence of the secreted NtPAP was obtained by PCR. Comparison of its deduced amino acid sequence among other plant PAP-phytases and the presence of the conserved metal-coordinating motifs (Li et al., 2002) confirm its PAP identity (Fig. 4).

There is a consensus that in the plant kingdom secreted acid phosphatases play a ubiquitous role in mineralizing external P-esters. In contrast to intracellular acid phosphatases (e.g., phosphatidic acid phosphatase, phosphoenolpyruvate phosphatase, etc.), which exhibit distinct substrate specificity and have clear metabolic functions, secreted acid

phosphatases are vaguely referred to as "non-specific" phosphatases due to their unclear substrate specificity (Duff et al., 1994). Several researchers have purified and characterized acid phosphatases from Arabidopsis (Coello, 2002), lupin (Ozawa et al., 1995), and tomato root exudates (Li and Tadano, 1996). The partial amino acid sequencing of two acid phosphatases that were secreted from phosphate-starved tomato cell cultures further identified these acid phosphatases as PAPs (Bozzo et al., 2002). However, none of them were reported to exhibit phytase activity. Li et al. (1997b) detected phytase activity in the root exudates of 16 plant species, and purified a phytase from tomato root exudates to homogeneity (Li et al., 1997a). Nonetheless, the identity of these secreted phytases was not verified. The present study verified the identity of a native phosphatase with phytase activity that is secreted from P-starved tobacco. This enzyme was secreted into the extracellular medium at high specific activity (10–12 nkat mg^{-1} protein) when tobacco plants were cultivated hydroponically under P deficiency, as revealed in our previous (Lung and Lim, 2006) and the present study (Table 1). Interestingly, while both HAP phytases (Dionisio et al., 2007; Mehta et al., 2006) and PAP phytases (Zhu et al., 2005; Rasmussen et al., 2003) were present in the plant kingdom, there are more PAP-like genes than HAP-like genes in each individual plant genome. For example, 29 PAP-like genes (Li et al., 2002) and one HAP-like gene (Mullaney and Ullah, 1998) are present in the Arabidopsis genome.

2.7. Physiological role of secreted acid phosphatases in soil

An insight into the catalytic properties of this secreted NtPAP and other secreted acid phosphatases demonstrates their potent functionality in the soil environment. Most of the secreted acid phosphatases, including the one reported in the present study (Fig. 3a), exhibit slightly acidic pH optima (pH 5–6; Bozzo et al., 2002; Coello, 2002; Li and Tadano, 1996), which are physiologically important for optimum enzyme activity in the rhizosphere because of the acidification of the root surfaces of many plants (Duff et al., 1994). In addition, the relatively high thermostability of the secreted NtPAP (Fig. 3b) is comparable to those of other secreted acid phosphatases, which exhibit at least 80% residual activity at or below 60 °C (Bozzo et al., 2002; Li and Tadano, 1996; Ozawa et al., 1995), thus suggesting the potential persistence of the activities of these enzymes against thermal degradation in soil.

Substrate specificity is also a paramount consideration. Like other known secreted acid phosphatases (Bozzo et al., 2002; Coello, 2002; Li and Tadano, 1996), the secreted NtPAP also displays remarkable activities toward a range of physiological substrates (Table 2). However, other known secreted acid phosphatases generally exhibit negligible hydrolytic activity toward $InsP_6$ (**1**) (Bozzo et al., 2002; Coello, 2002), despite the predominance of $InsP_6$ in soil (Anderson, 1980). Only a subgroup of PAP exhibits phytase activity and plants do not preferentially secrete PAP with phytase activity from roots, implying that soil $InsP_6$ (**1**) is not a readily available substrate for plant phytase. The invulnerability of $InsP_6$ (**1**) to enzyme degradation can be attributed to the extremely high charge density that renders the molecule highly insoluble in soil (Turner et al., 2002), which is in agreement with our previous study showing that the assimilation of $InsP_6$-P by the extracellular phytase activity of tobacco was affected by the availability of soluble $InsP_6$ (**1**) (Lung and Lim, 2006). In view of the three-dimensional structures of plant PAPs, the cleavage of phosphomonoester bonds requires the precise coordination of phosphate ions with the amino acid residues in the active sites (Schenk et al., 2005). Hence, such an explicit enzyme-substrate interacting model may preclude most of the secreted PAP-phytase from initiating a hydrolytic reaction toward $InsP_6$ (**1**) that has been tightly bound with cations or soil components (Tang et al., 2006). Like other secreted phosphatases, the secreted NtPAP was found to exhibit maximum activity for deoxyribonucleotide and ribonucleoside triphosphates (Table 2). These activities are more physiologically important than the phytase activity because P_o that is derived from microbial turnover accounts for up to 90% of soil solution P within the rhizosphere (Helal and Dressler, 1989). P_o from microbes occurs predominantly as nucleic acids (30–50% P in RNA and 5–10% P in DNA), phospholipids (<10% P), and a range of sugars, and is rapidly mineralized in soil environments (Macklon et al., 1997; van Veen et al., 1987). This may explain why plants do not universally secrete phosphatases with phytase activity and why IHP is the predominant P_o in soil.

3. Conclusions

In summary, the present study verifies the identity of the phosphatase with phytase activity in tobacco root exudates as a PAP. In previous studies, acid phosphatases that are secreted from plant roots have generally exhibited negligible phytase activity, and the identity of the secreted phytase is poorly known. The secreted NtPAP that exhibits phytase activity in root exudates exhibits broad substrate specificity and shows maximum activity for deoxyribonucleotide and ribonucleoside triphosphates. Since P_o from microbial turnover are predominantly nucleic acids, which account for up to 90% of soil solution P within the rhizosphere, it is suggested that nucleotides and nucleosides are the major target substrates of plant secreted phosphatases. In contrast, the insolubility of soil phytate limits its availability to plant phytases. This may explain why, in evolution, plants do not universally secrete phosphatases with phytase activity.

4. Experimental

4.1. Plant materials

Plants were cultivated hydroponically following the procedure described previously (Lung and Lim, 2006). Tobacco

(*N. tabacum*) variety 'GeXin No1' was obtained from the Shanghai Institute of Plant Physiology and Ecology. Seeds were surface-sterilized in 20% (v/v) Clorox for 20 min, followed by several rinses with sterile, deionized H_2O. The sterilized seeds were sown on 50 mL of liquid MS medium (ca. 120 seeds plate^{-1}) with lowered KH_2PO_4 concentration (0.1 mM), and maintained aseptically in controlled environment cabinet with a day/night regime of 16/8 h, 22/16 °C, and photon flux density of 100 μmol m^{-2} s^{-1}. After 14 days, the growth medium was collected and replenished with fresh MS medium. On day 28, the growth medium was collected and pooled for the purification of the secreted enzyme.

4.2. Purification of the secreted enzyme

The collected root exudates were filtered through a 0.45 μm membrane, and the filtrate was lyophilized. The resulting residue was resuspended in one-twentieth of its original volume in 50 mM NaOAc (pH 5.0) and dialyzed against the same buffer at 4 °C overnight. The dialysate was loaded on a Mono S HR 5/5 column (FPLC System, Pharmacia), which was equilibrated with 50 mM NaOAc (pH 5.0). The column was washed thoroughly with the same buffer, and the adsorbed proteins were eluted with a linear salt gradient of the same buffer that contained 0 to 0.5 M NaCl. The flow rate was maintained at 1 mL min^{-1} and the eluate was collected in 2 mL fractions and assayed for phytase activity and protein concentration. Subsequently, active fractions were pooled and loaded onto a Superose 12 HR 10/30 column (FPLC System, Pharmacia), which was equilibrated with 50 mM NaOAc, pH 5.0, 150 mM NaCl. Flow rate was maintained at 0.5 mL min^{-1}, and the eluate was collected in 0.5 mL fractions and assayed for phytase activity and protein concentration. The purified samples were fractionated by SDS–PAGE and visualized by silver staining (Yan et al., 2000).

4.3. Native molecular weight estimation

The native M_r of the secreted enzyme was estimated by Superose 12 HR 10/30 gel filtration (FPLC System, Pharmacia) during phytase purification as described above. It was calculated from a plot of K_{av} (partition coefficient) against log M_r, which was produced using five protein standards, including IgG (M_r 150 kDa), albumin (M_r 55 kDa), ovalbumin (M_r 45 kDa), chymotrypsinogen A (M_r 20 kDa), and ribonuclease A (M_r 15 kDa).

4.4. Phytase and protein assays

Phytase activity was assayed in 100 μL of 50 mM NaOAc (pH 5.0) or 100 mM Tris–HCl buffer (pH 8.0; Jog et al., 2005), using 1 mM Na-InsP_6 (**1**) (Sigma cat no. P3168) as the substrate. All reactions were carried out at 37 °C for 30 min, unless otherwise specified, and terminated by an equal volume of 10% (w/v) TCA. The liberated P_i was quantified by molybdenum-blue assay (Murphy and Riley, 1962). Enzyme activity is expressed in nkat, which is defined as the activity that releases 1 nmol of phosphate per sec under the specified assay conditions. The kinetic constants were determined from Lineweaver–Burk plots using 0.2, 0.4, 0.6, 0.8, and 1.0 mM Na-InsP_6 (**1**) and 0.3125, 0.625, 1.25, 2.5, and 5.0 mM *p*NPP (**2**). A molybdate-inhibition assay was performed according to a protocol described previously (Tang et al., 2006). The concentration of total soluble proteins was determined by the FluoroProfile™ Protein Quantification Kit (Sigma cat no. FP0010), according to the manufacturer's protocol.

4.5. In-gel trypsin digestion

The band of interest was manually excised from silver-stained SDS–PAGE. The gel plug was destained, reduced with 10 mM dithiothreitol, and alkylated with 55 mM iodoacetamide. The in-gel enzymatic digestion was performed overnight (~16 h) with trypsin (Promega, Madison WI) at 37 °C. The tryptic peptides were extracted with HCO_2H:CH_3CN:H_2O (5:50:45, v/v), and the volume of extract was reduced to ca. 20 μL by speed vacuum. The extract was then purified using pipette ZipTip u-C18 (Millipore, Bedford, MA). The peptides were eluted in 3 μL of HCO_2H:CH_3CN:H_2O (0.1:50:44.9, v/v), and stored at −20 °C for further MS analysis.

4.6. Protein identification by mass spectrometry analysis

The eluted peptide sample was mixed 1:1 with the matrix solution (10 mg α-cyano-4-hydroxycinnamic acid in 1 mL of HCO_2H:CH_3CN:H_2O (0.1:50:44.9, v/v), and then spotted on a 100-well stainless steel MALDI plate for MALDI-TOF MS and MS/MS analyses. For MALDI-TOF MS analysis, reflectron MS analyses were performed on a Voyager DE STR Biospectrometry™ Workstation (Applied Biosystems) using a pulsed laser beam (nitrogen laser, 337 nm). All ion spectra were recorded in the positive mode with the accelerating voltage of 20.0 kV. The spectrometer was externally calibrated using a Cal Mix 2 standard mixture. MS/MS experiments were performed using a QSTAR XL quadrupole orthogonal acceleration time-of-flight mass spectrometer equipped with an oMALDI ion source (Applied Biosystems). The instrument was controlled by the Analyst® QS software and was operated in the positive mode. The pulse rate was set to 30 Hz and the power level was 20% v/v. Ar was used as the collision gas at a recorded pressure of 60 psi. The collision energy was optimized for different ions by ramping up the energy over the *m/z* range proportional to one-tenth of the *m/z* value of the precursor ion. The MASCOT search engine (http://www.matrixscience.com) was used for searching both the National Center for Biotechnology Information (NCBI) database and the MS protein sequence DataBase (MSDB). The search was performed under the Viridiplantae (Green Plants) category, and a mass tolerance range of 0.13–0.18 Da was used. *De novo* sequencing was performed

Table 4
A list of oligonucleotides used for cDNA cloning

Name	Amino acid position	Nucleotide sequence	Orientation
TF-1	42–48	GTIGAYYTICCIGAYACIGA	Sense
PR-1	253–257	TCCCAICKIGGYTGRTAIGT	Anti-sense
dTA	N/A	AAGCAGTGGTATCAACGCAGAGTAC$(T)_{22}$VN	N/A
dTAP	N/A	AAGCAGTGGTATCAACGCAGAGT	N/A
dC	N/A	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	N/A
dCP	N/A	GGCCACGCGTCGACTAGTAC	N/A
T-Sta	5′NTR	GGATTTCTTGAGGTTTGGAG	Sense
T-End	3′NTR	CATCAAATTTGAGGGTTCTTG	Anti-sense
T379S	192–200	GCAATTGTAGGGGACCTCGGTCTAACG	Sense
T550S	249–256	CGTGCTCATTTAACGACACTCCGA	Sense
T478A	228–232	GTGACATCTCCAACCAATAGAAC	Anti-sense
T379A	192–199	GCTATTCTCTTTGGGTAGCTCTT	Anti-sense

manually using Analyst QS1.1 software, and sequence information was used for database searching.

4.7. cDNA cloning by degenerated PCR and RACE

All oligonucleotides used in the present study are listed in Table 4. The first-strand cDNA was synthesized from the tobacco root total RNA using dTA and used as a template for degenerated PCR with the primers TF-1 and PR-1, which were designed corresponding to the tryptic peptide sequences VDLPDTD and TYQPRWD, respectively. Based on the sequence of a 630 bp product, two sense (T379S and T550S) and antisense (T379A and T478A) primers were designed for 3′- and 5′-RACE, respectively. The 3′ sequence was obtained from a nested PCR using the sense primers (T379S and T550S) and an antisense primer dTAP. For 5′-RACE, a first-strand cDNA was first synthesized using the degenerated primer PR-1 which was then dC-tailed by T4 polynucleotide kinase after RNase treatment. Then the 5′ sequence was obtained from a nested PCR using the two pairs of primers, dC/T478A and dCP/T379A, respectively. The entire sequence was verified on a full length cDNA amplified by the primers T-Sta and T-End.

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