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Fungal Infection Alters Phosphate Level and Phosphatase Profiles in *Arabidopsis*

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Abstract

Phosphorus (P), in the form of phosphate ion (Pi), is a vital element contributing in biomolecule structures, metabolic reactions, signaling pathways and energy transfer within the living cells. The objective of the present study was to assess the influence of fungal infection on Pi metabolism in compare to the effects of phosphate stress in *Arabidopsis*. Quantification of total P contents showed higher storage of P in the shoots than in the roots of Pi-fed plants, while the homeostatic levels of soluble Pi was kept in a fairly narrow range in roots and shoots of both Pi-fed and Pi-starved. When the plants were subjected to Pi starvation, both total P and soluble Pi contents were reduced to minimal levels in roots and shoots. Total acid phosphatase (APase) activity was also affected by the level of available Pi such that it was higher in the starved plants than in the fed plants. When Pi-fed plants were subjected to fungal infections, a remarkable reduction was observed for the above indicators in roots but not shoots. Surprisingly, the analysis of APase expression profiling after inoculation with *Alternaria brassicicola* showed that the rates of transcription of several APase-encoding genes were affected by fungus infection. *Atpap9*, a fungal inducible gene, promoter analysis also indicated alterations in tissue-specific expression patterns upon the fungal infections. These data clearly illustrate that how a nutrient distribution is affected by environmental conditions, even regardless of available phosphate.

Keywords: Acid Phosphatase, Expression Profiling, Fungi Inducible Gene, Phosphate Starvation, Promoter Analysis.

Introduction

Phosphorus (P) is an essential macronutrient for plant growth and development. It is a structural element in nucleic acids and phospholipids and involved in many fundamental physiological and biochemical pathways such as photosynthesis, respiration, energy transfer and signal transduction in the form of inorganic phosphate ion (Pi; Morcuende et al., 2007, Lin et al., 2009, Yang et al., 2010, Shen et al., 2011). As a result, it is important to maintain Pi homeostasis in a narrow range in order to ensure biological reactions run properly. Therefore, a wide range of morphological and molecular adaptations have been evolved to increase uptake, reallocation and recycling as well as efficient utilization of Pi in plants, particularly when subjected to harsh environmental conditions (For a review see Plaxton and Tran., 2011). These adaptations include changes in the expressions of a number of genes such as acid phosphatases (APases; del Pozo et al., 1999, Haran et al., 2000, Baldwin et al., 2001, Miller et al., 2001, Li et al., 2002, Tran et al., 2010, Wang et al., 2011), ribonucleases (Nurnberger et al., 1990, Bariola et al., 1994) and high affinity phosphate transporters (Muchhal et al., 1996, Daram et al., 1999, Karthikeyan et al., 2002, Mudge et al., 2002, Wul et al., 2011, Jia et al., 2011) that are directly or indirectly involved in maintaining Pi homeostasis (Muller et al., 2007, Pratt et al., 2009, Plaxton and Tran., 2011). It is well known that APases play major roles by scavenging Pi from internal and external sources

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(for a review see Tran et al., 2010). The expression profiles of members of this relatively large family is not only affected by Pi availability, but also is largely influenced by other abiotic harsh conditions such as the lack of nutrients and high salt concentration (Berger et al., 1995, del Pozo et al., 1999, Lohrasebi et al., 2007).

Likewise, sophisticated mechanisms have been evolved in plants to deal with pathogenic attacks. Plants perceive the presence of pathogens and rapidly trigger defense responses that involve the expressions of several protein families including some APases (Goodman et al., 1994, Petters et al., 2002, Feng et al., 2003). For instance, Petters et al. (2002) identified an APase gene expressed locally in potato leaves in response to infection with *Pseudomonas syringae* bacterium as well as *Phytophthora infestans* fungus. The encoded protein has high homology to a Pi starvation-induced APase identified in tomato. The authors suggested that the soluble Pi availability affected by pathogenic attack may act as a signal for the induction of the APase gene, though no evidence for changed Pi level was provided. Jakobek and Lindgren (2002) isolated a cDNA clone with similarity to APases induced in bean during hypersensitive response to inoculation with *P. syringae*. A putative APase transcript accumulated in barley when exposed to chemical compounds that activate systemic resistance (Beber et al., 2000). Similarly, increased APase activity was observed in tobacco following inoculation with bacteria which induced hypersensitive response (Kenton et al., 1999). Plant responses to elicitors, wounding, nematodes and insects have also been reported to involve increased APase activity that are not necessarily associated with available Pi (Williamson et al., 1991, Jakobek et al., 2002, Feng et al., 2003, Liu et al., 2005, Lohrasebi et al., 2007).

Alternaria brassicicola, the causal agent of black spot disease of crucifers, is a necrotrophic pathogen that assimilates nutrients from dead host tissue and actually benefits from reactive oxygen species (ROS) production and programmed cell death (Govrin et al., 2000, Mayer et al., 2001). Inoculation of *Arabidopsis* with *A. brassicicola* leads to induced expression of a large number of genes while the lesion does not spread significantly beyond the inoculated site due to incompatible interaction (Van Wees et al., 2003). These criteria made the infection of *Arabidopsis* with *A. brassicicola* an attractive plant-fungus interaction model in this study. Besides, there are considerable amounts of data about this interaction using various *Arabidopsis* accessions and mutants (Van Wees et al., 2003, Mukherjee et al., 2009). For example, studies with *Arabidopsis* mutants defective for some hormone-dependent defense pathways showed that interactions with necrotroph fungi primarily employ jasmonic acids and phytoalexin dependent pathways (Thomma et al., 1998, Kim et al., 2004).

While Pi homeostasis has been the subject of many studies involving harsh abiotic conditions, particularly Pi starvation, no such a deliberation has been reported during biotic stresses. In this research, we have established a family-wide profiling of fungal inducible APases versus Pi-starved and Pi-fed ones with the use of DNA array and semi-quantitative RT-PCR methods. Then, the expression pattern of a highly induced gene, At*pap9*, was further elucidated in Pi-fed, Pi-starved and fungi treated plants by the use of a promoter::reporter gene fusion. The analysis of the obtained data indicated that fungus infections greatly influences Pi metabolism in plants.

Materials and Methods

Apase Sequence Retrieval and Primer Design

A phrase search was conducted on Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez) to get all *Arabidopsis* sequences annotated as "acid phosphatase". To ensure no locus encoding APases was neglected; the retrieved data were cross-checked with annotated AtAPases in relevant division of Map Viewer available at NCBI web site (http://www.ncbi.nlm.nih.gov/projects/mapview/) and TAIR (http://www.arabidopsis.org/). The retrieved sequences were grouped according to the results of preliminary studies. Then, the unique sequences of each family member were selected using a lab-written program which deleted segments with over 75% identities by considering BLASTN search results against *Arabidopsis* genomic database (Richmond et al., 1999, Grike et al., 2000, Hilson et al., 2004). A set of gene-specific primers were designed based on the unique sequences using

Oligo5 software (Molecular Biology Insights, USA).

Plant and Fungus Materials

Seeds of *Arabidopsis thaliana* ecotype Colombia were sterilized and hydroponic cultured as described by Malboobi et al. (1997) with minor modifications. 11-day old *Arabidopsis* seedlings grown on solid medium were transferred into liquid half-strength MS medium (Murashige and Skoog 1962) containing 1.2 mM KH_2PO_4 and supplemented with 1% sucrose for 3 days to achieve identical nutritional states. Then, the seedlings were subjected to 5 mM Pi-fed and Pi-depleted treatments for 7 days as described by Lohrasebi et al. (2007).

To prepare fungal propagules for inoculation, *Alternaria brassicicola* culture was grown in a growth chamber on potato dextrose agar (Difco) under continuous white light at 25°C for 7 days. Fungal inoculums with spores were added to liquid medium in order to infect 28-day old Pi-fed plants. The infected plants were placed in a growth chamber for 24 h before harvesting. A group of Pi-fed plants were treated by 100 µg/ml chitin as a general elicitor for 1 day as described by Shobbar et al. (2005).

Quantification of Total P and Soluble Pi

Total P content was measured using modified Ames's method (1966). To about 50 mg fresh tissue samples placed in a Pyrex tube, 10% nitrate magnesium in 95% ethanol was added to final volume of 1.5 ml. The biomaterial was dried and burned to ash by shaking the tube over a flame until the brown fumes disappear. The tube was then allowed to cool down before adding 500 µl of concentrated perchloric acid (60%). The capped tube was heated in a boiling water bath for 30 min to hydrolyze any pyrophosphate formed in the ash after which the final volume was brought to 4.5 ml by water. To 300 µl of this sample, 700 µl of assay reagent (1 volume of 10% ascorbic acid and 6 volume of 0.42% ammonium molybdate in 1N H_2SO_4) were mixed and incubated at 45°C for 20 min before spectrometric measurement.

For quantification of soluble Pi, 30-50 mg frozen biomaterials were ground in liquid nitrogen and homogenized in deionized water by shaking up to the final volume of 500 µl. After centrifugation at 10000 rpm for 10 min, 50 µl of the supernatant was mixed with 250 µl of water. To this, 700 µl of the above assay reagent was added and incubated at 45°C for 20 min.

Total P and soluble Pi contents were measured at A_{820} expressed as micromoles of P or Pi per mg fresh weight using relevant standard calibration curves.

Quantification of Total APase Activity

APase activity in crude protein extracts was measured as described by Naseri et al. (2004) with minor modifications. APase activity in crude extracts prepared for quantification of soluble Pi was measured in 50 mM sodium acetate pH 5.6 and 5 mM *para*-nithrophenyl phosphate as a colorimetric substrate. Assays were performed in 96-well plates in final volume of 200 µl reactions containing 10 µl crude protein extracts and stopped after 5 min by adding 100 µl 0.5 M NaOH that final volume was adjusted by 50 mM sodium acetate pH 5.6. The release of *para*-nithrophenolate (*p*NP) was measured at A_{405}. Total protein concentrations were estimated using Bradford's reagent (Bradford 1976) and used to calculate total APase activity as unit/mg protein. In this assay, each unit of enzyme activity was defined as the release of 1µmol *p*NP in 1 min at the above conditions.

Expression Profiling of APases

Fifty eight gene-specific tags (GST) derived from unique sequences of each member of *Arabidopsis* APase families were PCR-amplified using the relevant primers (Supplemented Table 1). Following isolation with PCR Products Purification Kit (Qiagen, West Sussex, UK), about 500 ng of alkaline-denatured GSTs were spotted onto duplicate H^+-nylon membrane. The amplified *Arabidopsis* α-tubulin cDNA was also spotted onto the membranes as the internal control.

To prepare labeled cDNA, 20 µg of total RNA plants subjected to Pi-fed, Pi starved, chitin and fungi treatments were used as template for first strand cDNA synthesis reactions 0.2 µl of 50 mM oligo-dT (15) were annealed to the RNA samples

denatured for 10 min at 70°C in a final volume of 11 µl. Labeling reaction of 20 µl were prepared by adding the above mixture to 10 mM DTT, 40 units of RNase inhibitor (Fermentas, Lithuania), 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dATP, 0.13 mM dTTP and 0.07 mM DIG-dUTP (Roche Applied Sciences, Germany) and 20 units of M-MulV reverse transcriptase (Fermentas, Lithuania). The reactions were incubated for 3 h at 42°C.

GST-DNA arrays were hybridized at 65°C for 20 h in hybridization buffer composed of 7% SDS, 0.25 M NaH_2PO_4 and 1 mM EDTA. Stringency washes and detection steps were carried out according to the DIG DNA Labeling and Detection Kit instructions (Roche Applied Sciences, Germany). The intensity of each dot was quantified by TotalLab software (Phoretix International, Newcastle, UK). The mean intensity value of each gene was computed for two biological replicates divided by that of the α-tubulin and used for statistical analysis.

RNA Extraction and Semi-Quantitative RT-PCR

Total RNA was extracted from at least two biological replicated samples of shoots and roots of Pi-starved, Pi-fed and fungi-infected plants using a commercially prepared guanidine reagent RNX-Plus™ (Cinagen, Tehran, I.R. Iran) according to the manufacturer's instructions. To eliminate genomic DNA contamination, the RNA samples were further treated with DNase free RNase A (Roche, Basel, Switzerland) according to the supplier's instruction. RNA integrity was checked on a 1.6% (w/v) agarose gel containing ethidium bromide.

For cDNA synthesis, 2 µg of each RNA sample was added to a reaction consisted of 1 µl of 100 µM oligo-dT (12-15) primer, and 1 mM of each dNTP in a volume of 10 µl. The reaction was heated at 70°C for 20 min and cooled on ice. 10 µl of master mix was added to each reaction which contained 2 µl 5x RT buffer, 0.1 M dithiothreitol, 50 mM $MgCl_2$, 40 units of RNAse inhibitor (Roche Applied Sciences, Germany) and 50 units of M-MulV reverse transcriptase (Fermentas, Lithuania). The reaction was incubated at 42°C for 1.5 h. This reaction was followed by PCR using gene-specific primers (Table 1) for the fungus-induced genes. The calibration of the cDNA contents of RT-PCR reactions were performed by comparing the amplified transcripts of α-tubulin.

Analysis of *Arabidopsis* APase Promoters

1500-bp upstream sequences from the translation start site of APase-encoding genes were downloaded from Map Viewer database and scanned for known *cis*-elements stored at PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE) for jasmonic acid (JA), salicylic acid (SA) and other pathogenesis responsive motifs (Higo et al., 1999). In addition, the frequency of each motif was determined by software developed in our lab which uses rapid scaning algorithm for finding *cis*-elements that are longer than 4-mers and repeated at least once within or between promoter sequences of APase genes (Supplemented Table 2)

Tissue-Specific Gene Expression Analysis

Genomic sequence located 2 kb upstream of the *Atpap9* translation start site was amplified by PCR and introduced into T/A cloning vector (Fermentas, Lithuania). The utilized oligonucleotides were: 5′CGGTGGAGGAGTG AGAGTGGGAAGT3′ and 5′TGTAAACGGCG GGGATCCTGATTT3′. The promoter fragment was digested with *BamH*I (within the reverse primer) and *Hind*III (at position -1830 of the promoter sequence) enzymes and ligated upstream of *uidA* gene within pAM194 binary vector (Han et al., 2000). The resulting construct, *Atpap9*-promoter::*uidA* was transferred into *Agrobacterium tumefaciens* strain GV3101 via freeze and thaw method (Holsters et al., 1978).

Arabidopsis thaliana ecotype Col plants were grown on soil to produce strong rosettes in the green house conditions (16 h light, 8 h darkness, 25°C). The first bolting shoot was removed to promote the growth of secondary ones. Seven days later, plants were used for *In planta* transformation by vacuum infiltration (Betchtold et al., 2000). Transgenic lines were selected on solid MS medium containing 8 g/l agar supplemented with 50 mg/l kanamycin and 100 mg/l cefotaxim. Resistant plants were transferred to soil for consecutive self pollination to produce

T3 homozygote seeds.
Sterilized seeds of transgenic plants containing *Atpap9*-promoter::*uid*A were cultured in solid MS medium containing 1.2 mM KH_2PO_4 for 7 days prior to transferring into solid MS medium containing none or 5 mM KH_2PO_4 for the next 7 days. A set of Pi-fed plants were inoculated with several fungi including *A. brassicicola*, *Trichoderma harzianum*, *Rhizoctonia solani*, *Sclerotonia sclerotiorum* and *Hyaloperonospora arabidopsidis* and co-cultivated for 3 to 5 days before histochemical staining according to Jefferson et al. (1987). In each case, at least 20 independent lines were examined to ensure consistent patterns of GUS staining under the same conditions.

Statistical Data Analysis

Statistical analyses of the data were conducted using SPSS v.16 (Statistical Package for the Social Science). Complete randomized-block design analysis of variance and Walter-Duncan K ratio or *t*-test were used to compare the means at $P<0.05$.

Results

The Effects of Available Pi and Fungus Inoculation on Pi Solubilization

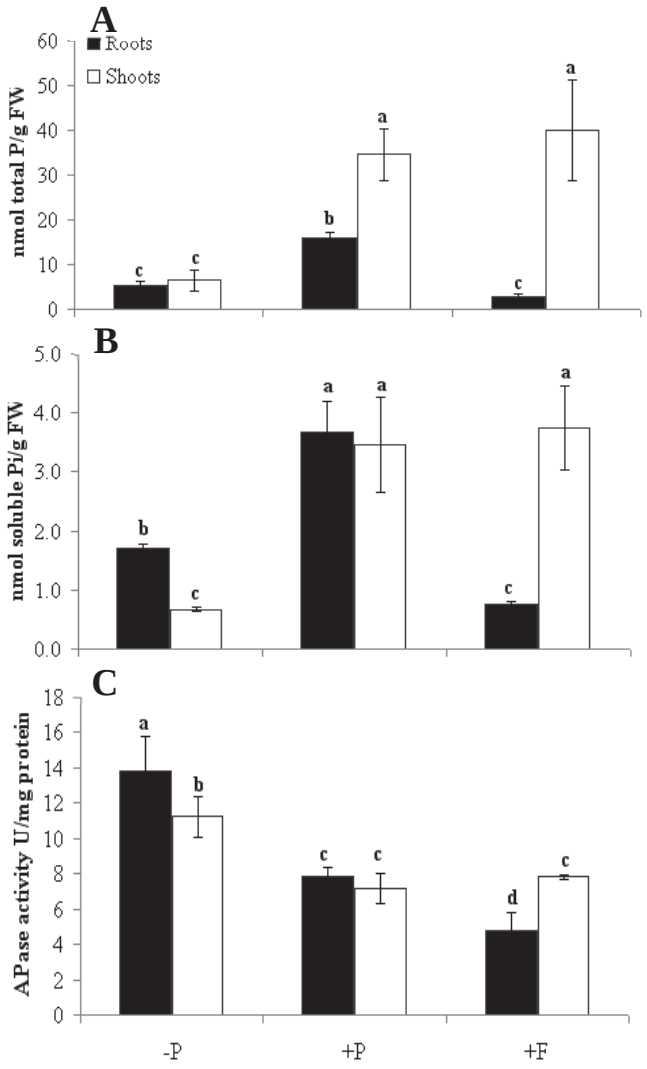
Three indicators were examined to find about Pi metabolism changes in the treated plants, total P, soluble Pi and total APase activity. Quantifications of total P contents showed high storage rate of excess P in the shoots rather than in the roots of Pi-fed *Arabidopsis* plants (Fig. 1A). In comparison, the levels of soluble Pi were almost the same in both of the Pi-fed roots and shoots (Fig. 1B). As a result, about 23 and 8.5 percent of P was in the form of soluble Pi in the roots and shoots, respectively. When grown in no-Pi condition, total P and soluble Pi levels decreased significantly in both roots and shoots (Fig. 1B). A reflection of soluble Pi status was observed as increased total APase activity in the starved roots and shoots while there were significant decreases in this indicator when plants supplied with sufficient Pi (Fig. 1C).
As compared to Pi-fed plants, inoculation with *A. brassicicola* fungus had no effect on total P and soluble Pi contents as well as APase activity in the shoots. However, marked reductions were observed for all three indicators in the roots (Fig. 1A-C).

Expression Profiling of *Arabidopsis* APase-Encoding Genes

Significant reductions in total P, soluble Pi and APase activity in fungi-infected roots prompted us to investigate the expression profiles of APase-encoding genes. A GST-DNA macroarray was constructed for all 58 *Arabidopsis* APases-encoding genes for which unique cDNA fragments were amplified and spotted on a nylon membrane. Labeled-cDNA derived from Pi-starved, Pi-fed, chitin treatment and as well as the fungal-infected *Arabidopsis* roots and shoots were used for hybridization to elaborate comparisons among the treatments as the instances of abiotic and biotic stresses further (Supplemented Table 3).
As shown in Table 2, the number of expressed APases in the roots was higher than those in the shoots, in general. Such that, 60 and 57 percent of the analyzed genes expressed at some levels in the Pi-fed and Pi-starved roots while 48 and 53 percent of the genes were on in the Pi-fed and Pi-starved shoots, respectively. One gene, At*pap*5, was specifically induced in the shoots subjected to Pi starvation. Eleven APases showed no detectable expression level in all examined conditions.
Surprisingly, inoculation of Pi-fed seedlings with *A. brassicicola* led to repression of a score of APases except for 8 and 14 genes expressed in the roots and shoots, respectively. Most of the expressed genes in the presence of this necrotroph fungus belong to PAP family. The highest expression levels were observed for At*hap*2, At*pap*9and At*pap*13 genes in both root and shoot samples of fungal-treated seedling. Interestingly, the collective expression values (Table 2, last row) showed the same patterns as the total APase activities (Fig. 1C). The differential expression patterns for the fungus-responsive genes were verified by comparative RT-PCR using gene-specific primers (Fig. 3).
In order to find out if the above outcomes are because of response to fungus presence or else (e.g. nutritional competition); Pi-fed seedlings

were treated with chitin only. As illustrated in Fig. 2, six out of eight induced APase genes in fungal inoculated roots are shared with chitin treatment. The expression of four other genes in chitin-treated roots was unexpected (Supplemented Table 3).

Figure 1. Changes in total P contents, soluble Pi contents and total APase activities in *Arabidopsis* plants grown in different conditions. 14-day old seedlings were grown hydroponically for 7 days with no Pi (-P) or 5 mM Pi (+P). A group of +P plants were inoculated with *Alternaria brassicicola* fungi for 24 hrs (+F). Total P (A) and soluble Pi contents (B) as well as APase activities (C) were measured in the root and shoot samples. All the values are the means for two biological replicates with 10 seedlings each. Vertical bars show standard errors and identical letters indicate no significant difference among treatments based on mean comparison by Duncan method at $P<0.05$.



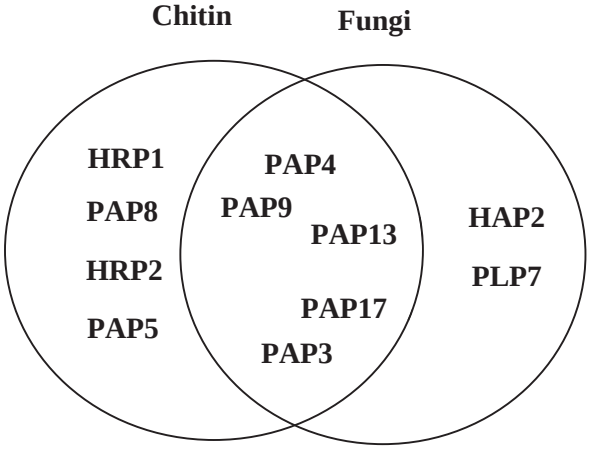


Figure 2. Comparing the list of expressed APases in response to chitin treatment and fungal inoculation of Pi-fed roots.

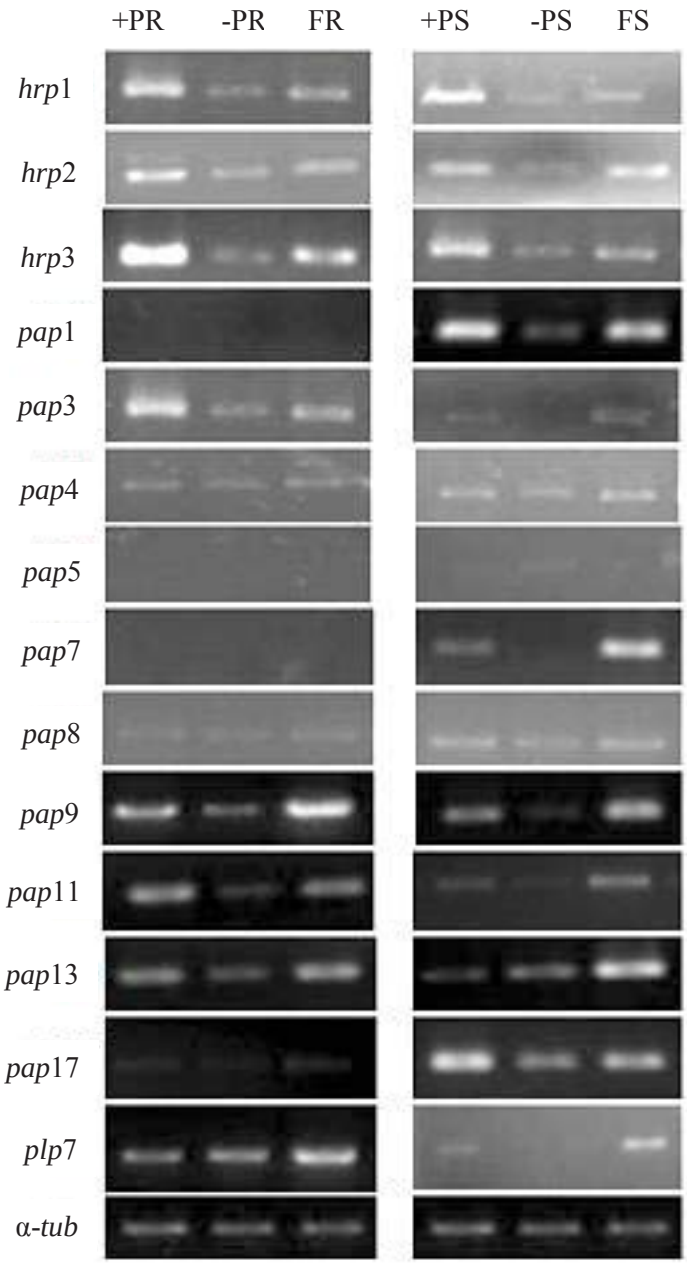


Figure 3. Verification of the expression levels of fungus-induced APase-encoding genes in shoots and roots of *Arabidopsis* by semi-quantitative RT-PCR. RNA isolated from shoots (S) and roots (R) of Pi-starved (-P) and Pi-fed (+P) plants as well as those infected with *A. brassicicola* (F) were reverse transcribed and amplified as described in Materials and methods. The level of amplification products of α-*tubulin* transcripts were used to adjust the cDNA contents prior to running RT-PCR reactions for APase-encoding genes.

Table 1. Genes and oligonucleotides used in the semi-quantitative RT-PCR.

Gene Name	Locus No.	Primer Name	Tm	Expected Band (bp)	Primer Sequence
HRP1	At1g04040	AP1F, R	53	673	5'tccaaaactcttctttcaaacat3' 5'tgatcttctagccccctgagcat 3'
HRP2	At1g73010	73010F, R	58	321	5' gaacttggcttcactgatttgt 3' 5' agcagctatgaggggatttagt 3'
HRP3	At1g17710	17710F, R	58	354	5'gaagatatggagaggatactaatgg 3' 5' aagaagtgatacgaaaaatgagc 3'
PAP1	At1g13750	uniPAP1F, R	52	458	5' caatatagttctccgaaaccatc 3' 5'aaggtcaatgttccagcag 3'
PAP3	At1g14700	uniPAP3F, AP6R	53?	860	5' agagacacaaagatcacaacaaa 3' 5' tccacgctgcttatgtgctcta 3'
PAP4	At1g25230	uniPAP4F, R	52	250	5' atgactctgcttatttgcttc 3' 5' cgatacttttcatcccgttat 3'
PAP5	At1g52940	AP9F, R	54.5	205	5'ggttataacgctcctgaac 3' 5'gaagataaccagatgtgtaatca 3'
PAP7	At2g01880	UniPAP7F, R	67	663	5'ctctcatatctacactcatcctagtc 3' 5'aatgaggatcggtgaagaacattgcc 3'
PAP8	At2g01890	UniPAP8F, R	52	455	5'gcctatcaaactcatattttctattttc 3' 5'gtaatacgcctctccaat3'
PAP9	At2g03450	Uni800F, R	65	232	5'atgatcgccgccgtttacac 3' 5'tgggtgaatcggagaggaat 3'
PAP11	At2g18130	AP18F, R	51	340	5'tgtttgcgctgcgattg 3' 5'ggtggcgtggtgaagataac 3'
PAP13	At2g32770	UniPAP13F, R	67	844	5'gtccttttcgtcattttcgcttcta 3' 5'ggttggaacattagccgtgagtg 3'
PAP17	At3g17790	AP29F, R	57.5	544	5'cgagtctgagtttgctgttgt 3' 5'acataagagttgcgagatggaac 3'
PLP7	At3g50920	AP34Fn, R	61	246	5'caaaaaaactttgaagattgatttgaa 3' 5'gttaccgtcattgctcctgcttc 3'

Table 2. Means of expression values of APase-encoding genes determined by GST-DNA macroarrays.

Gene name	-PR	-PS	+PR	+PS	FR	FS
PAP1		1.11 [a]		0.16 [b]		1.33 [a]
PAP2	1.10 [a]	1.21 [a]	1.15 [a]	0.82 [a]		
PAP3	1.25 [a]	0.16 [c]	0.12 [c]		0.54 [bc]	0.86 [ab]
PAP4	0.43 *ns*	0.73	0.18	0.20	0.69	0.96
PAP5		0.22 [a]				
PAP6						
PAP7						1.60 [a]
PAP8	0.53 [b]	1.30 [a]	0.18 [bc]	0.55 [b]		0.99 [a]
PAP9	0.71 [b]	0.50 [b]	0.39 [b]	0.28 [b]	2.22 [a]	1.96 [a]
PAP10	1.60 [a]		0.40 [b]	0.46 [b]		
PAP11	1.58 [a]		0.04 [b]		1.03 [a]	1.41 [a]
PAP12	0.60 [a]	0.18 [b]	0.27 [b]	0.18 [b]		
PAP13	0.31 [b]	0.12 [b]	0.20 [b]	0.10 [b]	1.52 [a]	2.05 [a]
PAP14	0.54 [a]		0.38 [a]	0.39 [a]		
PAP15	0.32 [b]	0.14 [c]	0.71 [a]			
PAP16	*ns*	0.06				
PAP17	*ns*	1.43		1.07	0.57	1.40
PAP18	0.06 [b]	0.63 [a]	0.29 [ab]	0.27 [b]		
PAP19	0.33 [ab]	0.22 [bc]	0.54 [a]	0.17 [bc]		
PAP20		0.20 [a]				
PAP21						
PAP22						
PAP23						
PAP24						
PAP25						
PAP26	0.99 [a]	0.65 [a]	0.66 [a]	0.48 [ab]		
PAP27	0.62 [a]	0.26 [b]	0.28 [b]	[c]		
PAP28			0.27 [a]			
PAP29						
PLP1						
PLP2	1.40 [a]	0.61 [bc]	0.97 [ab]	0.34 [cd]		
PLP3	0.62 [a]	0.23 [bc]	0.43 [ab]	0.38 [ab]		
PLP4		0.60 [a]	0.29 [ab]	0.52 [a]		
PLP5						
PLP6						
PLP7	0.84 [ab]		0.35 [c]		1.53 [a]	0.93 [ab]
PLP8			0.22 [a]			
PLP9	0.33 *ns*	0.17	0.06			
PLP10	0.36 [a]		0.38 [a]	0.25 [a]		
PLP11	0.48 [a]		0.12 [b]			
PLP12			0.07 [a]			
SAP1	0.73 [b]	1.44 [a]	0.87 [b]	0.60 [b]		
SAP2	0.34 *ns*	0.38	0.47	0.33		
HAP1	0.32 [a]		0.17 [ab]	0.19 [ab]		
HAP2	1.44 [b]	0.67 [b]	2.82 [a]	0.98 [b]	3.18 [a]	4.04 [a]
HAP3	0.44 *ns*		0.09			0.62
HRP1	1.42 [a]	0.65 [b]	0.39 [bc]	0.07 [c]		
HRP2	1.22 [a]	0.94 [bc]	0.50 [bc]	0.18 [c]		0.94 [ab]
HRP3	1.56 [a]	1.24 [a]	0.44 [bc]	0.19 [c]		0.99 [ab]
HRP4						
HRP5		0.33 [a]		0.24 [a]		
HRP6		0.44 [a]	0.21 [b]			
HRP7	0.71 [a]	0.38 [b]	0.47 [b]	0.37 [b]		
HRP8	0.24 [a]		0.10 [ab]	[b]		
HRP9		0.37 [a]	0.17 [b]	0.10 [bc]		
HRP10	0.68 [a]		0.51 [a]	[b]		
HRP11			0.27 [b]	0.31 [a]		
HRP12	0.19 [b]		0.60 [a]			
No. of expresses genes	33	31	40	28	8	14
Total expression values	24.98	17.19	17.13	16.77	6.59	20.11

Relative expressions values were computed by dividing the intensity values for spotted GSTs divided by that of alpha-tubuline gene. Abbreviations: S, shoot; R . root; -P, Pi starved; +P, Pi fed; F, Fungi treated. All treatments are as described in Materials and methods. All the values are the means for two biological replicates. Different letters indicate significant difference among treatments based on mean comparison by Duncan method at $P< 0.05$.

Table 3. Occurrence of known defense-related *cis*-elements within 1500-bp upstream of APase genes induced by fungal infection.

Gene Name	Locus No.	Region of Promoter Sequence on Genome	JA Responsive Elements	SA Responsive Elements	Other Defense Responsive Elements
HRP1	At1g04040	1043748-1045248	0	6	4
HRP2	At1g73010	27469771-27471270	2	11	6
HRP3	At1g17710	6091888-6093388	0	14	11
PLP7	At3g50920	1893544-18934444	0	8	6
PAP1	At1g13750	4718018-4719518	0	8	4
PAP3	At1g14700	5057105-5058605	0	12	9
PAP4	At1g25230	8842215-8843715	0	10	6
PAP5	At1g52940	19718640-19720140	0	16	4
PAP7	At2g01880	393896-395396	2	7	2
PAP8	At2g01890	399097-400597	2	9	6
PAP9	At2g03450	1040095-1041595	1	9	6
PAP11	At2g18130	7890947-7892447	0	13	6
PAP13	At2g32770	13901485-13902985	1	17	6
PAP17	At3g17790	6088355-6089855	2	8	7
HAP2	At3g01310	101540-103040	0	9	7

Jasmonic acid (JA) responsive elements: AACGTG, CACGTT
Salicylic acid (SA) responsive elements: GTCAA, CGTCA, WTTWYC
Other defense responsive elements: TGACA, ATCTTT, RGTCAAA

Promoter Analysis of the APase Genes Induced by Fungal Interactions

The presence and the frequency of known JA, SA and other defense responsive *cis*-elements deposited in PLACE database were analyzed within 1500-bp upstream promoter sequences of the fungi induced APase-encoding genes as presented in Table 3. Some of the APases promoters contain only one of the known JA-responsive motifs, AACGTG. In contrast, numerous recurrences of three SA-responsive elements were found in the promoters of APase genes (a total of 579 elements). Interestingly, these promoter sequences are extremely enriched for two motifs, GRWAAW (S000198; 438 elements) and TTGAC (S000390; 102 elements). However, we found no correlation between the frequencies of the defense-related motifs and the expression levels. For instance, the promoter of three highly expressed APases by fungi treatment, At*hap*2, At*pap*9 and At*pap*13, carry 16 to 24 defense responsive motifs, whereas a repressed gene such as At*sap*1 carry 26 defense related *cis*-elements.

Tissue-specific expression patterning of transformed plant with *Atpap9*-promoter:: GUS fusion construct revealed consistent results to RT-PCR and reverse northern blotting. As shown in Fig. 4, light and spotted patterns of expression of *uid*A gene under *Atpap9* promoter were detectable in the roots and shoots of Pi-starved and Pi-fed seedlings. However, when transgenic seedlings were inoculated with *A. brassicicola* uniform staining of shoot tissues (but not roots) was observed. The staining was much more intense when the transgenic plants were infected with pathogenic oomycete *H. arabidopsidis*. Such a high level of expression was detectable all over the roots and shoots tissues.

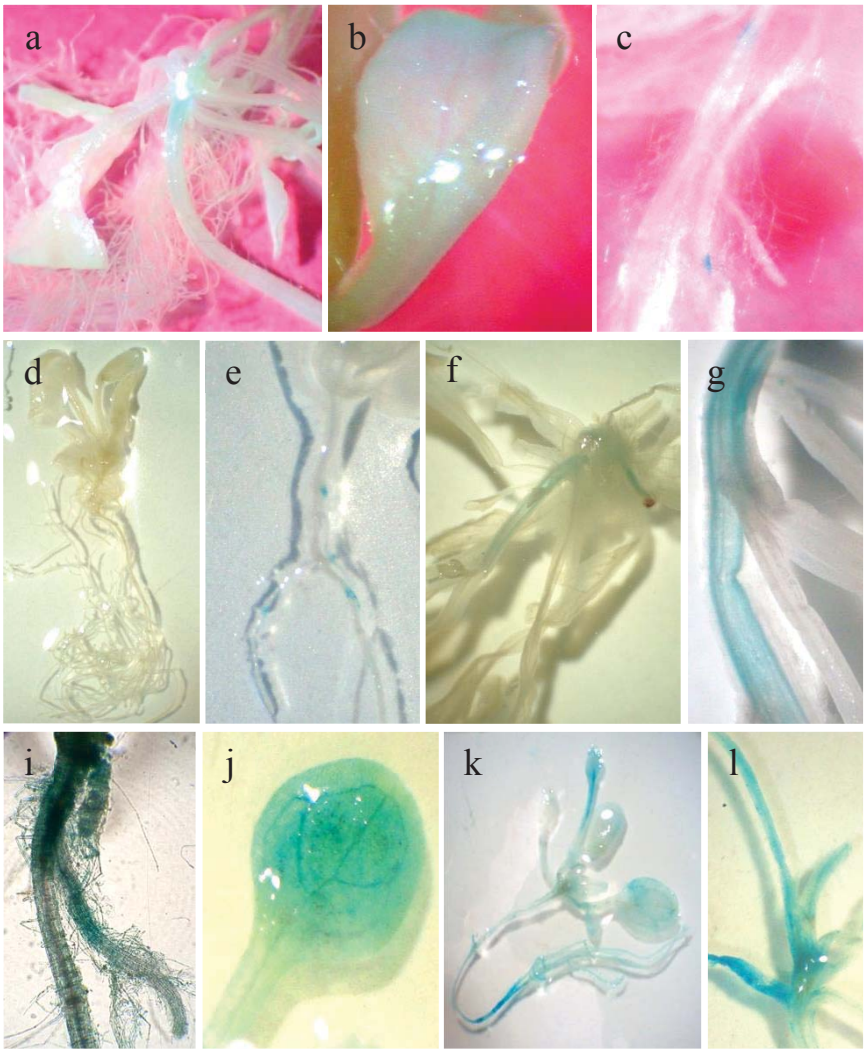


Figure 4. Tissue specific expression patterns of *Atpap9* promoter activity. Top, illustration of promoter-*uid*A fusion construct carrying 1830-bp promoter fragment of PAP9 gene cloned upstream of *uid*A gene in T-DNA segment in pAM194 binary expression vector. Bottom, histochemical GUS staining of transgeneic *A. thaliana* plants carrying the above construct. Light staining is visible in untreated Pi-fed shoots and roots (a-c) and Pi-starved shoots and roots (d-e) while shoots and roots of plants inoculated with *A. brassicicola* (f-g) and *H. arabidopsidis* (i-l) were stained intensely. All plants were cultured on solid medium as described Material and methods.

Discussion

Hydroponically cultured *Arabidopsis* seedling is a simple experimental system for examination of nutrients effects. When seedlings were subjected to Pi starvation for 7 days, total P content was reduced considerably in both roots and shoots. While the level of soluble Pi was also reduced, it was higher root than in the shoots in the starved. Apparently, high APase activity leads to higher rates of Pi remobilization in the roots. Increased APase activity in the Pi-starved roots and shoots was consistent with previous reports (Duff et al., 1989, Goldstein 1992, Green et al., 1994, Lohrasebi et al., 2007). Since a long time ago, it is known that the Pi level is highly controlled in cytoplasm and organelles. As shown by Mimura et al. (1990), the concentration of Pi in the cytoplasm is regulated within very narrow limits. This is more pronounced in chloroplasts since Pi is critical for photosynthesis (Lee and Ratcliffe., 1993). In contrast, it varies widely in the vacuoles depending on available Pi (Mimura et al., 1990). This implicates that compartmentalization in the vacuoles is a mechanism for rapid adjustment of Pi level in cytoplasm to reduce the impact of variations on Pi availability on cell metabolism. Here, we have shown that a homeostatic level of soluble Pi is kept within a narrow range, 0.5 to 5 nmole per gram fresh weight in the roots and shoots.

Higher APase activity could be due to increased expression levels of the same set of genes rather than higher number of expressed APase-encoding genes. As experienced, the highest numbers of APase genes are expressed in Pi-fed roots which include the same set plus seven more genes expressed in Pi-starved roots. However, the total APase activity of Pi-fed roots was about 7.5 units per mg protein while that was almost twice for Pi-starved roots (Fig. 1C). Of course, it is expected that distinct sets of APase types are expressed to release the required level of soluble Pi from certain sets of substrates when the available Pi level is varied.

In general, total APases activity corresponded well with collective expression values for the encoding genes in the root and shoots of treated seedlings. All the fungal-induced genes, except for At*pap*17, in the root and At*pap*7 and At*pap*11 in the shoots are also upregulated by Pi starvation condition. However, some genes, such as At*pap*7, At*pap*9, PAP11, PAP13 and PLP7 are expressed at higher levels in fungal infected plants than in Pi-starved ones. Whether these are the consequences of competitive nutrient consumption in the hydroponic culture or interaction between fungus and plant is still a valid question. Experimentally, we have found that all fungal-induced genes, except for At*hap*2 and At*plp*7, were affected by the addition of chitin as external elicitor. Also, chitin treatment led to upregulation of four other genes, At*pap*5, At*pap*8, At*hrp*1 and At*hrp*2. These discrepancies could be related to complicity of fungal infection as well as dosage of chitin.

Both DNA macroarray and RT-PCR methods revealed almost the same expression patterns for the induced genes (Tamaoki et al., 2008). Genes coding for At*pap*9, At*pap*13 and At*plp*7 in the root samples and At*pap*7, At*pap*9, At*pap*11, At*pap*13 and At*plp*7 in the shoots were highly transcribed after fungal inoculation. Despite providing excess Pi, almost all the induced genes had high expression levels in the Pi-starved tissues as well. However, since the expression levels differed greatly, one might rule out that these genes are induced in all stresses as general response. In addition, we have already monitored no detectable expression for the APase genes when plants are subjected to other abiotic stresses such as high salt, cold and heat stresses (Lohrasebi et al., 2007). For more than two decades, it is known that a number of APase genes are upregulated when plants are challenged with pathogens (Williamson et al., 1991, Beber et al., 2000, Jakobek and Lindgren, 2002, Petters et al., 2002, Stenzel et al., 2003). Different expression patterns suggest that the transcript levels of APase genes are regulated by distinct pathways. For example, *Arabidopsis* vegetative storage proteins (VSPs) with APase activity was rapidly induced by wounding, jasmonate or insect feeding on leaves (Berger et al., 1995, 2002, Stotz et al., 2000, Reymond et al., 2004). Among them, *vsp*1 (At5g24780) and *vsp*2 (At5g24770) genes exhibited different expression patterns in response to methyl jasmonate and Pi starvation while another VSP gene, At1g04040, responded to Pi starvation only. In this research, these three VSPs, named as *hrp*1 (At1g04040), *hrp*2 (At1g073010) and *hrp*3 (At1g17710), exhibited

higher induction by *A. brassicicola*.

Scanning the promoters of APase-encoding genes for JA, SA and other defense responsive motifs revealed that the regulatory regions of chitin/fungal induced genes are enriched for such elements, even though they are present in the other down-regulated APase genes too. Conceivably, the presence of other unknown *cis*-elements and interactions among the transcription factors would affect gene expression levels as well.

The presence of SA-responsive motifs in the At*pap*9 promoter could be responsible for higher expression when exposed to *H. arabidopsidis* biotrophic oomycete, as a pathogen, than to *A. brassicicola*, as a necrotrophic fungus. It is noteworthy that no activity was observed for At*pap*9 promoter::GUS fusion when transgenic plants were inoculated with other fungi such as *T. harzianum*, *R. solani* and *S. sclerotiorum* (data not shown).

In *Arabidopsis*, the APase-encoding genes have been well studied with respect to plant response to Pi starvation (Tran et al., 2010, Plaxton and Tran., 2011). Increased APase activity under Pi deficiency implies that these enzymes play major roles in retrieving Pi from soil as well as recycling it within plant. However, what are the possible roles of APases during plant-pathogen interactions remains to be shown. We may simply assume that there would be a competition for soluble Pi absorption when fungus is present. In such situation, we expect plant response to Pi starvation to occur which could be presumed only for total P and soluble Pi contents in the roots Nonetheless, the APase activities and expression levels were rather low in the infected roots. Besides, despite similar level of APase activity in the infected shoot and Pi-fed plants, the expression profiles for APase-encoding genes differed greatly. As already shown, APase genes may have multiple functions such as scavenging ROS accumulated during plant infection (Jones et al., 1994, del Pozo et al. 1999, Nimchuk et al., 2003, Mukherjee et al., 2009). Further elucidations are still needed.

Briefly, we have shown that the presence of fungus greatly affects the distributions of total P and soluble Pi in plant organs as well as the expression profiles of APase-encoding genes. Such alterations are extended to the levels and the locations of gene expression.

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