Differential expression of *Arabidopsis thaliana* acid phosphatases in response to abiotic stresses

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Abstract
The objective of this research is to identify Arabidopsis thaliana genes encoding acid phosphatases induced by phosphate starvation. Multiple alignments of eukaryotic acid phosphatase amino acid sequences led to the classification of these proteins into four groups including purple acid phosphatases (PAPs). Specific primers were degenerated and designed based on conserved sequences of PAPs isolated from plants, fungi and animals. RNA profiles of P_i-fed and P_i-starved A. thaliana roots were compared with two methods established for gene-family-directed differential display of pap genes. Having analyzed the differentially displayed fragments, seven pap encoding cDNA clones were isolated. One of the clones was a trans-splicing product of two genes that encodes an acid phosphatase carrying a zinc-finger domain. Six other clones were predicted to encode secretory phosphatases. Reverse-northern blotting and semi-quantitative RT-PCR revealed distinct expression patterns for each gene under diverse environmental conditions such as P_i starvation, high-salt concentration, cold shock, nitrogen and sulfur deprivation. The presented data can provide some clues for dissecting the possible roles of PAPs in P_i acquisition.
Keywords: Acid phosphatase; Purple acid phosphatase; differential display; gene expression; Arabidopsis thaliana; Abiotic stress.

INRTODUCTION

Phosphorous is one of the most essential macronutrients that contributes to the structure of key biomolecules such as DNA, RNA, phospholipids and phosphoproteins as well as energy transfer components such as pyrophosphate, ATP, ADP or AMP (Marshner 1995). It also plays vital roles in metabolic reactions, gene expression and signaling pathways (Marshner 1995).

In soil, high quantity of phosphorous is present in various forms of inorganic and organic compounds ranging from 400 to 1200 mg/kg (Rodriguez and Fraga 1999). Despite, plants only absorb soluble inorganic phosphate (P_i) which is the least readily available nutrient in the rhizosphere (Marshner 1995; Robinson *et al*., 1999). To deal with sub-optimal P_i concentrations in soil, a number of adaptive strategies have been evolved in plants. Recent genome-wide transcriptional analysis by the use of cDNA microarrays and oligonucleotide chips, have shown that about one third of the *Arabidopsis* genes exhibit altered expression patterns within the first three days of P_i starvation (Wu *et al*., 2003; Hommand *et al*., 2003; Misson *et al*., 2005). These include many genes encoding enzymes of nitrogen assimilation and carbon fixation indicating that cell rescue system is turned on when the P_i starvation is prolonged.

Among these, the up-regulation of acid phosphatases (Apases) is believed to be the primary means for the releasing, recycling and scavenging of P_i from both internal and external resources (Goldstein *et al*., 1988a and b; Duff *et al*., 1994). Purple acid phosphatases (PAPs) are a group of APases that catalyze the hydrolysis of a wide range of phosphate esters and anhydrides in plants, fungi and animals (Shenck *et al*., 2000a; Li *et al*., 2002; Flanagan *et al*., 2006). Structurally, plant PAP proteins are categorized to high molecular weight (HMW) and low molecular weight (LMW) phosphatases all of which share metal-ligating conserved sequences (Shenck *et al*., 2000a). The former is thought to be functional in homodimeric form and carries two β-α-β-α-β motifs in its carboxyl end; while the later is typically monomeric with a sequence similar to carboxyl segment of HMW proteins but carrying only one of the structural motif (Klabunde *et al*.,

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1996; Zhang *et al*., 1996; Huang *et al*., 1997; Mertz *et al*., 1997; Uppenberg *et al*., 1999). Comparison of primary sequences of PAP proteins from both eukaryotic and prokaryotic organisms has revealed five blocks with seven invariant residues required for metal coordination (**D**XG/G**D**XX**Y**/G**N**H(D/E)/VXX**H**/GHX**H**; bold letters represent metal ligating residues). Using metal-ligating conserved sequences as motifs, Li and colleagues (2002) reported the identification of 29 PAPs in *A. thaliana* complete genome sequence. Later, Zhu *et al*. (2005) compared the expression pattern of 28 PAP encoding genes in five organs.

In this research, we have established a couple of methods for gene-family-directed differential display (DD) with the use of degenerate primers designed based on PAP proteins conserved sequences. Using these methods, we have reported the identification of PAP genes that are responsive to environmental conditions, particularly P_i starvation.

MATERIALS AND METHODS

Plant materials, culture conditions and treatments: The seeds of *Arabidopsis thaliana* ecotype Col-1 were obtained from *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, Ohio). Seed sterilization and hydroponics growth conditions were as described by Malboobi and Lefebvre (1997). Prior to the treatments, 14-day old seedlings were transferred into 15 ml of half-strength MS medium (Murashig and Skooge, 1962) supplemented with 1% sucrose and incubated for two days to achieve identical nutritional states. For all investigated stresses other than P_i starvation, the initial concentration of KH_2PO_4 was kept at 5 mM. For P_i deprivation, 5 mM KCl replaced KH_2PO_4 in half-strength MS medium. For high-salt treatment, NaCl solution was added to a final concentration of 100 mM, a sub-lethal concentration for *A. thaliana* seedlings (Saleki *et al*., 1993). For minus nitrogen treatment, KNO_3 and NH_4NO_3 were replaced by equal molarity of KCl. For medium lacking sulfur, equal molarity of $MgCl_2$ was added in place of $MgSO_4$. Cold treatment was applied by incubating 27 days plants at 4°C for 24 hr. For elicitor treatment, 28 days plants were incubated in the 20 ml fresh medium containing 100 µg/ml chitin for 4 hrs. In all cases, the culture medium was refreshed twice a week. All plants were harvested after a 14 day treatments; except for plants grown without nitrogen which were harvested after 9 days of treatment due to onset of severe deprivation symptoms. All tissue samples were stored at -70°C.

Database search and clustering of acid phosphatases: Initially, a key word search was performed using the phrase “acid phosphatase” against Swiss-PROT and PIR databases. Second , PAP1 (GenBank Acc. No. ATU48448) protein sequence isolated from *A. thaliana* Var. Landsberg erecta (Patel K.S., Lockless S.W and McKnight T.D. unpublished data) was used for BLASTP search in the above databases using the default setting. Sequences with identities above 95% were deleted, a total of forty amino acid sequences of Apases isolated from plants, fungi and animals’ species were compilied by the end of year 1998. The retrieved Apase amino acid sequences were aligned using several softwares including ClustalW 1.8 (http://www.ebi.ac.uk/clustalw/) method with BLOSUM matrix. For clustering, the alignment outcomes were imported to PhyloDraw 0.8 software (http://pearl.cs.pusan.ac.kr/phylodraw/) and analyzed with the use of neighbor-joining algorithm.

Primer design: The protein alignment was used to identify the conserved motifs, that were selected to design degenerate primers PAF1, PAF2 , PAF3 and specific primers P1 and P2 (Table 1). Specific primers for the identified PAP genes were designed within the unique sequences of each one (Table 1). To sort out the unique parts, the predicted open reading frame nucleotide sequences were searched against *Arabidopsis* genome database and segments with over 75 percent similarities were deleted.

RNA extraction and differential display: Total RNA was isolated from treated and non-treated *A. thaliana* roots as described by Chirgwin and colleagues (1979). 20 µg of total RNA was reverse transcribed in a 20-µl reaction containing 50 units of Expand reverse transcriptase (Roche Biochemical, Mannheim, Germany), 40 units of RNase inhibitor (Roche Biochemical) and 40 pmole of either P2 or oligo$(dT)_{15}$ primers which was incubated at 42°C for 2 hrs. Two optimized methods used for gene-family- directed differential display were as below:

1) *Low-stringency DD:* PCR amplification of cDNA molecules synthesized with oligo$(dT)_{15}$ primer was performed using three combinations of degenerate and specific primers including PAF1and P2, PAF2 and P2 or PAF3 and P2 pairs. PCR reaction was composed of

0.1 µl of cDNA products, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, 10 pmole of each primer, 1X *Taq* polymearase buffer and 1 unit *Taq* DNA polymerase (Cinnagen, Tehran, Iran) in a total volume of 20 µl. The first PCR program was 4 cycles of 1 min denaturation at 94°C, 1 min annealing at 45°C and 1 min extension at 72°C. This was followed by the second PCR program which was 1 min denaturation at 94°C, 1 min annealing at 50°C, 1 min extension at 72°C for 36 cycles and a final extension at 72°C for 5 mins.

2) *Slow-ramping DD:* two-step PCR amplification of cDNA molecules synthesized with P2 primer was performed with the above combinations of degenerate and specific primers. The initial PCR reaction was consisted of 0.2 µl cDNA products, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, 20 pmole of each primer, 1X *Taq* polymearase buffer and 0.5 unit *Taq* DNA polymerase in a total volume of 20 µl. The first round of PCR reaction was carried out for 30 cycles with the following program: 1 min denaturation at 94°C, ramping from 94°C to 40°C at the rate of 8 sec/°C, 1 min annealing at 40°C and 1 min extension at 72°C. After addition of 10 µl solution containing 0.25 mM of each dNTP, 1X *Taq* polymearase buffer and 0.5 unit *Taq* DNA polymerase, the second round of PCR was performed for 20 cycles of 94°C for 1 min, 50°C for 1 min (without slow ramping) and 72°C for 1 min proceeded. The amplified DNA fragments were separated on 1% agarose gel and stained with ethidium bromide.

Cloning and sequence analysis: Differentially displayed bands were cut and eluted by the use of Agarose gel DNA extraction Kit (Roche Biochemical). Purified cDNA fragments were cloned into pTZ57RT vector supplied in T/A cloning Kit (Fermentas, Vilnius, Lithuania) and recombinant plasmids were used to transform competent E. coli strain DH5α cells (Sambrook, Fritsch and Maniatis 1998). Specific features of the sequenced PAP clones were predicted by the use of online databases and relevant software including Pfam (Bateman *et al*., 2000), Prints (Attwood *et al*., 2000), Blocks (Pierrokovski *et al*., 1996), SMART (Schultz *et al*., 2000; Letunic *et al*., 2002), InterPro (http://www.ebi.ac.uk/InterProScan/) and PSORT (Nakai and Horton, 1999).

Reverse-northern blotting: Reverse-northern blotting was performed following the procedure of

Table 1. A list of primers used for gene cloning and semi-quantitative RT-PCR.

Primer name[1]	Primer sequence	Protein motif [2]	Annealing temperature
PAF1	[GA], A[CT], ACN, A[AC]N, TA[TC], TA[TAC]TA	[DN]T[KT]Y[YI]Y	–
PAF2	GGN, GA[TC], [CAT][TA]N, [AT][GCT][TC], TA[TC], [CT]C	GD[LIN][SF]YAD	–
PAF3	[AGC][CTN], [GCCN], GGN, AA[TC], CA[TC] GA	[TALML][AP]GNH[IAH][DE]	–
PAF4	[AT] [AG], N[TGC] [CAT], [AG]GT, NAC, [AG]TG, NCC	[GS]HV[HD][AS]YE	–
P1	GGAGA C TTG TCT TTA CGC G	GDLSYA	–
P2	ATA GGC ATG AAC ATG ACC	GHVHAY	–
UPAP9F	ATGATCGCCGCCGTTTACACTCTCTTC		56°C
UPAP9R	TACAAACCACTATACCCTACAATATGT		
UPAP12F	CGAACCTTCCCCAAGTATCCCAT		55°C
UPAP12R	CCAGATGACATGCCACTAGACAGCG		
UPAP18F	GAAGGATCCTCGAGTAAAGCTCGCAGAGATGGAAA		68°C
UPAP18R	GTCAAGCTTGAATTCCCCCAAACGTGTCCCACTTA		
UPAP5F	CAGGTCGCTCCACTAGACAATTCAACT		58°C
UPAP5R	CCTATGCTTTGGGCGTAATCTATCT		
UPAP26F	GAAGGATCCTCGAGTATAGGCGATATGGGTCAGACATTC		62°C
UPAP26R	GTCAAGCTTGAATTCCAGCGTACCAAAGAGGACTGC TAC		
E7F	TAGGAATTTCTTGTTAGTGCTTCTA		58°C
E7R	CCGAGACAAGTGTTGGATTTGAGAGT		
TubR	CATCGTACCACCTTCAGCAC		56°C
TubF	GCTTTCAACAACTTCTTCAG		

[1] PAF primers are degenerates and the others are gene specific ones. Letter U shows primers designed to amplify the unique sequences of each gene.
[2] Amino acid motifs corresponding to degenerate and specific Apase primers.

Mohsenzadeh and colleagues (2005). Duplicate H^+ nylon membranes (Roche Biochemical) were prepared by placing dots of reamplified, alkaline-denatured DNA inserts of the isolated clones. To synthesize labeled cDNA molecules, 10 μg heat-denatured total RNA isolated from P_i-fed and P_i-starved plants were added into cDNA synthesis reactions consisted of 80 units of Expand reverse transcriptase, 50 mM Tris-HCl, pH 8.5, 8 mM $MgCl_2$, 150 mM DTT, 1.25 units/μl RNase inhibitor, 0.5 mM dNTP mixture, 0.13 mM dTTP, and 0.07 mM DIG-dUTP (Roche Biochemical) incubated at 42°C for 2 hrs. Prehybridization, hybridization, stringency washes, and detection steps were carried out according to the DIG Labeling and Detection Kit instructions (Roche Biochemical) using hybridization buffer composed of 7% SDS, 0.25 M NaH_2PO_4, 1 mM EDTA. The intensity of each dot was quantified by TotalLab software (Phoretix International, Tyne House, Newcastle, UK). All data were normalized by dividing the given volume value for each dot by that of alpha-tubulin. The relative expression level was calculated as the ratio of dot intensities hybridized with labeled cDNA molecules derived from P_i-starved plants by those of the P_i-fed ones.

Semi-quantitative RT-PCR: In order to evaluate the transcriptional responses of the isolated pap genes to P_i deprivation and other stress conditions, a series of semi-quantitative RT-PCR were conducted. For all samples, the same amounts of total RNA (10 μg) were used in reverse transcription reactions. Pairs of specific primers that anneal to the unique sequences of pap genes and alpha-tubulin were used (Table 1) in the PCR reactions. PCR reactions were performed in a solution of 50 mM KCl, 15 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 0.25 mM dNTP mix, 2 pmole of each primer, 0.1-1 μl cDNA and 1unit *Taq* DNA polymerase (CinnaGen) on a thermocycler (Perkin-Elmer, GeneAmp 9600) at the appropriate annealing temperature (see Table 1). Prior to PCR amplification of Arabidopsis PAPs, the cDNA content of all reverse transcription reactions were normalized by amplification of alpha-tubulin transcript using primers TubF and TubR (Table 1). Ethidium bromide stained gel images were analyzed by TotalLab software (Phoretix International) to obtain numeric data representing the band intensities. All calculations were as mentioned above and performed on Microsoft Excel software.

RESULTS

Classification of acid phosphatases: In the first attempt, we tried to retrieve as many Apase protein sequences as available by the year 1998. At this stage, the main goal was to identify the conserved motifs in eukaryotic Apase sequences for subsequent primer design. Removing redundant sequences with more than 95% identities, 40 Apase sequences were compiled by databases searches, 6 from plants, 19 from animals and 15 from fungi. Multiple sequence alignments by Clustal W led to classification of Apases into four groups: group 1, mainly animal histidine phosphatases (15 sequences); group 2, fungi histidine phosphatases (9 sequences); group 3, high and low molecular weight PAPs (12 sequences) and group 4, class B acid phosphatases (3 sequences).

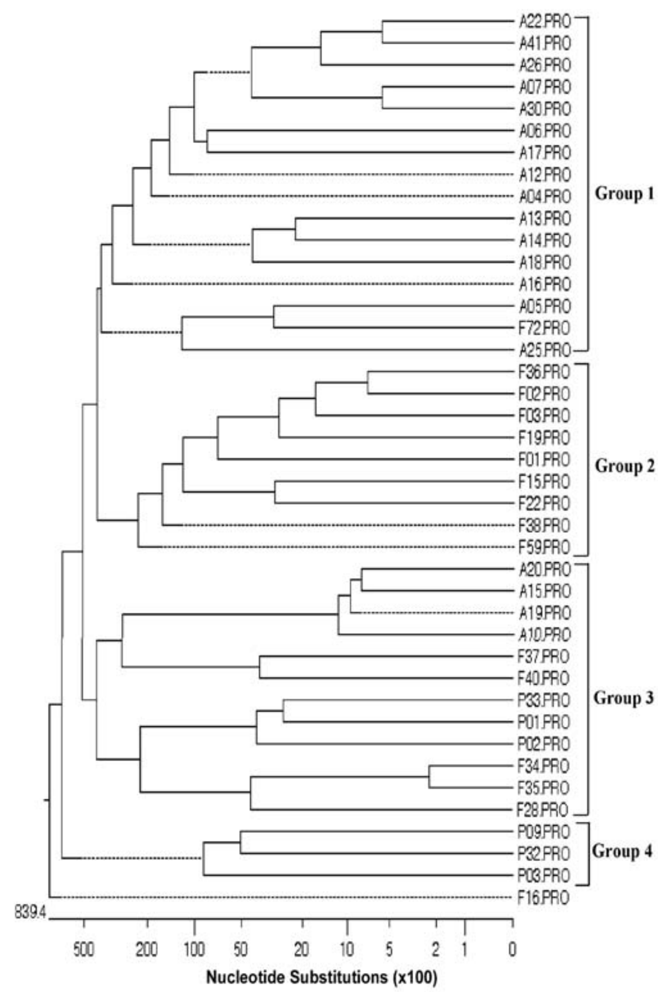

Figure 1. Classification of APase proteins based on multiple alignments of the available amino acid sequences with ClustalW method using BLOSUM30 matrix. Four major homology groups are animal histidine phosphatases, fungi histidine phosphatases, low and high molecular PAPs and class B acid phosphatases.

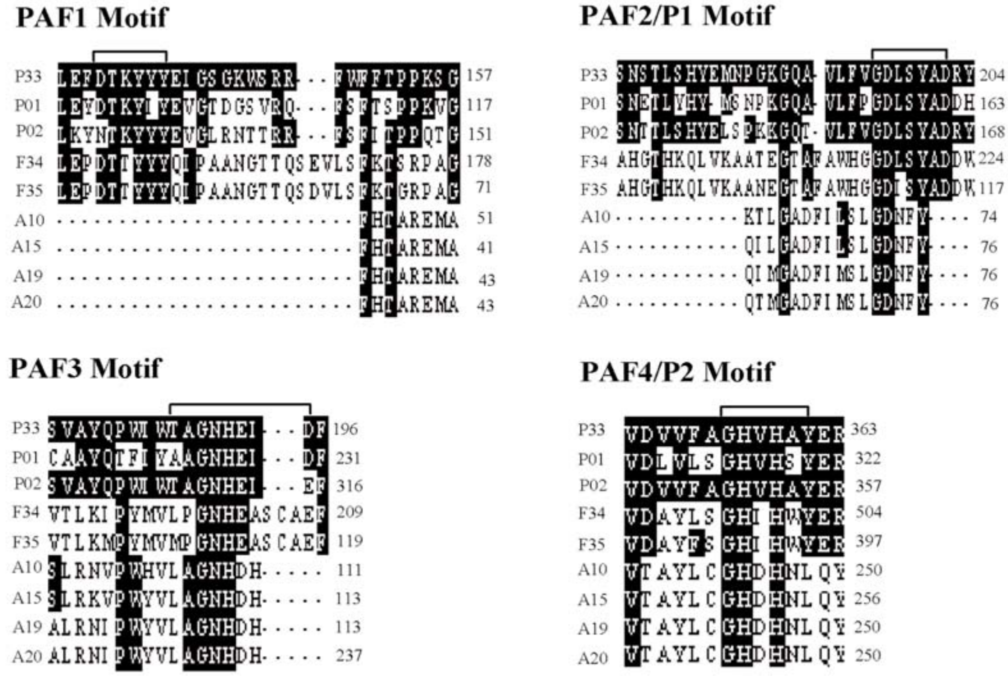

Figure 2. Amino acid conserved sequences of animal (A), fungi (F) and plant (P) PAPs. Sequences used for designing of degenerate primers PAF1, PAF2 and PAF3 and specific primers P1 and P2 are shown by brackets. Blocked residues are identical to the sequence of P33 which is a PAP protein isolated from *A. thaliana* ecotype Landsberg erecta.

The alignments of the PAP sequences (Figure 1, group 3) revealed at least four conserved motifs with some invariant amino acid residues (Figure 2). A part of PAF1 motif was used for degenerate primer design (Table 1) which is located at N-terminal sequence of plants and fungi PAPs. This motif is also a part of fibronectin type III domain commonly found in many plant and animal proteins as well as extracellular enzymes of soil bacteria (Tsyguelnaia and Doolittle, 1998). Three other degenerate primers corresponded to the short sequences of PAF2, PAF3 and PAF4 motifs (Table 1, Figure 2) known to contain metal- ligating residues (Schenk *et al*., 2000a). P1 and P2 specific primers were designed based on nucleotide sequences of PAP1 gene encoding GDLSYA and GHVHAY motifs that are highly conserved among plant PAPs (Figure 2).

Differential display of PAP family members: In order to identify PAP genes preferentially expressed in *Arabidopsis* plants in response to long term harsh P_i stress, two methods for gene-family-directed DD were established to illustrate the expression profiles of a subpopulation of transcripts encoding PAP family members. In both methods, total RNA samples were prepared from both P_i-fed and P_i-starved roots after 14 days of treatments. For the first method, the starting material was cDNA molecules prepared by reverse transcription with oligo(dT)$_{15}$ primer that were used in the PCR reactions conducted at low stringency. As shown in Figure 3A, only a few bands were amplified with this method. Four combinations of degenerate and specific primers yielded five differentially expressed cDNA fragments. For the second method, reverse transcription was carried out with a PAP-specific primer (P2) proceeded with slow ramping (8 sec/°C from 95°C to 40°C) using pairs of degenerate and gene-specific oligos. After addition of fresh Taq DNA polymerase, another round of PCR was performed at higher annealing temperature. With this method, an average of 8-12 bands per lane was displayed (Figure 3B). Six other preferentially expressed cDNA fragments in P_i -starved samples were identified with this method.

Sequence analysis of the identified PAPs: Differentially expressed fragments were cloned for further analysis. The partial cDNA sequences were searched against *A. thaliana* genomic sequence and expressed sequence tag (EST) databases. An inventory of relevant data for the isolated gene fragments is presented in Table 2. Only two fragments isolated by the

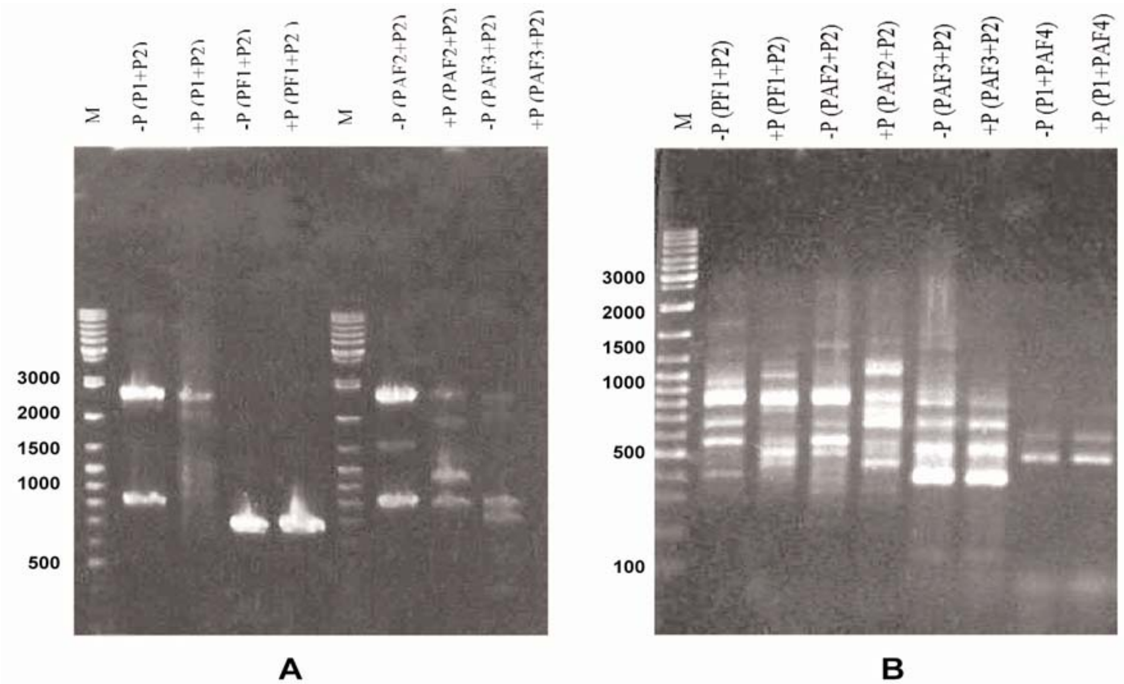

Figure 3. Typical images of differential display of PAP gene family amplified with PCR reaction at low stringency (A) or slow ramping (B) visualized by agarose gel. RNA samples were extracted from 14–day Pi-fed (+P) or P_i-starved (–P) *Arabidopsis* roots. After reverse transcription with oligo$(dT)_{15}$ (A) or P2 (B) primers, PCR reactions were performed with different combinations of degenerate and specific primers as shown in parenthesis. Lanes M are 100-bp ladder molecular weight markers.

first DD method were similar to the predicted *pap* genes whereas five fragments obtained through the second method were representing *pap* gene sequences. Those two fragments, L8 and M3, isolated by the first method correspond to *pap9* and *pap18* genes sequences, respectively. Four fragments, F1, G4, H5 and K6, isolated by the second method were almost identical to *pap5-1*, *pap11*, *pap12* and *pap26* genes, respectively, as named by Li and colleagues (2002). The full-length coding sequence of the identified pap genes predicted by AGI were double checked with EST and TC sequences available until year 2005 on TIGR database.

Interestingly, E7 fragment was found to be identical to a segment of *pap5-1* gene on chromosome 1 and a segment of a gene on chromosome 4 encoding an

Table 2. An inventory of available data for differentially expressed pap genes isolated from Arabidopsis roots that are responsive to phosphate starvation.

Clone Name	Gene Name	DD Method[1]	Chromosome No.	Locus No.	Predicted Transcripts[2]	Transcript size (bp)	Protein size (aa)	Relative expression[3]	Predicted cellular location
F1	*pap 5-1*	2	1	At1g52940	TC257811	1191	396	1.84	Secretory
E7	*pap 5-2*	2	1&4	–	*trans*-spliced	2646	880	3.00	Nuclear
L8	*pap9*	1	2	At2g03450	TC168270	2195	646	1.72	Membrane
G4	*pap 11*	2	2	At2g18130	TC160273	1377	441	4.44	Secretory
H5	*pap 12*	2	2	At2g27190	TC271923	1602	496	9.3	Secretory
M3	*pap18*	1	3	At3g20500	TC153375	1463	389	3.3	Secretory
K6	*pap 26*	2	5	At5g34850	TC261416	1823	475	3.6	Secretory

[1] DD methods are as described in Material and Methods.
[2] Tentative consensus (TC) stored in TIGR gene index representing both ESTs and predicted transcripts.
[3] Relative expression levels are the ratios of gene expression in P_i- starved roots in compare to P_i- fed roots as judged by reverse-northern blotting. Each value is the mean of 3 biological replicates.

unknown protein carrying a zinc finger domain, designated as *zf4*. The full length *trans*-spliced product, called *pap5-2*, is composed of 13 exons carrying a ~2120 bp open reading frame encoding an ~708 amino acid sequence. It is notable that there would be an early stop codon within the resulting mRNA of *pap5-2* gene unless a frame shift close to *trans*-splicing site is presumed.

The predicted protein sequence of the identified PAP genes carry all five blocks of conserved amino acid sequences encompassing seven metal-ligating residues as shown by Schenk and colleagues (2000). As well, fibronectin type III domain was found in the N-terminal parts of PAP5-1, PAP12, PAP18, and PAP26. Analysis with PSORT program collection indicated that all identified could be secretory proteins except for PAP9 and AP5-2. PAP9 sequence includes several hydrophobic regions that are common characteristics of membrane proteins. Search in Blocks and PROSITE databases showed the presence of C_2H_2 type zinc-finger domain and two nuclear targeting motifs within the N-terminal sequence of PAP5-2 both of which are indicative of being a nuclear protein.

Gene expression analysis: In order to illustrate and quantify the relative expression levels of the identified genes in various environmental conditions two approaches were taken:

1) *Reverse-northern blot analysis:* Reamplified cDNA fragments were arrayed on nylon membrane and hybridized to labelled cDNA molecules derived from total RNA extracts of P_i-fed and P_i-starved roots. It was shown that all seven cDNA clones were up-regulated considerably in response to long-term P_i starvation (Figure 4). After normalization of data against alpha-tubulin as constitutively expressed gene, relative levels of transcripts were calculated to be 2.5 to 4.6 times higher in P_i-starved roots in compare to P_i-fed ones (Table 3). It is noteworthy that it is not possible to differentiate the expression level of *pap5-2* from *pap5-1* gene and *zf4* genes since both genes could be expressed.

2) *Semi-quantitative RT-PCR:* After reverse transcription, initial cDNA template level for each sample was adjusted with respect to alpha-tubulin expressed transcripts prior to semi-quantitative PCR amplifications. Primers were designed based on specific unique sequences of each gene to ensure distinctions among gene family members. In general, the relative expression levels measured by this technique were consistent with the results of reverse-northern blots (Table 3).

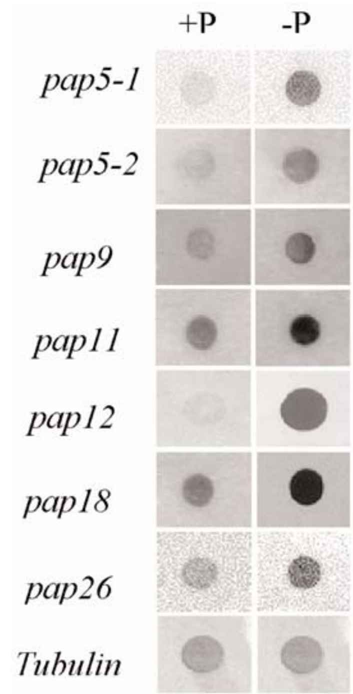

Figure 4. The illustration of responsiveness of the *pap* genes toward P_i starvation condition by reverse-northern blot analysis. After fixation of the cDNA fragments on nylon membrane, it was then hybridized with Pi-fed (+P) and Pi-starved (–P) total cDNA probes. *Alpha-tubulin* dots were used as internal controls.

Figure 5 illustrates comparisons among the expression patterns of the isolated *pap* genes in various environmental conditions. To calculate relative expressions in each condition, the band intensities were divided by that of the control plants which were grown in half-strength MS medium with sufficient amount of P_i (Table 3). *Pap5-1* gene was highly induced by P_i deprivation condition while it was repressed by other abi-

Table 3. Relative expressions of the identified pap genes in various environmental conditions measured by semi-quantitative RT-PCR.

Gene Name	Relative Expressions					
	– P/C	Cold/C	HS/C	Chitin/C	– S/C	– N/C
pap5-1	5.1	ND	ND	ND	ND	ND
pap5-2	6.3	ND	6.6	ND	ND	ND
pap9	6.8	3.5	3.2	5.9	2.0	1.0
pap12	3.0	2.00	0.6	0.49	0.5	0.5
pap18	2.6	ND	ND	ND	ND	ND
pap26	5.9	ND	1.0	ND	ND	ND

Relative expression was calculated as described in Materials and Methods. C, control plants grown in half strength MS medium with 5 mM P_i ; all other abbreviations are as mentioned in Figure 5; ND, not detected.

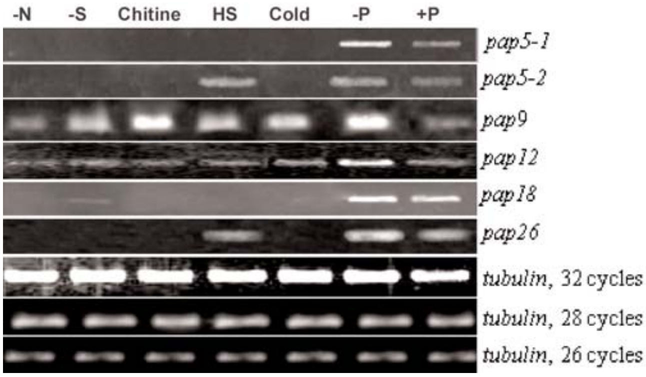

Figure 5. Differential transcriptional responses of **pap** genes toward different stressful conditions examined by semi-quantitative RT-PCR. Samples were taken from plants treated with no P_i (–P), 5 mM P_i (+P), no nitrogen (–N), no sulfur (–S) or 100 ìg ml–1 chitin (Chitin) and 100 mM NaCl (HS). Semi-quantitative RT-PCR assay was conducted as described in MATERIALS AND METHODS. In these experiments, the amplification of *alpha-tubulin* transcripts were used to normalize the cDNA template in PCR reactions (three bottom panels).

otic and biotic stresses. The transcript levels of *pap9* in all examined environmental conditions were higher than the control plants, except for the nitrogen starvation treatments. This was more pronounced in chitin-treated and P_i-starved plants. The expression of *pap12* was only increased in response to P_i starvation and cold stress, though it was constitutively expressed in all the other environmental conditions at low levels. In comparison, the expression of *pap18* gene was repressed in all environmental stresses except for the P_i-starved plants. The expression of *pap26* gene was repressed in all environmental conditions other than no-P_i and high-salt treatments. Interestingly, *pap5-2* transcripts were accumulated in these two conditions too. For this experiment, primers specific to 5′-region of *zf4* and 3′-end of *pap5-1* genes were used such that it is expected only *trans*-spliced products are amplified. We consistently obtained a 675-bp band when conducting RT-PCR with Pi-fed mRNA. However, Southern blotting of the RT-PCR products confirmed that this band is not related to *pap5-2* while the others are (data not shown).

DISCUSSION

Altogether, 59 Apases with divergent protein sequences were identified in Arabidopsis plant that could be classified into at least five major groups (Lohrasebi T. and Malboobi M.A., unpublished data). While 29 PAP protein sequences aligned well, they are barely aligned with the members of other Apase families.

The examination of gene expression patterns may provide some clues about the biological roles of PAP. Zhu and colleagues (2005) studied the expression profiles of *A. thaliana pap* genes in five organs using semi-quantitative RT-PCR method. Based on the clustered data, four groups of *pap* genes were recognized with respect to their expression in root, stem, leaf, flower and silique. Sixteen genes were constitutively expressed in all organs while others were expressed in one or more organs. Interestingly, all of the genes, except for *pap5-1*, were expressed in flowers. In another effort, Misson *et al.,* (2005) reported differential expression of 11 out of 27 *pap* genes represented on ATH1 DNA chip in response to either short term or long term Pi starvation stress.

Altogether, seven Pi-starvation induced cDNA fragments encoding PAPs, six secreted PAPs and one nuclear were isolated (Table 2). Both sequences of differentially-displayed DNA fragments and subsequent RT-PCR reactions with primers specific for the unique sequences verified the transcription of open reading frames predicted by *Arabidopsis* Genome Initiative (AGI). However, there was an error in recognition of the second exon-intron boundary of *pap5-1*. Other researchers have also reported such erroneous predictions in *pap7* and *pap8* coding sequences (Li *et al*., 2002). Despite using different sets of primers and RNA preparations, we were unable to amplify *pap11* transcripts while Li *et al (*2002) have shown that the expression of this gene is highly induced after 3 days of P_i starvation treatment. Also, Zhu and colleagues (2005) did not detect the expression of *pap5-1* in seven examined organs including roots of *A. thaliana* grown in pots, whereas both we (Figure 5) and Misson *et al* (2005) showed its expression in roots particularly when P_i was limited. These discrepancies could be due to growth conditions indicating complex patterns of Apase expression in distinct locations and in response to environmental conditions.

Li *et al* (2002) used specific primers to amplify the coding region of individual PAPs. They found that the transcript levels of *pap7*, *pap8*, *pap9*, *pap10* and *pap13*, were not affected after 1, 3 and 5 days growth in low phosphate medium while the expression of *pap11* and *pap12* were elevated. In our experience, the transcript levels of *pap5*, *pap9*, *pap12*, *pap18 and pap26* were highly induced after 14 days of P_i starvation. The discrepancy in these results could be related to the duration of P_i-starvation treatments. Consistently, we found that the expression of *pap12* increased proportionally after 6 hrs, 5, 8, 14 and 21 days of low phosphate treatment (data not shown).

We have also shown that several environmental conditions such as no sulfur, no nitrogen, high salt and cold affected the expression of the *pap* genes differentially (Figure 5, Table 3). The induction of Apases of other plant species in response to high salt has been shown as well (delPozo *et al*., 1999; Mimura *et al*., 2003; Parida and Das 2004). The expression data stored in TAIR website (http://www.arabidopsis.org/; Yamada, *et al*., 2003) also include the effect of several environmental conditions (but not P_i starvation). Based on these data, osmotic stresses (e.g. high salt, ABA, drought and monitol treatments) induce the expression of *pap5-1*, *pap12* and *pap18*. Exposure to UV-B increased the expression of *pap5-1* in root while repressed it in the shoot. Responsiveness to cold was exceptionally high (seven times) for *pap18* in one-hr treated plants grown on soil while no major effect was observed for long term-treated plants, nor for the other *pap* genes (Yamada *et al*., 2003).

The emerging data in recent years have signified the involvement of acid phosphatases in response to biotic stresses too. Jakbek *et al*. (2002) identified an Apase gene that accumulates during the hypersensitive reaction in bean. Similarly, Petters *et al*. (2002) identified an Apase expressed locally in potato leaves in response to bacterial infiltration. This gene is highly homologous to a P_i starvation induced Apase in tomato. We have also found that *pap9* expression was highly induced by chitin treatment as an elicitor (Figure 5).

Concluding Remarks

Why plants have so many Apases is still an interesting question that requires accumulative efforts for compilation of data describing the structural features, expression patterns, intermolecular relations, sub-cellular localizations and biochemical characteristics that lead to exploration of functions and roles of the Apases. Three independent *Arabidopsis* mutants defective in Apases activity have already been characterized. *pho3* with reduced Apase activity phenotype is also defective in various Pi deficiency responses (Zakhleniuk *et al*., 2001). Reduced Apase activity on the root surface but not in whole cell extract of *pup1* is indicative of restriction of Apase activites to certain locations (Trull and Deikman 1998). Tomscha *et al*. (2004) have introduced an *Arabidopsis* mutant, *pup3*, with 25 to 49 percent APase activity reduction mainly related to PAP26 and PAP12 isozymes. Based on the position of mutation locus, they suggested possible defect in *pap26*. As the transcript level of *pap12* was not affected, they presumed posttranscriptional modifications in a ground of mutual relations among Apases could be the cause of observed phenotypes. The availability of T-DNA insertional mutant (Pan *et al*., 2003) and gene-specific silenced lines (Hilson *et al*., 2004) are other great resources for functional analysis of Apases. Overexpression of the isolated Apases genes, mutation phenotyping and complementation of the mutants would pave the way for understanding the distinct roles of Apases in P_i homeostasis in plants.

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