Advances in Molecular Biology (1-2): 37-42, 2009
İstanbul Kültür University, Printed in Turkey
www.advmolbiol.org

Heat stress and wounding response in expression and RNAi impact on *Arabidopsis thaliana* soluble pyrophosphatase isoforms

Z. Neslihan Ergen[1]*, Steffen Greiner[2] and Thomas Rausch[2]

[1] Istanbul Kültür University, Faculty of Science and Letters, Department of Molecular Biology and Genetics, Atakoy Campus, 34156 Bakirkoy, İstanbul, Turkey

[2] Heidelberg Institute of Plant Sciences (HIP), Molecular Ecophysiology, Im Neuenheimer Feld 360, D-69120 Heidelberg, Germany

Abstract

Mature plants are assumed to lack cytoplasmic soluble pyrophosphatase activity and vacuolar membrane-bound proton-pumping pyrophosphatase is generally accepted as the sole enzyme responsible for the removal of pyrophosphate accumulated in the cytosol as a by-product of several biosynthetic pathways. On the contrary, *Arabidopsis thaliana* genome encodes six soluble pyrophosphatase isoforms, which were shown to be highly conserved both in nucleic and in amino acid sequences. There is only one isoform that is shown to be localized in plastids and there is no data available on the localization and/or possible expressional regulation of other isoforms. The analyses of the change in transcription of all *A. thaliana* soluble pyrophosphatases showed isoform-specific regulation, indicating the role of these isoforms during stress response. Furthermore, we have selected two isoforms through Digital Northern analysis derived from UniGene database and specifically knockdown these isoforms by RNAi approach. The results indicated that *A. thaliana* soluble pyrophosphatases have specific function *in vivo* and the loss of function of one or more isoforms can not be compensated by the function of others. Data obtained from this study indicates the possible critical role of soluble pyrophosphatases during plant development and stress response.

Key Words: Inorganic pyrophosphate, expression analysis by Real-time PCR, abiotic stress, mechanical wounding, double RNAi knockdown, Digital Northern analysis

* Correspondence author:
İstanbul Kültür University, Faculty of Science and Letters,
Department of Molecular Biology and Genetics
Atakoy Campus, 34156 Bakirkoy, İstanbul, Turkey
E-mail: n.ergen@iku.edu.tr

Received: June, 15, 2009, Accepted: June, 19, 2009

Introduction

Inorganic pyrophosphate (PPi) contains a pyrophosphate bond, which makes it an important molecule with respect to cellular bioenergetics. It is even speculated that PPi can be the predecessor of ATP as an energy currency during the early stages of biochemical evolution (Perez-Castineria *et al.*, 2001a; Perez-Castineria *et al.*, 2001b).

There is at least one PPi-utilizing step in major activation or polymerization reactions of nucleic acid, carbohydrate, protein, carotenoid, amino acid and fatty acid biosynthesis in the plant cell (Geigenberger *et al.*, 1998; Stitt, 1998; Rojas-Beltran *et al.*, 1999; Farre et al., 2001; Sonnewald, 2001; Lopez-Marques *et al.*, 2004). These steps are reversible and operate at near-equilibrium; therefore, the accumulation of pyrophosphate should be prevented to make biosynthesis thermodynamically irreversible (Geigenberger *et al.*, 1998; Lopez-Marques *et al.*, 2004). This is achieved by two structurally and functionally different pyrophosphatases (EC 3.6.1.1) that have been characterized to date; (i) membrane-bound proton-translocating inorganic pyrophosphatases and (ii)

soluble inorganic pyrophosphatases (sPPases) (Rojas-Beltran *et al*., 1999; Perez-Castineira *et al*., 2001b; Lopez-Marques *et al*., 2004).

Membrane-bound proton-translocating pyrophosphatases were shown to be localized on tonoplast and Golgi membrane in eukaryotes, the former being unique to plant kingdom (Perez-Castineira *et al*., 2001b; Lopez-Marques *et al*., 2004). They catalyze proton translocation from cytosol to the lumen, thus generate an electrochemical gradient that can be used for secondary active transport (Rea and Pole, 1993; Kim *et al*., 1994). Soluble pyrophosphatases localized in cytosol, plastids or mitochondria, in contrast, hydrolyze PPi and dissipate the energy simply as heat (Perez-Castineira *et al*., 2001b; Lopez-Marques *et al*., 2004). The activity of plastidial sPPase isoform is believed to be essential for plant metabolism, since PPi accumulating purine, pyrimidine and starch synthesis reactions occur in this organelle (Stitt, 1998). Measurements of PPi pool of mature spinach leaves indicated the presence of low PPase activity and high PPi concentration (200-300 μM) in cytosol, whereas plastids were characterized by high PPase activity and scarce concentration of PPi (Weiner *et al*., 1987; Stitt, 1998). Therefore, it is widely accepted that cytosolic sPPase activity in plants is mostly limited to young plants and dividing tissues where macromolecule synthesis occurs at high rates. In older tissues, the activities of sPPases are exclusively specific to plastids, and tonoplast-bound pyrophosphatase is the sole enzyme responsible from the removal of cytosolic PPi pool in mature plants (Weiner *et al*., 1987; Geigenberger *et al*., 1998; Stitt, 1998; Rojas-Beltran *et al*., 1999; Farre *et al*., 2000; Schulze *et al*., 2004). Interestingly, *Arabidopsis thaliana* genome encodes six sPPase isoforms (http://www.tair.org), only one of which was shown to be localized in plastids (Schulze *et al*., 2004). The significance of the presence of five different isoforms and their localization has not been studied.

Although PPi metabolism is a central and distinctive feature of plants, the role and significance of PPi in plant growth and development and the reason for tight control of cytosolic PPi concentration in plants are largely unknown. For a long time PPi was considered to be a waste product of anabolism, however, it is highly possible that it contributes to the flexibility of plant metabolism to respond to a changing environment. Provided that adequate PPi supply is present, substitution of ATP-utilizing reactions with PPi-utilizing steps under energy limiting conditions may allow ATP to be conserved and might improve the performance of the plant cell (Stitt, 1998). In order to understand isoform specific expressional regulation upon abiotic (heat) and mechanical (wounding) stress conditions, we used *A. thaliana* as a model organism. The effect of knockdown of sPPases on plant growth and development was observed by a double RNAi knockdown approach to selected isoforms by Digital Northern analysis.

Materials and methods

Plant growth

Arabidopsis thaliana (L) ecotype Columbia was used for all experiments. The growth of *A. thaliana* in hydroponic culture (25 mM H_3BO_3, 0,06 mM $CuSO_4$, 0,14 mM $MnSO_4$, 0,03 mM Na_2MoO_4, 0,001 mM $CoCl_2$, 0,1 mM $ZnSO_4$, 20 mM Fe-EDTA, 2 mM KNO_3, 1 mM $Ca(NO_3)^2$, 1 mM KH_2PO_4 and 1 mM MgSO4) was performed according to Noren *et al*. (2004). In short, *A. thaliana* seeds were surface sterilized and inoculated onto pipette tips filled with hydroponic culture solidified with 1 % plant agar. The pipette boxes were sealed and transferred to climate chambers [14 hours light (24°C), 8 hours dark (18°C), light intensity 125 μmol $m^{-2}s^{-1}$, 50 % relative humidity] after two days of incubation at 4°C. The seals were opened 10 days after transfer and the *A. thaliana* seedlings were grown until the age of 4 leaves under these conditions. The seedlings were then transferred to specially designed boxes that can hold 6 l of hydroponics solution. The plants were grown under these conditions without flowering up to six weeks with weekly renewal of hydroponics solution.

Stress treatments in hydroponic culture

Stress treatments of *A. thaliana* grown in hydroponic culture were performed five weeks after transfer to hydroponics. Before each treatment, the hydroponic culture was renewed. The heat treatment was performed

by incubating whole plants at 37°C for 2 hours and 16 hours of recovery at growth chamber conditions. The control and stress treated samples were collected both after 2 hours of treatment and after recovery.

For mechanical wounding, the rosette leaves were punched with a leaf-piercing device opening approximately 100 holes in 1 cm^2 area. The control and wounded leaf samples were collected after 1 and 5 hours of treatment.

Semi-quantitative Real Time PCR

Total RNA was isolated using RNeasy Plant Kit (Qiagen) and reverse transcription of 2 μg of total RNA was performed by Omniscript Reverse Transcriptase (Qiagen) according to the manufacturer's instructions. In order to prevent degradation of RNA, RNaseOUT (Invitrogen) was added to the reaction mixture. The real time PCR was prepared in 25 μl volume and performed using iCycler (Biorad). The annealing temperature was optimized for each gene specific primer pair (Asp1, Asp2A, Asp2B and Asp4 at 60°C, and Asp3 and Asp5 at 58°C). Following gene specific primer pairs spanning one intron sequence were used for amplification of *A. thaliana* sPPases; *Asp1* (5´-ACA ATC GGC TGT TTC GTT TC-3´; 5´-TTC CTT TAG TGA TCT CAA CAA CCA C-3´), *Asp2A* (5´-GAT TCT CTG CTT CGG TTT CG-3´; 5´-CAG TAG GAG CTT CTG GAC CAA TC-3´), *Asp2B* (5´-CAA ATG CTC TGT TTT CTT CTG C-3´; 5´-CCT TTG TGA TCT CAA CCA CCA C-3´), *Asp3* (5´-TGA GAT CTG TGC TTG CGT TT-3´; 5´-TGG GGC TTC AGG TCC TAT C-3´), *Asp4* (5´-CTC CAC ACT TTC CGC AAG AT-3´; 5´-ACT GGA GCT CCA GGT CCG-3´), *Asp5* (5´-GAG ACA AAC CAG CAA ACA AAG AC-3‘; 5´-AAA CAA AAT CCA AAT CCC AAT G-3´), and actin (5´-GGT AAC ATT GTG CTC AGT GGT GG-3´; 5´-CTC GGC CTT GGA GAT CCA CAT C-3´). The data normalized according to Muller *et al.* (2002) using actin expression as the reference.

Digital Northern analysis

Digital Northern analysis of *A. thaliana* sPPase isoforms was performed according to Koo and Ohlroge (2002) based on Unigene database (http://www.ncbi.nlm.nih.gov/UniGene).

Generation of RNAi1:2A constructs and stable transformation of *A. thaliana*

For genomic DNA isolation, 100 mg of grinded *A. thaliana* leaf material was suspended in 500 μl extraction buffer (200 mM Tris-HCl, pH 9, 400 mM LiCl, 25 mM EDTA, 1 % SDS) and extracted twice with phenol:chloroform:isoamylalcohol (25:24:1). The supernatant was precipitated with isopropanol and genomic DNA was collected by centrifugation at 15000 g for 10 min at 4°C. After drying, the pellet was resuspended in 500 μl TNE (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8) and total RNA was removed by RNaseA digestion. Genomic DNA was then precipitated and dissolved in TE buffer.

3'-UTRs of *Asp1* and *Asp2A* isoforms were amplified using following primer pairs containing Gateway compatible ends and BamHI/Spe1 digestion site; *Asp1* (5´-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG CTT CTC CTC AGA AGA TTT C-3´; 5'-GAA GGG ATC CAC TAG TGA ACT CTT CAA ACA AAT TAA ACC-3´) and *Asp2A* (5´-GTC GAC TAG TGG ATC CGA AGA AAC CAG TCC TTT TCC-3´; 5´-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG ATT GTA CTG GAA ATT TTT CAT C-3´). PCR products were digested with BamHI/SpeI, ligated together and cloned to the Entry vector pDONR201 (Invitrogen) via the BP reaction (Invitrogen) according to manufacturer's instructions. The reactions were transformed into *E. coli* XL1-Blue strain by electroporation and inserts were verified by DNA sequencing. The LR reaction (Invitrogen) was used to transfer inserts from entry clone pDONR201 into destination vector pB7GWIWG2(I) (Invitrogen). The products of LR reactions were transformed into *E. coli* DH5α strain and inserts were confirmed by restriction digestion (Figure 1). The constructs were then mobilized in *Agrobacterium tumefaciens* strain C58C1 containing Ti plasmid pGV2260 by electroporation.

The *A. tumefaciens* cells were grown in YEB medium supplemented with 100 μg/ml rifampicin, 50 μg/ml carbenicillin and 100 μg/ml spectinomycin at 28°C. The cells were collected by centrifugation at

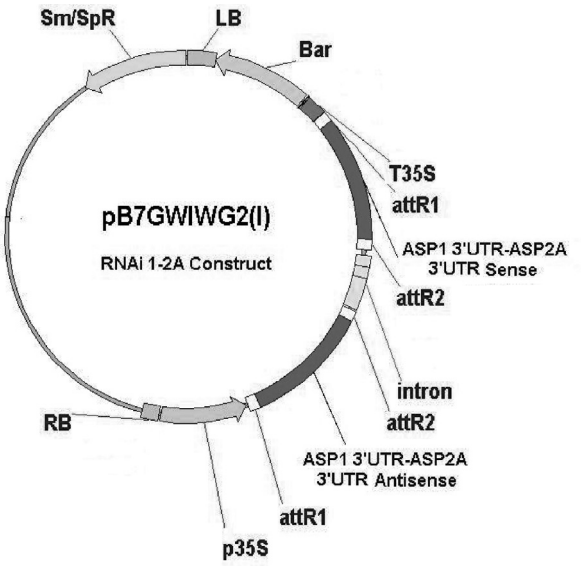

Figure 1. Scheme of pB7GWIWG2(I) vector carrying ASP1 3´UTR-ASP2A 3´UTR construct.

4000 g for 10 min and suspended in DIP-medium [½ MS, 5 % sucrose, pH 5,8, 10 ml/l BAP, 0,05 % Vac-In Stuff (Silwet L-77, Lehle Seeds)] to achieve OD600=0,9. The *A. thaliana* plants were transformed by floral dip according to Clough and Bent (1998). Positive plants from T1 generation were selected by BASTA™ screening (0,02 % v/v).

Conventional RT-PCR for double RNAi1:2A knockdown mutants

Total RNA for cDNA synthesis was isolated using RNeasy Plant Kit (Qiagen) and reverse transcription of 2 μg of total RNA was performed by Omniscript Reverse Transcriptase Kit (Qiagen) both according to manufacturer's instructions. In order to prevent degradation of RNA, RNaseOUT (Invitrogen) was added to the reaction mixture. Five hundred nanograms of cDNA and the following primer pairs were used for verification of double RNAi knockdown with conventional reverse transcription; *Asp1* (5´-TGG CAT GAT CTT GAG ATT GGA C-3´; 5'-CAC ACA ATG TGC GAG GAA CAA-3´) and *Asp2A* (5´-GGA TGT CCT GGT ACT GAT GCA GG-3´; 5´-GGT GAG GGG GAA GCT CTT TG-3´).

Results

The *A. thaliana* soluble pyrophosphatase family

The *Arabidopsis thaliana* genome sequence is available on the Arabidopsis Information Resource (TAIR) web-based interface (http://www.tair.org). Search of the database reveals that the *A. thaliana* genome encodes six soluble inorganic pyrophosphatase (sPPase) isoforms, one of which was shown to be localized in plastids (Schulze *et al.*, 2004). The *A. thaliana* soluble pyrophosphatase (ASP) isoforms are abbreviated as ASP1 (At1g01050), ASP2A (At2g18230), ASP2B (At2g46860), ASP3 (At3g53620), ASP4 (At4g01480) and ASP5 (At5g09650-the plastidial isoform).

The alignment of *A. thaliana* sPPase amino acid sequences indicates the high homology with respect to both amino acid (Table 1) and coding DNA sequences (data not shown). The main divergence of nucleotide sequences is observed at 5´- and 3´-UTRs.

Response of *A. thaliana* sPPases to heat stress and mechanical wounding

There is no data available in the literature with respect to the changes in expression of soluble plant pyrophosphatases upon different types of stresses. We analyzed the possible expressional regulation of *A. thaliana* sPPases upon heat stress and mechanical wounding by semi-quantitative Real Time PCR using six weeks old plants grown in hydroponics (Figure 2). It is important to note that the mean normalized expression levels of ASP2A, ASP2B, ASP3 and ASP4 in leaf and root tissues are very low; therefore, the changes in the percent expressions can be very high when the gene expression is induced with treatment even though the increased expression level is still comparable to actin expression.

Figure 2. shows that the induction of expression of ASP1 in heat-treated leaf tissues is observed only after recovery. The response in heat-treated root tissues is completely opposite to that of leaf tissues, where the 80 % induction in root tissue occurs after 2 hrs at 37°C and returns to initial level during recovery.

Table 1. The percent homology of *A. thaliana* sPPase isoforms based on amino acid sequence alignments performed according to Nikolaev *et al.* (1997). Note that the plastidial isoform (ASP5) sequence used in the alignment includes the signal peptide.

	ASP1	ASP2A	ASP2B	ASP3	ASP4	ASP5
ASP1	100	77	91	84	91	37
ASP2A	77	100	76	80	75	35
ASP2B	91	76	100	83	86	35
ASP3	84	80	83	100	83	35
ASP4	91	75	86	83	100	37
ASP5	37	35	35	35	37	100

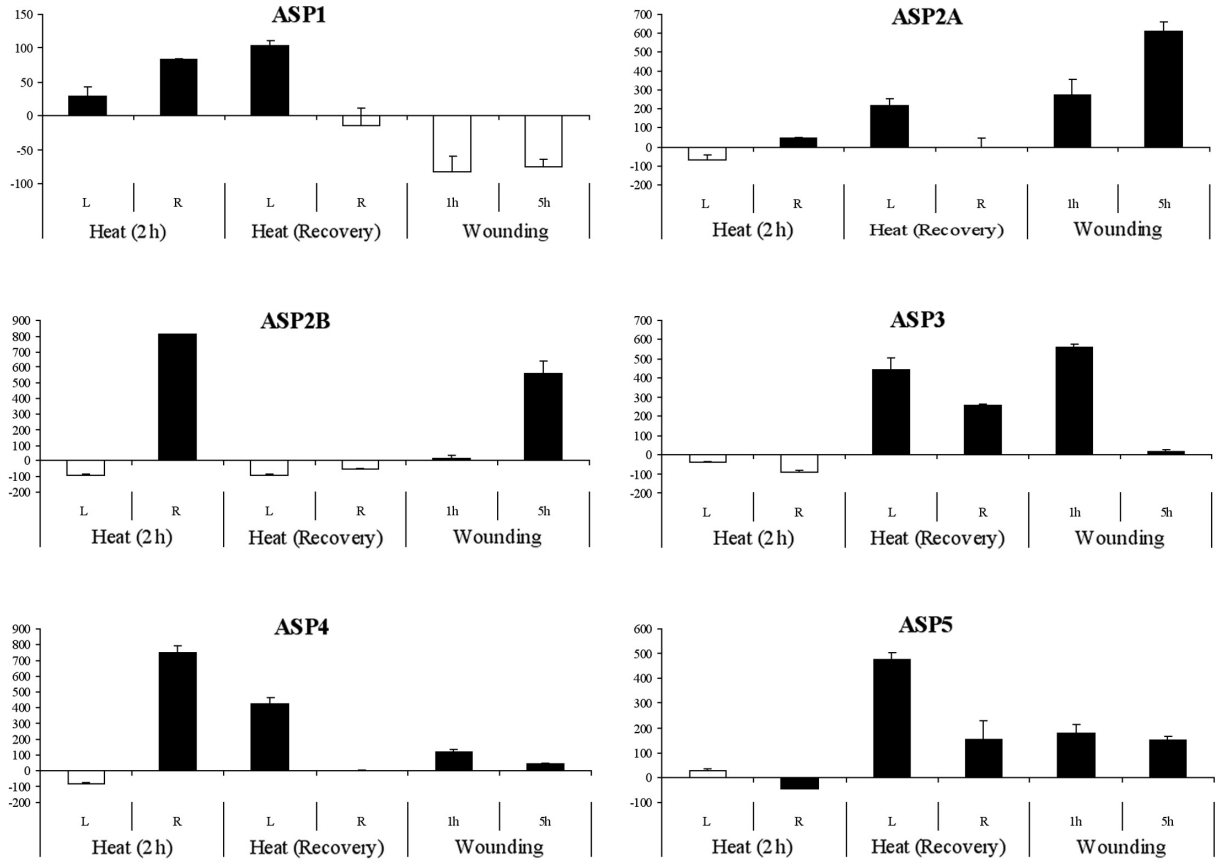

Figure 2. Percent change in the expression of ASPs in response to heat stress and mechanical wounding. The data normalized (Muller *et al.*, 2002) using actin as the reference gene and averaged from two independent experiments. Error bars indicate ± SD. L, leaf tissue; R, root tissue. Stress treatments were performed as follows: Heat (2h), 2 hours at 37°C; Heat (Recovery), 2 hours at 37°C and 16 hours recovery under normal growth conditions; Wounding; by wounding rosette leaves with 100 needle holes in 1 cm^2 area.

There is about 80 % decrease in ASP1 expression in response to mechanical wounding. The expression of ASP2A in leaf tissue is repressed by 70 % after 2 hrs at 37°C and induced by about 200 % during recovery. On the other hand, the 45 % induction in root tissue returns to initial level during recovery after 2 hrs of heat treatment. The wounding causes a significant induction in the ASP2A expression, which is observed to be increasing by time (274 % after 1 hr and about 600 % after 5 hrs). The gene expression of ASP2B is responsive to all stress treatments with distinct differences between leaf and root tissues. In the case of heat treatment, the difference between the response of leaf and root tissues is clearly visible after 2 hours at 37°C; 91 % decrease in gene expression in leaf tissue and almost 800 % increase in root tissue. The transient induction in gene expression in root tissue is diminished during 16 hours of recovery after heat treatment and the leaf and root responses becomes more close to each other with 90 % and 56 % decrease in gene expression, respectively. The expression of ASP2B is not affected during the first hour of mechanical wounding, whereas strongly induces after 5 hours (by approximately 550 %). The response of gene expression of ASP3 to heat stress is time-dependent; that is, after 2 hours at 37°C, both leaf and root expression is downregulated (by 40 % in leaf and by 90% in root), whereas during recovery the expression is significantly induced both in leaf (by about 440 %) and root (by 257 %) tissues. The wounding of leaves causes a transient induction in the transcript amount of ASP3 which is increased by 558 % one hour after wounding and returns to initial level after 5 hours. The changes in the ASP4 expression in response to heat stress and mechanical wounding are shown in Figure 2. The heat treatment causes a repression of gene expression of ASP4 in leaf tissue (by 81 %) and induces that of root tissue by about 750 %. During recovery after 2 hours at 37°C, the ASP4 expression in leaf tissue is up regulated by approximately 420 %, whereas the root induction after 2 hours returns to normal level of gene expression. Wounding transiently induces the transcript amount of ASP4, which is increased by about 120 % at the first hour of wounding and reduced to 43 % after 5 hours. The change in expression of ASP5 upon heat stress is clearly visible after 16 hours of recovery, where transcript amount is significantly increased both in leaf (by 475 %) and root (by approximately 150 %) tissues. The wounding induces the ASP5 expression regardless of the time frame, i.e., upregulation is 178 % after 1 hour and 151 % after 5 hours of wounding.

RNAi-mediated knockdown of *A. thaliana* sPPase isoforms 1 and 2A

The selection of *A. thaliana* sPPase isoforms to be used in RNAi approach was based on the Digital Northern analysis (i.e., the abundance of ESTs showing homology to ASP isoforms in each library pool) (Koo and Ohlrogge, 2002) using data downloaded from UniGene database (http://www.ncbi.nlm.nih.gov/UniGene). The results are given on Table 2. The mostly encountered *A. thaliana* isoform was ASP5 (plastidial isoform; by 24 ESTs out of 196000; Table 2), however, since the activity of the plastidial isoform is essential for plant growth (Weiner *et al*., 1987; Farre *et al*., 2001), it was not used in functional knockout approach. The other significantly expressed *A. thaliana* sPPase isoforms were ASP1 (by 21 ESTs out of about 196000) and ASP2A (by 16 ESTs out of 196000) according to Digital Northern analysis. Therefore, both of the isoforms were selected to be used in RNAi approach.

The intention was to be able to specifically knockdown both isoforms with a single transformation. In order to achieve that we focused on 3'-UTRs of selected isoforms considering high similarity of the coding sequences of *A. thaliana* sPPase isoforms (Table 1). The 3'-UTRs of ASP1 (184 bp) and ASP2A (176 bp) were amplified from genome with the addition of an artificially created restriction digestion site (BamHI/SpeI) and ligated together as a single construct (Figure 1). After three independent transformation trials of RNAi1/2A construct to *A. thaliana*, only two T1-lines (R1 and R2) with successful knockdown of transcripts of both ASP1 and ASP2A isoforms were obtained (Figure 3).

Figure 4 shows the seedlings of the T1 generation R1 and wild type *A. thaliana* germinated on non-selective plates either horizontally or vertically ten days after germination. The comparison of seedlings

Table 2. The percent homology of *A. thaliana* sPPase isoforms based on amino acid sequence alignments performed according to Nikolaev *et al.* (1997). Note that the plastidial isoform (ASP5) sequence used in the alignment includes the signal peptide.

			ASP1		ASP2A		ASP2B		ASP3		ASP4		ASP5	
Library	Total Number of ESTSs	% Total EST	#ESTs	%	#ESTs	%	#ESTs	%	#ESTs	%	#ESTs	%	#ESTs	%
Mixed	36.396	18.6	8	0.0	7	0.0					2	0.0	9	0.0
Flower buds	5.827	3.0	1	0.1										
Root untreated	23.023	11.7	4	0.0	3	0.0					4	0.0	3	0.0
Siliques	13.436	6.9	1	0.1	2	0.0			1	0.0	1	0.0	3	0.0
Not known	8.049	4.1	2	0.1										
Seedling	4.552	2.3	2	0.2					5	0.1				
Rosette dehydration	7.025	3.6	3	0.1	1	0.0							1	0.0
Whole plant stress	184	0.1			1	0.5			3	1.6	3	1.6	1	0.5
Rosette tissue	2.723	1.4			1	0.0			2	0.1	1	0.0	1	0.0
Inflorescence	2.478	1.3			1	0.0								
Silique and flower	3.404	1.7					2	0.1						
Rosette cold stress	5.102	2.6									1	0.0		
Leaf senescence	6.164	3.1											1	0.0
Leaf untreated	1.714	0.9											1	0.1
Leaf infected	3.719	1.9											3	0.1
Germinating seeds	12.424	6.3											1	0.0
Others	72.205	36.8												
Total	196.001		21	0.0	16	0.0	2	0.0	11	0.0	12	0.0	24	0.0

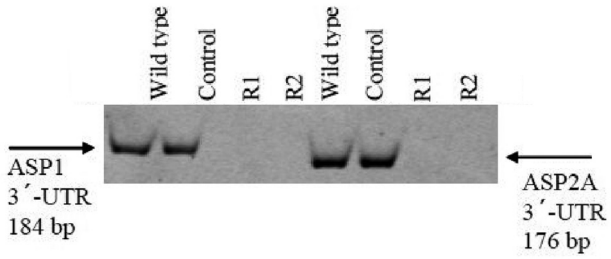

Figure 3. Conventional reverse transcriptase PCR of RNAi1/2A lines R1 and R2 proving loss of both ASP1 and ASP2A transcript.

in horizontally grown plants shows that there are a number of relatively smaller plants with no true leaves in the transgenic line. The same comparison in vertically grown seeds reveals that there are a number of plants having much shorter root lengths in the R1 line. These small plants were carefully collected and shown to be positive with respect to knockdown effect of both ASP1 and ASP2A transcripts by RT-PCR (data not shown). The selected representatives of these small plants were transferred to soil and observed to be dead, whereas wild type seedlings transferred at the same time were well-adapted to soil and grown to rosette leaves.

Discussion

Responses of ASP isoforms to heat stress are time dependent

In nature, plants are subjected to shifts of temperatures, both during seasonal changes or more rapidly over the course of individual days in one season. Therefore, they have evolved strategies for preventing and repairing the damage caused by increased temperatures (Larkindale and Knight, 2002). The heat stress response is characterized by a rapid reprogramming of gene expression, leading to a transient accumulation of heat shock proteins that are correlated with enhanced thermotolerance (Lee *et al*., 1995; Busch *et al*., 2005). The induction of genes in different functional classes such as proteins of the degradation pathways, enzymes of carbohydrate metabolism, membrane transporters, transcription factors, and signaling components are included in the transcriptional responses to heat stress (Busch *et al*., 2005).

The *A. thaliana* sPPase isoforms were shown to be differentially regulated upon heat stress by semi-quantitative real time PCR analyses (Figure 2.) indicating the specificity of response of isoforms. Heat treatment was performed and sampled at two steps; incubating the plants at 37°C for 2 hours followed by 16 hours recovery under normal climate conditions. The effect after 2 hours of treatment is upregulation of the expressions of ASP2A and ASP2B in root tissue. The expressions of ASP2A, ASP2B and ASP4 in leaf tissue, and ASP3 and ASP5 in root tissue are down regulated with the same treatment. The ASP1, ASP2A, ASP3 and ASP4 transcript amounts are significantly increased in leaf tissue during recovery from heat stress, and that of ASP5 is upregulated in both leaf and root tissues. The ASP2B is the only isoform that shows a downregulation after recovery to heat treatment in leaf tissue. These indicate that the heat stress response of *A. thaliana* sPPases is not only isoform-specific but also strongly time-dependent.

Differential gene expression of soluble pyrophosphatases to mechanical wounding

Wounding is a common damage that occurs in plants as a result of abiotic stress factors such as wind, rain, hail, and of biotic factors, especially insect feeding (Cheong *et al*., 2002). It causes localized cell death, loss of water and solutes from exposed surfaces, provides a point of entry for pathogens, and can disrupt the vascular system (Cheong *et al*., 2002; Quilliam *et al*., 2006). Therefore, mechanical wounding activates many signaling pathways either directly by the process or subsequent to changes in several response pathways (Reymond *et al*., 2000; Meyer et al., 2004; Quilliam *et al*., 2006). Mechanical wounding was reported to induce expressions of genes involved in water stress, cellular repair and metabolism, and defense against pathogens (Quilliam *et al*., 2006). The activation of defense and repair mechanisms places a high metabolic demand upon the wounded region; carbon skeletons are required for the synthesis of new molecules and an energy source is required to fuel biosynthetic reactions (Reymond *et al*., 2000; Cheong *et al*., 2002; Quilliam *et al*., 2006).

The effects of mechanical wounding of source leaves on the expression of ASP isoforms imply isoform specific and time-dependent changes in the expressions upon wounding. The period of induction of ASP isoforms may reflect whether the enzyme is regulated by wounding or regulation occurs due to induction of anabolic pathways. That is, the induction in the expressions of early responsive genes like ASP2A and ASP3 may reflect the regulation by signaling molecules that are activated directly after the recognition of the stress. On the other hand, the delayed changes in the expression of, for example ASP2B, might indicate that the enzyme is not directly regulated by wounding, but rather the expression is altered due to adaptive changes in the metabolism.

Functional knockdown of two *A. thaliana* sPPase isoforms implies specific function *in vivo*

Functional knockdown of two of six *A. thaliana* sPPase isoforms strongly impaired with growth (Figure 4.) This supports the isoform-specific regulation of gene expression upon heat stress and mechanical wounding and shows the specificity of in vivo function of each isoform. The activity of plant soluble pyrophosphatases can be highly specialized in their function and loss of function of one or more isoform can not be compensated with the others.

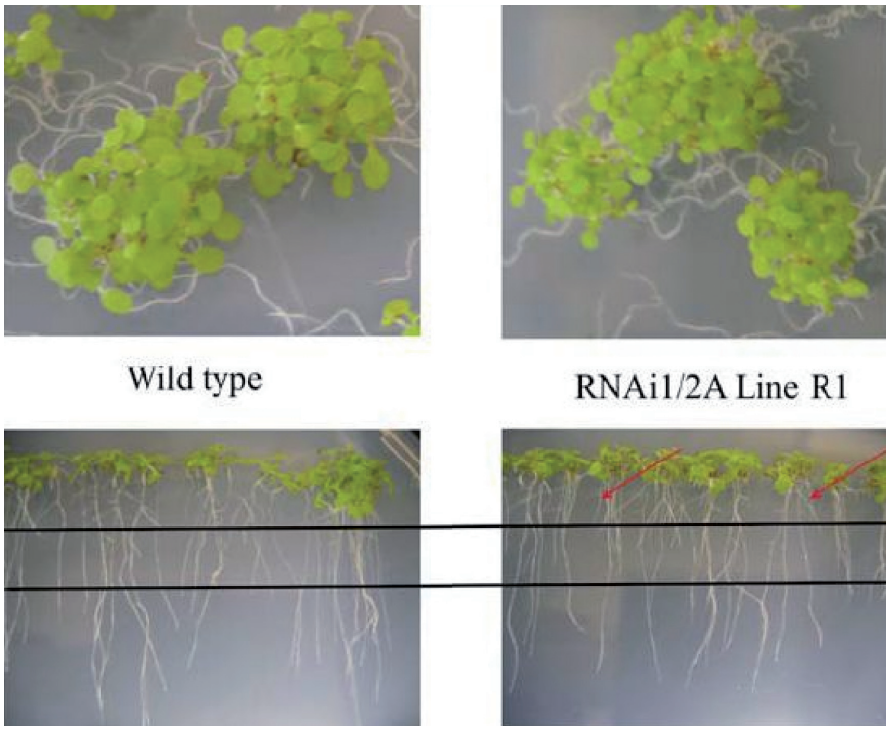

Figure 4. Comparison of wild type and R1 seedlings grown in non-selective ½ MS plates. The figure shows the status of seedlings grown either horizontally (top line) or vertically (bottom line) ten days after germination. The lines were drawn to differentiate two distinct root length populations in wild type and transgenic line. The lower line to the bottom is the range where the primary roots of wild type were found. The upper line is the range where plants with extremely short root length lie (only for transgenic line). Red arrows indicate the R1 seedlings with a much shorter root length compared to that of wild type.

These preliminary results are indicated that plants possess cytoplasmic soluble pyrophosphatase activity, which can be essential throughout plant development and stress response, and the changes in cytoplasmic PPi concentration carried out by sPPase activity may act in fine tuning of several PPi-dependent biosynthetic reactions.

References

Busch W, Wunderlich M and Schoffl F. Identification of novel heat shock factor-dependent genes and biochemical pathways in Arabidopsis thaliana. Plant J. 41: 1-14, 2005.

Cheong YH, Chang H, Gupta R, Wang X, Zhu T and Luan S. Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal response in Arabidopsis. Plant Physiol. 129: 661-677, 2002.

Farre EM, Geigenberger P, Willmitzer L and Trethewey RN. A possible role for pyrophosphate in the coordination of cytosolic and plastidial carbon metabolism within the potato tuber. Plant Physiol. 123: 681-688, 2000.

Farre EM, Bachmann A, Willmitzer L and Trethewey RN. Accelera-tion of potato tuber sprouting by the expression of a bacterial pyrophosphatase. Nat Biotechnol. 19: 268-272, 2001.

Geigenberger P, Hajirezaei M, Geiger M, Deiting U, Sonnewald U and Stitt M. Overexpression of pyrophosphatase leads to increased sucrose degradation and starch synthesis, increased activities of enzymes for sucrose-starch

interconversions, and increased levels of nucleotides in growing potato tubers. Planta 205: 428-437, 1998.

Kim Y, Kim EJ and Rea PA. Isolation and characterization of cDNAs encoding the vacuolar H(+)-pyrophosphatase of Beta vulgaris. Plant Physiol. 106: 375-382, 1994.

Koo AJ and Ohlrogge JB. The predicted candidates of Arabidopsis plastid inner envelope membrane proteins and their expression profiles. Plant Physiol. 130: 823-836, 2002.

Larkindale J and Knight MR. Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. Plant Physiol. 128: 682-695, 2002.

Lee JH, Hubel A and Schoffl F. Derepression of the activity of genetically engineered heat shock factor causes constitutive synthesis of heat shock proteins and increased thermotolerance in transgenic Arabidopsis. Plant J. 8: 603-612, 1995.

Lopez-Marques RL, Perez-Castineira JR, Losada M and Serrano A. Differential regulation of soluble and membrane-bound inorganic pyrophosphatases in the photosynthetic bacterium Rhodospirillum rubrum provides insights into pyrophosphate-based stress bioenergetics. J Bacteriol. 186: 5418-5426, 2004.

Meyer S, Lauterbach C, Niedermeier M, Barth I, Sjolund RD and Sauer N. Wounding enhances expression of AtSUC3, a sucrose transporter from Arabidopsis sieve elements and sink tissues. Plant Physiol. 134: 684-693, 2004.

Muller PY, Janovjak H, Miserez AR and Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 32: 1372-1379, 2002.

Nikolaev VK, Leontovich AM, Drachev VA and Brodsky LI. Building multiple alignment using iterative analyzing biopolymers structure dynamic improvement of the initial motif alignment. Biochemistry 62: 578-582, 1997.

Noren H, Suensson P and Anderson B. A convenient and versatile hydroponic cultivation system for Arabidopsis thaliana. Physiol Plantarum 121: 343-348, 2004.

Perez-Castineira JR, Lopez-Marques RL, Losada M and Serrano A. A thermostable K(+)-stimulated vacuolar-type pyrophosphatase from the hyperthermophilic bacterium Thermotoga maritima. FEBS Lett. 496: 6-11, 2001a.

Perez-Castineira JR, Gomez-Garcia R, Lopez-Marques RL, Losada M and Serrano A. Enzymatic systems of inorganic pyrophosphate bioenergetics in photosynthetic and heterotrophic protists: remnants or metabolic cornerstones? Int Microbiol. 4: 135-142, 2001b.

Quilliam RS, Swarbrick PJ, Scholes JD and Rolfe SA. Imaging photosynthesis in wounded leaves of Arabidopsis thaliana. J Exp Bot. 57: 55-69, 2006.

Rea PA and Poole RJ. Vacuolar H+-translocating pyrophosphatase. Annu Rev Plant Physiol Plant Mol Biol. 44: 157-180, 1993.

Reymond P, Weber H, Damond M and Farmer EE. Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell 12: 707-720, 2000.

Rojas-Beltran JA, Dubois F, Mortiaux F, Portetelle D, Gebhardt C, Sangwan RS and du Jardin P. Identification of cytosolic Mg2+-dependent soluble inorganic pyrophosphatases in potato and phylogenetic analysis. Plant Mol Biol. 39: 449-461, 1999.

Schulze S, Mant A, Kossmann J and Lloyd JR. Identification of an Arabidopsis inorganic pyrophosphatase capable of being imported into chloroplasts. FEBS Lett. 565: 101-105, 2004.

Sonnewald U. Control of potato tuber sprouting. Trends Plant Sci. 6: 333-335, 2001.

Stitt M. Pyrophosphate as an energy donor in the cytosol of plant cells: an enigmatic alternative to ATP. Bot Acta 111: 167-175, 1998.

Weiner H, Stitt M and Heldt HW. Subcellular compartmentation of pyrophosphate and alkaline pyrophosphatase in leaves. Biochim Biophys Acta 893: 13-21, 1987.