

Differential expression of soluble pyrophosphatase isoforms in *Arabidopsis* upon external stimuli

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Abstract: In plants, pyrophosphate (PP_i), generated in a wide range of reversible anabolic reactions, is hydrolyzed by pyrophosphatases. The presence of tonoplast- and Golgi-integral H⁺-translocating pyrophosphatases in plants led to the conclusion that plant cytosol has no soluble pyrophosphatase activity. However, the *Arabidopsis thaliana* (L.) Heynh. genome also encodes five soluble pyrophosphatase isoforms (PPases). There are almost no data in the literature on their redundancy in plant metabolism; therefore, we performed expression analyses of *A. thaliana* soluble pyrophosphatase isoforms in response to external stimuli including carbohydrate status, hormone action, and stress exposure. The results revealed pronounced specificity for each isoform. Interestingly, one isoform (*PPa3*; At2g46860) was specifically induced during seedling etiolation, and in the presence of the nonmetabolizable sugar 3-O-methylglucose. Based on quantitative PCR analyses, *PPa1* (At1g01050) and *PPa4* (At3g53620) appeared to be regulated by sugars. Quantitative PCR analyses indicated isoform- and tissue-dependent responses of *PPa* isoforms to ABA, salt, and cold stresses. *PPa2* (At2g18230) and *PPa5* (At4g01480) responded differentially to salinity, cold treatment, and ABA. We conclude that plant cytosolic pyrophosphatases perform multiple, so far overlooked functions during plant development and stress exposure.

Key words: Cytosolic pyrophosphatase, *Arabidopsis thaliana* (L.) Heynh., salt stress, cold stress, ABA response, phosphate starvation, carbohydrate regulation, etiolation

1. Introduction

The cytosolic inorganic pyrophosphate (PP_i) pool is generated by numerous metabolic reactions, including syntheses of polymers like DNA, RNA, proteins, and polysaccharides (Geigenberger et al., 1998; Stitt, 1998; Sivula et al., 1999; Sonnewald, 2001). Soluble (sPPases) and membrane-bound proton-pumping inorganic pyrophosphatases (especially vacuolar pyrophosphatase, vPPase) are responsible for the removal of excess PP_i to provide a pull for biosynthetic reactions by making them thermodynamically irreversible (Jelitto et al., 1992; Rea and Poole, 1993; Maeshima et al., 1994; du Jardin et al., 1995; Maeshima, 2000; Ferjani et al., 2011).

The importance of high cytosolic PP_i concentration and low cytosolic sPPase activity was proven by overexpression of *Escherichia coli* sPPase in the plant cytosol, which decreased cytoplasmic PP_i concentration to a level that impaired plant growth and development (Weiner et al., 1987; Jelitto et al., 1992; Sonnewald, 1992). Therefore, it is generally well accepted that plant cytosol lacks sPPase

activity (Geigenberger et al., 1998; Stitt, 1998; Farré et al., 2001). However, this hypothesis has some drawbacks. Weiner et al. (1987) measured the cytoplasmic PP_i concentration of fully developed spinach leaves as 200–300 μM, whereas for potato tuber cells it was 23 μM (Farré et al., 2001). In other words, there are still not enough data on in vivo cytoplasmic PP_i levels depending on the plant species, tissue, and developmental stage. In addition, Chen et al. (1990) suggested the presence of a posttranslational mechanism in *E. coli* to regulate the enzymatic activity of sPPase to keep cytoplasmic PP_i concentration at a certain level. Therefore, the higher decrease in the cytoplasmic PP_i concentration through ectopic expression of the *E. coli* sPPase gene might be due to the lack of the regulatory mechanisms of a bacterial enzyme in plants and does not necessarily prove the lack of sPPase activity in the plant cytosol.

The *Arabidopsis thaliana* (L.) Heynh. genome has six sPPase isoforms, one of which was shown to be localized in the plastids and the others in the cytosol and/or nucleus

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(Schulze et al., 2004; George et al., 2010; Öztürk et al., 2014). Navarro-De la Sancha et al. (2007) reported the possibility of ubiquitous expression of *A. thaliana* sPPase isoforms based on the GENEVESTIGATOR database and suggested that changes in cytosolic PP_i concentration by differential expression of sPPase isoforms might be important in the regulation of plant metabolism (Navarro-De la Sancha et al., 2007; Meyer et al., 2012). Here, we aimed to analyze the differential expression of *A. thaliana* soluble pyrophosphatase isoforms in response to external stimuli to get further insight into their possible roles in the plant metabolism.

2. Materials and methods

2.1. Plant growth and heterotrophic suspension-cultured cells

Arabidopsis thaliana (L.) Heynh. ecotype Columbia was used for all experiments. The growth of *A. thaliana* in hydroponic culture (25 µM H₃BO₃, 0.06 µM CuSO₄, 0.14 µM MnSO₄, 0.03 µM Na₂MoO₄, 0.001 µM CoCl₂, 0.1 µM ZnSO₄, 20 µM Fe-EDTA, 2 mM KNO₃, 1 mM Ca(NO₃)₂, 1 mM KH₂PO₄, 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 then sealed and, after 2 days of incubation at 4 °C, transferred to climate chambers [14 h light (24 °C), 8 h dark (18 °C), light intensity 125 µmol m⁻² s⁻¹, 50% relative humidity]. The seals were opened after 10 days and seedlings were grown in the pipette boxes until they reached the fourth leaf growth stage. They were then transferred to large pots filled with hydroponic solution (6 L) to be grown for up to 4 weeks with weekly renewal of the nutrient solution and no aeration.

The heterotrophic *Arabidopsis thaliana* (L.) Heynh. suspension-cultured cells were grown in cell suspension medium [4.3 g/L MS (basal salt mixture), 20 g/L sucrose or glucose, 1 mg/L 2.4 D, 1 mL/L vitamin solution (5 g of inositol, 25 mg of nicotinic acid, 25 mg of pyridoxin-HCl, 25 mg of thiamin-HCl in 5 mL), pH 5.7] in the dark (25 °C) with slight shaking. The cultures were renewed every 7 days by inoculating approximately 5 g of cells into fresh medium.

2.2. *Arabidopsis thaliana* (L.) Heynh. cell culture and in planta sugar treatments

Five grams of *Arabidopsis thaliana* (L.) Heynh. suspension-cultured cells in exponential growth were washed twice with sugar-free medium and then inoculated into fresh cell suspension medium containing glucose, sucrose, or 3-*O*-methylglucose (20 g/L). Cultures were grown in the dark (25 °C) with slight shaking. Five grams of samples were collected 7 days after inoculation for sucrose or glucose grown cells and 3 h, 1 day, and 3 days after for cell

cultures inoculated in suspension culture supplemented with 3-*O*-methylglucose. They were washed twice with sugar-free medium, directly frozen in liquid nitrogen, and stored at -80 °C for real time PCR analyses.

For in planta sugar treatment, *Arabidopsis thaliana* (L.) Heynh. plants grown in hydroponic cultures were used. Disks 0.05 cm in diameter were removed from fully developed rosette leaves (5 weeks after germination, tenth or eleventh leaf counted from the youngest developed sink leaf) and incubated in 100 mM or 300 mM sucrose, glucose, or 3-*O*-methylglucose dissolved in deionized water overnight in the dark at room temperature with slight shaking. The control tissue was incubated in deionized water under the same conditions. The disks were directly frozen in liquid nitrogen after the treatments, and stored at -80 °C for quantitative PCR analyses.

2.3. Etiolation of *Arabidopsis thaliana* (L.) Heynh. seedlings

Arabidopsis thaliana (L.) Heynh. seeds were surface sterilized and inoculated to ½ MS medium (pH 5.8) without sucrose. The plates were incubated at 4 °C overnight and transferred to climate chambers [14 h light (24 °C), 8 h dark (18 °C), light intensity 125 µmol m⁻² s⁻¹, 50% relative humidity]. Half of the plates were covered with aluminum foil, whereas the control plates were kept fully illuminated. Whole seedlings were collected 7 days after germination of control seeds, directly frozen in liquid nitrogen, and stored at -80 °C for quantitative PCR analyses.

2.4. External stimuli in hydroponic culture

External stimuli treatments of *Arabidopsis thaliana* (L.) Heynh. grown in hydroponic culture were performed with 5-week-old plants after transfer to hydroponics. The ABA and salt treatments were performed for 16 h by adding 100 µM ABA or 200 mM NaCl to the hydroponic culture. The cold treatment was performed by keeping whole plants at 4 °C for 2 h following 16 h in growth chamber conditions for recovery. The phosphate starvation (5 days in climate chambers) was performed by removing the phosphate source from hydroponic culture (by replacing KH₂PO₄ with K₂SO₄). The control samples were incubated in normal hydroponic culture. The control and stressed plant whole leaf and root tissues were collected at the same time points, directly frozen in liquid nitrogen, and stored at -80 °C for quantitative PCR analyses.

2.5. Quantitative PCR

Total RNA was isolated using an RNeasy Plant Kit (Qiagen, Valencia, CA, USA) and reverse transcription of 2 µg of total RNA was performed according to the manufacturer's instructions (Omniscript Reverse Transcriptase, Qiagen, Valencia, CA, USA). In order to prevent degradation of RNA, RNaseOUT (Invitrogen, Carlsbad, CA, USA) was added to the reaction mixture. Quantitative PCR was prepared in 25 µL volume and performed using iCycler

(Bio-Rad Laboratories, San Francisco, CA, USA). The annealing temperature was optimized for each gene specific primer pair (*PPa1*, *PPa2*, *PPa3*, and *PPa5* at 60 °C, and *PPa4* and *PPa6* at 58 °C). The following gene specific primer pairs spanning one intron sequence were used for amplification of *Arabidopsis thaliana* (L.) Heynh. soluble pyrophosphatases: *PPa1* (At1g01050; 5'-ACA ATC GGC TGT TTC GTT TC-3'; 5'-TTC CTT TAG TGA TCT CAA CAA CCA C-3'), *PPa2* (At2g18230; 5'-GAT TCT CTG CTT CGG TTT CG-3'; 5'-CAG TAG GAG CTT CTG GAC CAA TC-3'), *PPa3* (At2g46860; 5'-CAA ATG CTC TGT TTT CTT CTG C-3'; 5'-CCT TTG TGA TCT CAA CCA CCA C-3'), *PPa4* (At3g53620; 5'-TGA GAT CTG TGC TTG CGT TT-3'; 5'-TGG GGC TTC AGG TCC TAT C-3'), *PPa5* (At4g01480; 5'-CTC CAC ACT TTC CGC AAG AT-3'; 5'-ACT GGA GCT CCA GGT CCG-3'), *PPa6* (At5g09650; 5'-GAG ACA AAC CAG CAA ACA AAG AC-3'; 5'-AAA CAA AAT CCA AAT CCC AAT G-3'), and actin (At3g18780; 5'-GGT AAC ATT GTG CTC AGT GGT GG-3'; 5'-CTC GGC CTT GGA GAT CCA CAT C-3'). Stable expression of the actin gene was confirmed under all experimental conditions (data not shown) and used to normalize expression levels of *PPa* isoforms according to Muller et al. (2002). Actin Ct values outside the limits of 16 to 18 were not considered in calculations and amplification efficiencies of both target and reference genes were confirmed to be between 1.9 and 2.1 for each quantification. Results were calculated

as mean ± standard deviation of three replicates of three independent experiments.

3. Results

3.1. Differential expression of *Arabidopsis thaliana* (L.) Heynh. soluble pyrophosphatase isoforms upon external stimuli

The full promoter regions of *Arabidopsis thaliana* (L.) Heynh. sPPases from the TAIR website (The Arabidopsis Information Resource) were analyzed for the presence of known cis-acting elements related to the response to changes in environmental conditions using the plant web-based programs PLACE (Higo et al., 1999) and TFSearch (Heinmeyer et al., 1998), and Softberry PlantProm database tool (Softberry PlantProm DB). The results are summarized in the Table. Accordingly, the changes in the expression of *PPa* isoforms (*PPa1*, At1g01050; *PPa2*, At2g18230; *PPa3*, At2g46860; *PPa4*, At3g53620; *PPa5*, At4g01480; *PPa6*, At5g09650) in response to etiolation, ABA treatment, salt stress, low temperature, phosphate deficiency, and sugars were quantified by PCR.

3.2 Response to different carbohydrate sources in cell culture

Since promoter analyses suggested transcriptional regulation of all *Arabidopsis thaliana* (L.) Heynh. *PPa* isoforms with sugar and/or in response to sugar starvation (Table), the effects of differential carbohydrate sources

Table. Environmental stress responsive transcriptional factor binding motifs found in the promoter regions of *Arabidopsis thaliana* (L.) Heynh. soluble pyrophosphatases.

Regulatory element	Motif	Responsive to	PPa
ABRELATERD1	ACGTG	Etiolation	3, 5, 6
AGCBOXNPLB	AGCCGCC	Wounding	3
CCAATBOX1	CCAAT	Heat	All
CDA1ATCAB2	CAAAACGC	Dark	3
CGACGOSAMY3	CGACG	Sugar starvation	2, 4
DRE1COREZMRAB17	ACCGAGA	Drought	1
ELRECOREPCR1	TTGACC	Wounding	3, 5, 6
GARE1OSREP1	CAACTC	Gibberellin and ABA	3
GATABOX	GATA	Light	All
GCCCORE	GCCGCC	Wounding	3
GT1CORE	GGTTAA	Light	2, 6
MYCATERD1	CATGTG	Drought	4, 5, 6
TATCCAOSAMY	TATCCA	Sugar and hormone	1, 3, 5, 6
TATCCAYMOTIFOSRAMY3D	TATCCAY	Sugar	1, 2
TCA1MOTIF	TCATCTTCTT	Salicylic acid	2

Web-based tools PLACE (Higo et al., 1999) and TFSearch (Heinmeyer et al., 1998), and Softberry PlantProm database tool (Softberry PlantProm DB) were used for the detection of possible regulatory motifs.

on the gene expression of the *PPa* family were analyzed using *A. thaliana* suspension-cultured cells grown in medium supplemented with glucose, sucrose, or 3-*O*-methylglucose. *PPa2* and *PPa6* transcripts could not be detected by quantitative PCR; therefore, these isoforms can be considered as not expressed in cell cultures. Mean normalized expression levels of *PPa1*, *PPa3*, *PPa4*, and *PPa5* upon different carbohydrate stimuli are summarized in Figure 1. Sucrose as carbohydrate source induced expression levels of *PPa1* and *PPa4*, while *PPa3* transcript level did not considerably change (Figure 1). Expression level of *PPa4* was strongly induced 3 days after inoculation to medium containing glucose and stayed at the same level for 7 days after transfer (Figure 1). Transcripts of other *PPa* isoforms did not respond to the presence of glucose (Figure 1).

The effect of sugar starvation on the gene expression of *PPa* isoforms was analyzed using a nonmetabolizable glucose analogue, 3-*O*-methylglucose (Cortes et al., 2003), and quantified by PCR (Figure 1). Expression of *PPa1* was repressed after transfer from sucrose to 3-*O*-methylglucose containing medium (Figure 1). *PPa3* and *PPa4* transcription levels were induced in the presence of 3-*O*-methylglucose at different time points (Figure 1). Interestingly, *PPa3* showed no detectable expression in glucose fed cell culture, but was strongly induced by both etiolation and in response to 3-*O*-methylglucose (Figure 1).

3.3. Response to different carbohydrate sources in planta

The effects of different carbon sources on expression levels of *Arabidopsis thaliana* (L.) Heynh. *PPa* isoforms were confirmed in planta as described by Schulze et al. (2004). In planta mean normalized expression levels of most of the *PPa* isoforms were quite low (data not shown). *PPa1*, found to be expressed in the cell culture (Figure 1), was almost undetectable in response to different carbohydrate sources in planta. Expression levels of *PPa2*, *PPa4*, and

PPa5 were differentially changed in response to glucose, sucrose, or 3-*O*-methylglucose (Figure 2). Interestingly, *PPa3* transcript level was induced in response to 3-*O*-methylglucose in both cell culture (Figure 1) and in planta (Figure 2) experiments. The level of expression of *PPa6* (the plastidial isoform) was induced in the presence of 100 mM glucose, and otherwise repressed to different levels (Figure 2).

3.4. Response to etiolation

Levels of expression of *PPa* transcripts in both dark and light grown 7-day-old whole seedlings were shown to be quite low, except for the plastidial isoform in light grown seedlings (data not shown). In response to etiolation, expression of most of *PPa* isoforms was repressed to different levels, except for *PPa3*, which showed an induction, and the transcription of *PPa6*, the plastidial isoform, was almost completely downregulated (Figure 3). Promoter analyses (Table) suggested response of *PPa3*, *PPa5*, and *PPa6* transcription to etiolation and *PPa3* to dark, and quantitative PCR analyses revealed induction in expression of *PPa3* and repression of *PPa6* (Figure 3).

3.5. Response to external stimuli

Promoter analyses suggested transcriptional regulation of *Arabidopsis thaliana* (L.) Heynh. soluble pyrophosphatase isoforms in response to external stimuli (Table); therefore, changes in gene expression of the *PPa* family were analyzed by quantitative PCR in response to ABA, cold, salt, and phosphate deficiency. Percent changes in expression levels of stress treated leaf and root samples are summarized in Figure 4, where the expression level of several *PPa* isoforms differentially responded to ABA, cold, salt, and phosphate deficiency specifically in leaf or root tissues, or both.

The responses of *A. thaliana* sPPases to ABA treatment were clearly isoform-specific and quite complex (Figure 4). For example, *PPa1*, *PPa5*, and *PPa6* expression was induced in leaf tissue, but not changed in roots; on the other hand, the expression of *PPa2* and *PPa3* was induced

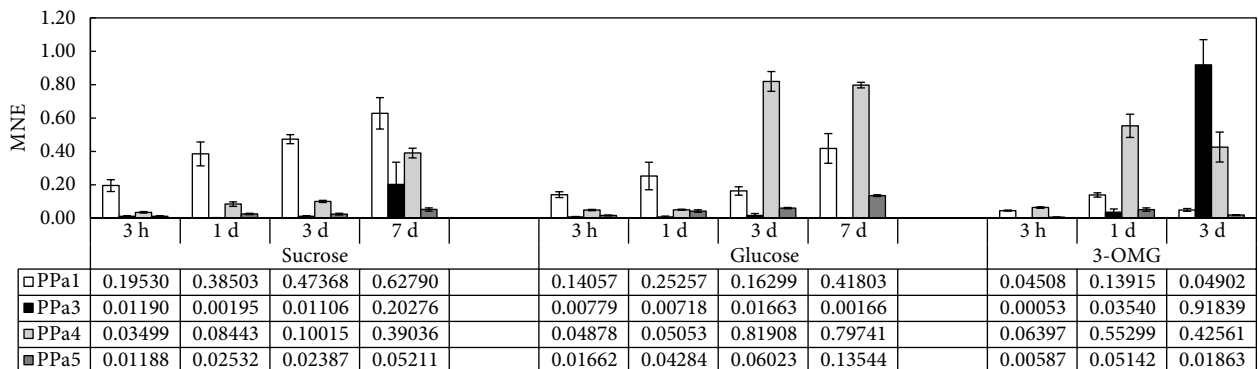


Figure 1. Isoform-specific real time PCR analysis of *PPa* expression in heterotrophic, exponentially growing suspension cultured cells. Data were normalized (Muller et al., 2002) using actin gene expression as the reference. Data were given as the means of three independent experiments and error bars indicate ± SD. 3-OMG: 3-*O*-methylglucose; MNE: mean normalized expression.

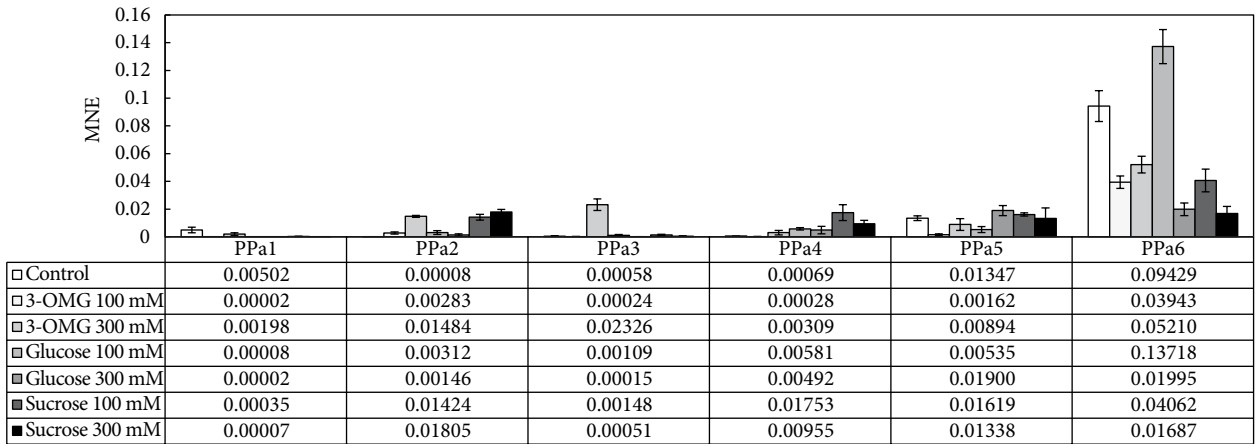


Figure 2. Isoform-specific real time PCR analysis of *PPa* expression upon incubation of leaf disks on media supplemented with sucrose, glucose or the non-metabolizable sugar 3-O-methylglucose at 100 mM or 300 mM concentrations. Data were presented as percent of expression values at time of cell transfer to fresh medium, and were normalized (Muller et al., 2002) using actin as the reference gene. Data were given as the means of three independent experiments and error bars indicate \pm SD. 3-OMG: 3-O-methylglucose; G: glucose; S: sucrose; MNE: mean normalized expression.

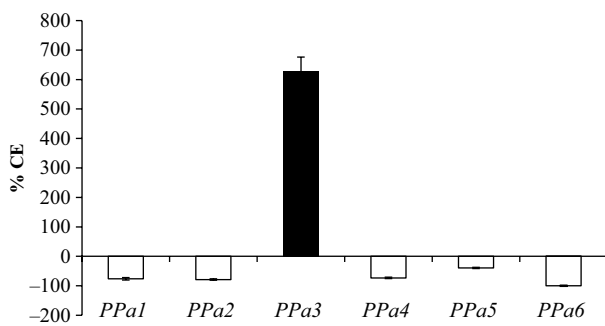


Figure 3. Expression analyses of *PPa* isoforms during seedling germination in the dark (7 days of etiolation). Data were normalized (Muller et al., 2002) using actin gene expression as the reference and expressed as percent of expression values in light grown control seedlings. Data were given as the means of three independent experiments and error bars indicate \pm SD. %CE: % change in expression.

in root and repressed in leaf. Among the *PPa* isoforms, only *PPa4* was upregulated by ABA treatment both in leaf and in root tissues.

The responses of *A. thaliana* sPPase transcripts to cold and salt stresses emphasized the isoform- and tissue-specificity of expression (Figure 4). For example, the gene expression of *PPa1* was upregulated in leaf tissue, whereas *PPa3* and *PPa5* were highly induced in root tissue upon cold treatment. Similar to the ABA responses, only the gene expression of *PPa4* was upregulated in both leaf and root tissues upon cold treatment. Salt stress resulted in induction of *PPa2*, *PPa3*, and *PPa4* transcripts in leaf tissues and *PPa2* and *PPa4* transcripts in root tissues. *PPa1* transcripts in leaf tissues were almost completely downregulated upon salt treatment.

The expression of most *A. thaliana* sPPases was found to be repressed upon phosphate deficiency for 5 days (Figure 4). While the expression of *PPa1*, *PPa3*, and *PPa6* was completely repressed in root tissues, the response in leaf tissues showed unique levels of downregulation, the highest of which were in the transcript levels of *PPa3* and *PPa6*.

4. Discussion

Arabidopsis thaliana (L.) Heynh. has six sPPase isoforms (Schulze et al., 2004; Öztürk et al., 2014). The presence of a vacuolar membrane localized proton-pumping PPase (Geingenberger et al., 1998; Farre et al., 2001) and data implying the importance of a large cytosolic PP_i pool for plant growth and development (Jelitto et al., 1992; Sonnewald, 1992) suggested lack of cytosolic sPPase activity. Recent data, however, imply possible roles of cytosolic sPPases in the regulation of plant metabolism (Meyer et al., 2012; Öztürk et al., 2014). In this study, we summarize real time PCR analyses of expressional changes of *PPa* isoforms in response to etiolation, different carbon sources, and environmental stresses including ABA, cold, salt, and phosphate deficiency.

4.1. Expression of *Arabidopsis thaliana* (L.) Heynh. PPa isoforms changes depending on the carbohydrate source
 Besides playing a central role in metabolism, soluble sugars like glucose and sucrose have been shown to be important signaling molecules regulating many developmental and physiological processes in plants including carbohydrate metabolism (Koch, 1996; Gibson, 2000). The suspension-cultured cell studies in the presence of different carbohydrate sources available in the growth medium revealed that transcripts of *PPa2* and *PPa6* were

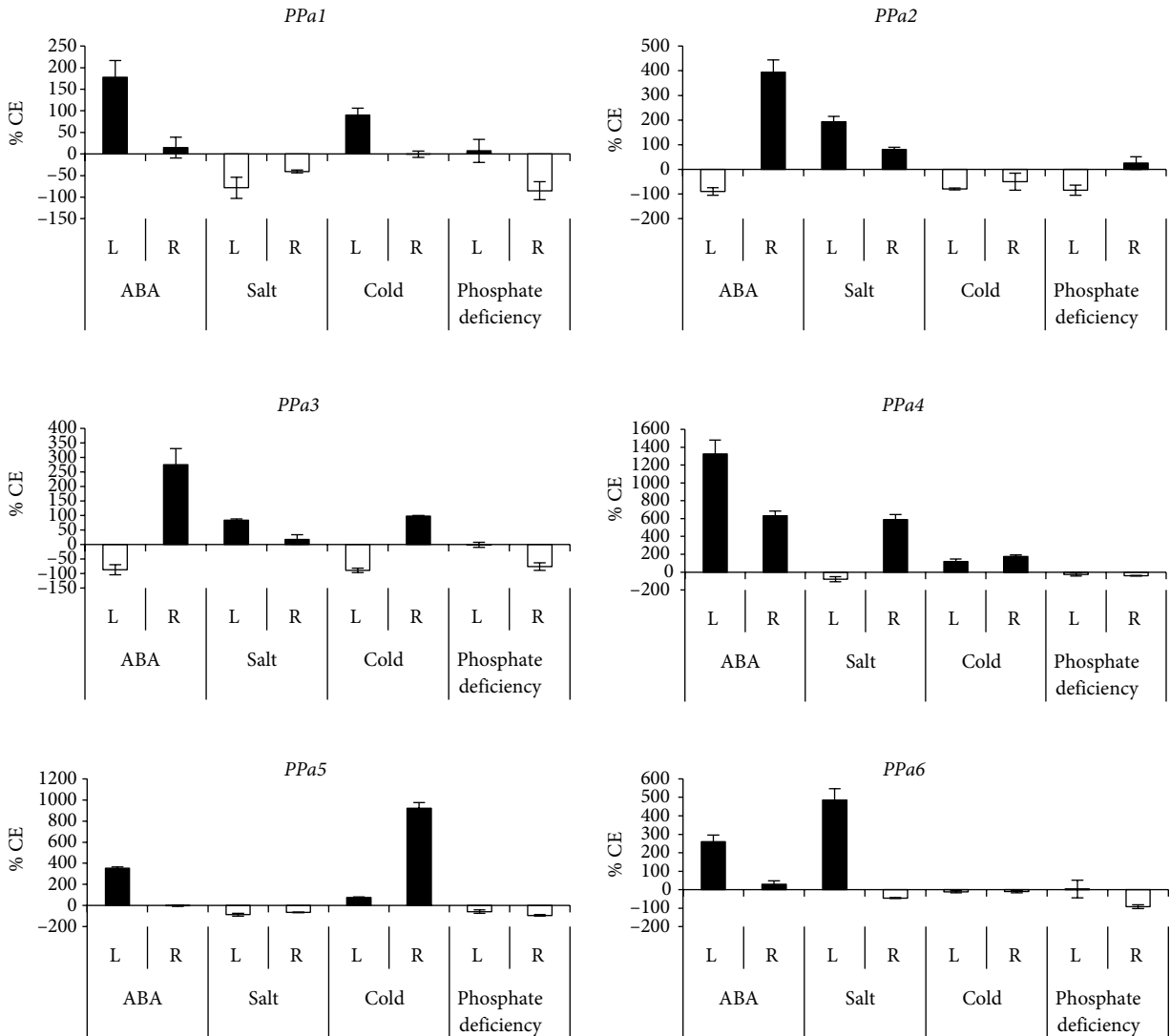


Figure 4. Impact of abscisic acid (ABA), salt exposure, cold treatment, and phosphate deficiency on relative expression of *PPa* isoforms in leaf (L) and root (R) tissues. Percent change in expression of *Arabidopsis thaliana* (L.) Heynh. sPPase isoforms in response to 100 μ M ABA (16 h), 2 h 4 °C (followed by 16 h recovery), 200 mM NaCl (16 h), and 5 days cultivation on phosphate-depleted medium are presented. Data were normalized (Muller et al., 2002) using actin as the reference gene expressed as percent of expression values under control conditions. Data were given as the means of three independent experiments and error bars indicate \pm SD. %CE: % change in expression.

not detectable by real time PCR (Figure 1). Among the other *PPa* isoforms, *PPa1*, *PPa3*, and *PPa4* transcripts indicated sugar-dependent expressional regulation (Figure 1). While *PPa1* expression was induced by sucrose, the transcript level of *PPa4* increased in response to glucose and 3-*O*-methylglucose (Figure 1). In addition, real time PCR analyses of in planta experiments indicated induction of *PPa4* transcript level in response to 100 mM sucrose (Figure 2). The expressional increases in *PPa1* (At1g01050) in response to sucrose in cell culture and *PPa4* (At3g53620) in response to sucrose and glucose in both cell culture

and in planta experiments support the previous finding on the possible roles of these isoforms in regulation of cytosolic glycolysis (Meyer et al., 2012). In planta studies revealed specific induction of *PPa6*, the plastidial isoform, in response to 100 mM glucose (Figure 2). Schulze et al. (2004) showed in planta induction of *PPa6* transcript by northern blot analysis in response to 300 mM glucose and 300 mM sucrose. Our results, on the other hand, showed an induction by 100 mM glucose only. The difference in the results presented in this study and the report by Schulze et al. (2004) might be related to using leaves from different

developmental stages (Schulze et al. did not specify the age of the plant used for collecting leaf disks). However, 300 mM sucrose and glucose are high concentrations of sugars; therefore, the induction observed by Schulze et al. (2004) might be independent of carbohydrate-mediated regulation since higher concentrations of both glucose and sucrose (300 mM) were reported to severely interfere with development (Gibson, 2000).

4.2. *PPa3* is induced by sugar starvation

While most *Arabidopsis thaliana* (L.) Heynh. sPPase isoforms showed different levels of repression (the strongest of which was observed in the plastidial isoform *PPa6*), expression level of *PPa3* showed a strong induction during etiolation (Figure 3). Interestingly, the expression of *PPa3* was induced both in seedlings germinated in the dark without exogenous sugar supply (Figure 3) and in cell cultures grown in medium with 3-*O*-methylglucose as carbon supply (Figure 1). Plants experience starvation due to repression of photosynthesis when grown in the dark. This is also true for long-term feeding with 3-*O*-methylglucose, since it is a nonmetabolizable glucose analogue (Cortes et al., 2003). The gene expression of *PPa3*, therefore, might be concluded to be induced by sugar starvation, indicating a possible role of this soluble pyrophosphatase isoform in starvation response. Interestingly, the sugar-mediated regulation of *PPa3* gene expression is surprisingly similar to the regulation of enzymes responsible for starch breakdown; they were shown to be significantly upregulated by carbohydrate depletion and completely repressed by glucose (Koch, 1996; Jang and Sheen, 1997).

4.3. ABA, salt, and cold specifically induce some *Arabidopsis thaliana* (L.) Heynh. *PPa* isoforms, while repressing others

The real time quantification of changes in expression of *PPa* isoforms showed isoform- and tissue-dependent responses to ABA, salt, and cold stresses (Figure 4). Expression levels of *PPa1*, *PPa5*, and *PPa6* in leaf tissues and *PPa2* in root tissues were induced upon exposure to ABA, and salt stress treatment significantly induced *PPa6* transcription only (Figure 4). ABA is a plant hormone functioning in response to environmental stress conditions by adjusting osmotic homeostasis or, at the cellular level, by regulating a set of genes to promote tolerance to stress conditions (Kang et al., 2002). Since the endogenous ABA level is known to accumulate upon salt stress and has a regulatory effect on gene expression, salt and ABA responses were reported to have similarities, i.e. there is crosstalk between these two stress responsive pathways (Zhu, 2002). However, no clear similarities were observed in changes in expression levels of *PPa* isoforms upon ABA treatment or salt stress in this study. Genes that are regulated by photosynthetic activities were reported to be affected but

not regulated by salt stress per se (Zhu, 2002); therefore, increased expression of *PPa6* transcripts upon salt stress (Figure 4) cannot easily be explained as a stress response but might be related to changes in cellular metabolism. Cold treatment creates energy stress due to mitochondrial dysfunction and consequently PP_i -dependent pathways were reported to be favorable under this ATP limiting condition (Carystinos et al., 1995). The induction of gene expression of *PPa1* in leaf tissue and *PPa5* in root tissue in response to low temperature may imply tissue-specific roles of these isoforms in cold stress response (Figure 4).

4.4. Phosphate starvation represses the expression of *Arabidopsis thaliana* (L.) Heynh. sPPases

Orthophosphate (P_i) plays a central role in the plant metabolism as an educt of ATP generation, and as a key component of the carbon exported from chloroplasts during photosynthesis. Therefore, P_i deficiency affects photosynthesis, carbon fixation, glycolysis, and respiration (Raghothama, 2000; Sánchez-Calderón et al., 2005). Phosphate starvation causes a large decline in the cytoplasmic P_i concentration, which is followed by large reductions in the intracellular levels of ATP and related nucleoside phosphates (Palma et al., 2000). This may hinder carbon flux mediated by enzymes of glycolysis that are dependent upon adenylates or P_i as co-substrates. At least six P_i - and adenylate-independent glycolytic 'bypass' enzymes (sucrose synthase, UDP-glucose pyrophosphorylase, PP_i -dependent protein kinase, nonphosphorylating NADP-glyceraldehyde-3-phosphate dehydrogenase, phosphoenolpyruvate carboxylase, and phosphoenolpyruvate phosphatase) were reported to be induced upon phosphate starvation (Palma et al., 2000). Thus, PP_i -dependent processes may be a crucial aspect of the metabolic adaptations of plants to phosphate deficiency, which is characterized by reduced ATP pools (Palma et al., 2000).

Interestingly, phosphate deficiency for 5 days repressed expression levels of all *PPa* isoforms, except the plastidial isoform *PPa6* (Figure 4). There are, however, reports on induction of expression levels of sPPase isoforms upon phosphate deprivation (Wasaki et al., 2003; Misson et al., 2005; Hernández-Domínguez et al., 2012). These contradictory findings could reflect the influence of the developmental stage of plants, different growth conditions, or treatments. For example, Hernández-Domínguez et al. (2012) determined changes in expression levels of sPPases of 6-week-old common bean grown in hydroponics with or without phosphate for 3 weeks. It is important to note that there are also studies reporting high cytoplasmic PP_i pools during energy-limiting stress treatments like anoxia (Dancer et al., 1990; Geigenberger et al., 2000); therefore, repression of expression levels of *PPa* isoforms may be important to limit sPPase activity upon phosphate

deprivation in order to maintain the cytoplasmic PP_i pool for the fate of PP_i -dependent metabolic pathways under ATP limiting conditions.

4.5. Real time PCR analyses of *Arabidopsis thaliana* (L.) Heynh. *PPa* isoforms imply tissue specific expression and specific roles of sPPase isoforms upon external stimuli

Here, we analyzed changes in expression levels of *PPa* isoforms during etiolation, different carbohydrate sources in cell suspension cultures and in planta, and upon ABA treatment, cold and salt stress, and phosphate deficiency. Responses to external stimuli implied specific in vivo function and/or regulation of each *PPa* isoform. Most interestingly, *PPa1* and *PPa4* appeared to be regulated by sugars, and *PPa3* was specifically induced in response to

sugar starvation. Increases in the expression levels of *PPa2* and *PPa5* were observed only in response to environmental stress conditions. All of these findings suggest specialized in vivo functions of *Arabidopsis thaliana* (L.) Heynh. soluble pyrophosphatase isoforms in regulation of the plant metabolism.

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References

- Carystinos GD, MacDonald HR, Monroy AF, Dhindsa RS, Poole RJ (1995). Vacuolar H(+)-translocating pyrophosphatase is induced by anoxia or chilling in seedlings of rice. *Plant Physiol* 108: 641–649.
- Chen J, Brevet A, Fromant M, Leveque F, Schmitter J, Blanquet S, Plateau P (1990). Pyrophosphate is essential for growth of *Escherichia coli*. *J Bacteriol* 172: 5686–5689.
- Cortes S, Gromova M, Evrard A, Roby C, Heyraud A, Rolin DB, Raymond P, Brouquisse RM (2003). In plants, 3-O-methylglucose is phosphorylated by hexokinase but not perceived as a sugar. *Plant Physiol* 131: 824–837.
- Dancer J, Veith R, Feil R, Komor E, Stitt M (1990). Independent changes of inorganic pyrophosphate and the AT/ADP or UTP/UDP ratios in plant cell suspension cultures. *Plant Sci* 66: 59–63.
- du Jardin P, Rojas-Beltran J, Gebhardt C, Brasseur R (1995). Molecular cloning and characterization of a soluble inorganic pyrophosphatase in potato. *Plant Physiol* 109: 853–860.
- Farré EM, Tiessen A, Roessner U, Geigenberger P, Trethewey RN, Willmitzer L (2001). Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. *Plant Physiol* 127: 685–700.
- Ferjani A, Segami S, Horiguchi G, Muto Y, Maeshima M, Tsukaya H (2011). Keep an eye on PP_i : the vacuolar-type H⁺-pyrophosphatase regulates postgerminative development in *Arabidopsis*. *Plant Cell* 23: 2895–2908.
- Geigenberger P, Fernie AR, Gibon Y, Christ M, Stitt M (2000). Metabolic activity decreases as an adaptive response to low internal oxygen in growing potato tubers. *J Biol Chem* 275: 723–740.
- Geigenberger P, Hajirezaei M, Geiger M, Deiting U, Sonnewald U, Stitt M (1998). 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.
- George GM, van der Merwe MJ, Nunes-Nesi A, Bauer R, Fernie AR, Kossmann J, Lloyd JR (2010). Virus-induced gene silencing of plastidial soluble inorganic pyrophosphatase impairs essential leaf anabolic pathways and reduced drought stress tolerance in *Nicotiana benthamiana*. *Plant Physiol* 154: 55–66.
- Gibson SI (2000). Plant sugar-response pathways. Part of a complex regulatory web. *Plant Physiol* 124: 1532–1539.
- Heinmeyer T, Wingender E, Reuter I, Hermjakolo H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA et al. (1998). Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. *Nucleic Acids Res* 26: 364–370.
- Hernández-Domínguez EE, Valencia-Turcotte LG, Rodríguez-Sotres R (2012). Changes in expression of soluble inorganic pyrophosphatases of *Phaseolus vulgaris* under phosphate starvation. *Plant Sci* 187: 39–48.
- Higo K, Ugawa Y, Iwamoto T, Korenaga T (1999). Plant cis-acting regulatory DNA elements (PLACE) database:1999. *Nucleic Acids Res* 27: 297–300.
- Jang JC, Sheen J (1997). Sugar sensing in higher plants. *Trends Plant Sci* 2: 208–214.
- Jelitto T, Sonnewald U, Willmitzer L, Hajirezaei M, Stitt M (1992). Inorganic pyrophosphate content and metabolites in potato and tobacco plants expressing *E. coli* pyrophosphatase in their cytosol. *Planta* 188: 238–244.
- Kang J, Choi H, Im M, Kim SY (2002). Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell* 14: 343–357.
- Koch KE (1996). Carbohydrate-modulated gene expression in plants. *Ann Rev Plant Physiol* 47: 509–540.
- Maeshima M (1991). H(+)-translocating inorganic pyrophosphatase of plant vacuoles. Inhibition by Ca^{2+} , stabilization by Mg^{2+} and immunological comparison with other inorganic pyrophosphatases. *Eur J Biochem* 196: 11–17.

- Maeshima M (2000) Vacuolar H(+)-pyrophosphatase. *Biochim Biophys Acta* 1465: 37–51.
- Maeshima M, Mimura T, Sato T (1994). Distribution of vacuolar H⁺-pyrophosphatase and a membrane integral protein in a variety of green plants. *Plant Cell Physiol* 35: 323–328.
- Meyer K, Stecca KL, Ewell-Hicks K, Allen SM, Everard JD (2012). Oil and protein accumulation in developing seeds is influenced by the expression of a cytosolic pyrophosphatase in *Arabidopsis*. *Plant Physiol* 159: 1221–1234.
- Misson J, Raghothama KG, Jai A, Jouhet J, Block MA, Bligny R, Ortet P, Creff A, Sommerville S, Rolland N et al. (2005). A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. *Proc Natl Acad Sci USA* 102: 11934–11939.
- Muller PY, Janovjak H, Miserez AR, Dobbie Z (2005). Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* 32: 1372–1379.
- Navarro-De la Sancha E, Coello-Coutiño MP, Valencia-Turcotte LG, Hernández-Domínguez EE, Trejo-Yepes G, Rodríguez-Sotres R (2007). Characterization of two soluble inorganic pyrophosphatases from *Arabidopsis thaliana*. *Plant Sci* 172: 796–807.
- Noren H, Suensson P, Anderson B (2004). A convenient and versatile hydroponic cultivation system for *Arabidopsis thaliana*. *Physiol Plantarum* 121: 343–348.
- Öztürk ZN, Greiner S, Rausch T (2014). Subcellular localization and developmental regulation of cytosolic, soluble pyrophosphatase isoforms in *Arabidopsis thaliana*. *Turk J Bot* 38: 1036–1049.
- Palma DA, Blumwald E, Plaxton WC (2000). Upregulation of vacuolar H(+)-translocating pyrophosphatase by phosphate starvation of *Brassica napus* (rapeseed) suspension cell cultures. *FEBS Lett* 486: 155–158.
- Raghothama KG (2000). Phosphate transport and signaling. *Curr Opin Plant Biol* 3: 182–187.
- Rea PA, Poole RJ (1993). Vacuolar H⁺-translocating pyrophosphatase. *Ann Rev Plant Physiol* 44: 157–180.
- Sánchez-Calderón L, López-Bucio J, Chacón-López A, Cruz-Ramírez A, Nieto-Jacobo F, Dubrovsky JG, Herrera-Estrella L (2005). Phosphate starvation induces a determinate developmental program in the roots of *Arabidopsis thaliana*. *Plant Cell Physiol* 46: 174–184.
- Schulze S, Mant A, Kossmann J, Lloyd JR (2004). Identification of an *Arabidopsis* inorganic pyrophosphatase capable of being imported into chloroplasts. *FEBS Lett* 565: 101–105.
- Sivula T, Salminen A, Parfenyev AN, Pohjanjoki P, Goldman A, Cooperman BS, Baykov AA, Lahti R (1999). Evolutionary aspects of inorganic pyrophosphatase. *FEBS Lett* 454: 75–80.
- Softberry PlantPromDB. Website:<http://www.softberry.com> [accessed 21 Jan 2014].
- Sonnenwald U (1992). Expression of *E. coli* inorganic pyrophosphatase in transgenic plants alters photoassimilate partitioning. *Plant J* 2: 571–581.
- Sonnenwald U (2001). Control of potato tuber sprouting. *Trends Plant Sci* 6: 333–335.
- Stitt M (1998). Pyrophosphate as an energy donor in the cytosol of plant cells: an enigmatic alternative to ATP. *Bot Acta* 111: 167–175.
- The Arabidopsis Information Resource (TAIR). Website:<http://www.arabidopsis.org> [accessed 21 Jan 2014].
- Wasaki J, Yonetani R, Kuroda S, Shinano T, Yazaki J, Fujii F, Shimbo K, Yamamoto K, Sakata K, Sasaki T et al (2003). Transcriptomic analysis of metabolic changes by phosphorus stress in rice plant roots. *Plant Cell Environ* 26: 1515–1523.
- Weiner H, Stitt M, Heldt HW (1987). Subcellular compartmentation of pyrophosphate and alkaline pyrophosphatase in leaves. *Biochim Biophys Acta* 893: 13–21.
- Zhu JK (2002). Salt and drought stress signal transduction in plants. *Ann Rev Plant Biol* 53: 247–273.