DISSERTATION

Submitted to the

Combined Faculties

for the NATURAL SCIENCES and for MATHEMATICS

of the RUPERTO-CAROLA UNIVERSITY OF HEIDELBERG, GERMANY

for the degree of

DOCTOR OF NATURAL SCIENCES

Presented by

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8 June 2006

A FUNCTIONAL GENOMICS APPROACH TO THE PLANT SOLUBLE PYROPHOSPHATASE FAMILY

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LIST OF ABBREVIATIONS

3D	:	Three dimensional
% v/v	:	Volume percentage
% w/v	:	Weight percentage
A. thaliana	:	*Arabidopsis thaliana*
ABA	:	Abscisic acid
ABRE	:	ABA-related element
ADP	:	Adenosine diphosphate
A. tumefaciens	:	*Agrobacterium tumefaciens*
$AgNO_3$	:	Silver nitrate
AGPase	:	ADP-glucose pyrophosphorylase
ASP	:	*Arabidopsis thaliana* soluble pyrophosphatase
ATP	:	Adenosine triphosphate
AVP	:	*Arabidopsis thaliana* vacuolar pyrophosphatase
BAP	:	Benzyl amino purine
bp	:	Base pair
B. vulgaris	:	*Beta vulgaris*
Bsp1	:	*B. vulgaris* soluble pyrophosphatase isoform 1
Bvp1	:	*B. vulgaris* vacuolar pyrophosphatase isoform 1
Ca^{2+}	:	Calcium ion
CaMV	:	Cauliflower mosaic virus
$Ca(NO_3)_2$	:	Calcium nitrate
cDNA	:	Complementary DNA
Cl^-	:	Chloride ion
CLSM	:	Confocal laser scanning microscopy
cm	:	Centimeter
CO_2	:	Carbon dioxide
$CoCL_2$	:	Carbonyl chloride
$CuSO_4$	:	Copper sulfate
DEPC	:	Diethylpyrocarbonate
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP)
DTT	:	1,4-Dithiothreitol
E. coli	:	*Escherichia coli*

EDTA	:	Ethylenediaminetetraacetic acid
EGTA	:	Ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ER	:	Endoplasmic reticulum
EST	:	Expressed sequence tag
Fe-EDTA	:	Iron-ethylenediaminetetraacetic acid
Fru	:	Fructose
Fru1P	:	Fructose 1-phosphate
Fru6P	:	Fructose 6-phosphate
FW	:	Fresh weight
g	:	Standard acceleration of gravity
g	:	Gram
GFP	:	Green fluorescent protein
Glu	:	Glucose
Glu1P	:	Glucose 1-phosphate
Glu6P	:	Glucose 6-phosphate
GUS	:	β-glucuronidase
hr(s)	:	Hour(s)
H_3BO_3	:	Boric acid
HCl	:	Hydrochloric acid
H^+	:	Proton
Hepes	:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H^+-PPase	:	Membrane-bound inorganic pyrophosphatase
HRP	:	Horseradish peroxidase
IPTG	:	Isopropyl-β-D-thiogalactopyranoside
K^+	:	Potassium ion
KCl	:	Potassium chloride
$K_3[Fe(CN)_6]$	:	Potassium ferricyanide
$K_4[Fe(CN)_6]$	:	Potassium ferrocyanide
KH_2PO_4	:	Potassium dihydrogen phosphate
K_2SO_4	:	Potassium sulfate
kb	:	Kilo base
kDa	:	Kilo Dalton
KH_2PO_4	:	Potassium dihydrogen phosphate
K_m	:	Michaelis-Menten constant
KNO_3	:	Potassium nitrate
LB	:	Luria-Bertani (Growth medium)

M	:	Molarity
MES	:	2-(N-Morpholino)ethanesulfonic acid
Mg^{2+}	:	Magnesium ion
$MgCl_2$	:	Magnesium chloride
$MgSO_4$	:	Magnesium sulfate
min	:	Minutes
$MnSO_4$	:	Manganese sulfate
MOPS	:	Morpholinepropanesulfonic acid
mRNA	:	Messenger RNA
MS	:	Murashige & Skoog (Growth medium)
MW	:	Molecular weight
Na^+	:	Sodium ion
NaCl	:	Sodium chloride
Na_2CO_3	:	Disodium carbonate
NADP	:	Adenine dinucleotide phosphate
Na_2(EDTA)	:	Disodium EDTA
Na_2HPO_4	:	Disodium hydrogen phosphate dodecahydrate
$Na_2H_2P_2O_7$	:	Dinatrium dihydrogen pyrophosphate
Na_2MoO_4	:	Sodium molybdate
NaN_3	:	Sodium azide
NaOCl	:	Sodium hypochlorite
NaOH	:	Sodium hydroxide
$Na_2S_2O_3$ x $5H_2O$	:	Sodium thiosulfate
NO^{3-}	:	Nitrate
OD	:	Optical density
P	:	Phosphate
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PFK	:	Phosphofructokinase
PFP	:	Pyrophosphate:fructose 6-phosphate phosphotransferase
pH	:	Ionic strength
pI	:	Isoelectric point
P_i	:	Inorganic phosphate
PKC	:	Protein kinase C
PMSF	:	Phenylmethylsulphonylfluoride
PPase	:	Inorganic pyrophosphatase

PP_i	:	Inorganic pyrophosphate
R	:	Radius
RNA	:	Ribonucleic acid
RT	:	Room temperature
s	:	Seconds
SDS	:	Sodiumdedocylsulfate
$SO4^{2-}$	:	Sulfate anion
sPPase	:	Soluble pyrophosphatase
SuSy	:	Sucrose synthase
TAIR	:	The Arabidopsis information resource
TEMED	:	N,N,N,N'-Tetramethyl-ethylenediamine
T_m	:	Melting temperature (Annealing temperature)
tRNA	:	Transfer RNA
U	:	Units
UDP	:	Uridine diphosphate
UDPGlu	:	UDP-glucose
UGPase	:	UDP-glucose pyrophosphorylase
UTP	:	Uridine triphosphate
UTR	:	Untranslated region
UV	:	Ultraviolet
V-ATPase	:	Vacuolar ATPase
vol	:	Volume
vPPase	:	Vacuolar pyrophosphatase
WT	:	Wild type
X-GlcA	:	5-Bromo-4-chloro-3-idolyl-β-D-glucuronic acid
$ZnSO_4$	:	Zinc sulfate

ACKNOWLEDGEMENTS

I would like to thank to my supervisor Prof. Dr. Thomas Rausch and my project leader Dr. Steffen Greiner for giving me a chance to prove myself as a future scientist at their laboratory. I want to acknowledge their help and guidance throughout every step of this study.

I would like to thank to Prof. Dr. Markwart Kunz, Dr. Mohammad Munir, and Dr. Karsten Harms from Südzucker AG, Mannheim, Germany for their financial support and discussions on the project. I want to thank to Dr. Hinrich Harling from KWS Saat AG for providing transgenic sugar beet lines. I am grateful of Prof. Dr. Masayoshi Maeshima (Nagoya University, Japan) for supplying vacuolar pyrophosphatase antibody as a kind gift.

I would like to thank to Dr. Andreas Wachter and Sebastian Wolf for their patience on answering my endless questions and to my colleagues Jan Eufinger, Rolf Stratmann, Mirsada Kurtisi, Ulrike Halvax, Manuella Link and Roland Gromes for their help during experimental procedures.

I want to acknowledge our technicians Emilia Sancho-Vargas, Heike Steininger and Cornelia Walter and my friends Vladislava Galovic and Slobodanka Grsic-Rausch for their understanding and support throughout my three years stay in Germany.

My special thank is to my family; my father Yılmaz and my mother Nurhayat for their endless love and support during my journey in life, and to my husband Erhan, the guiding light of my life. Without them, I would not be where I am now.

1 SUMMARY

Although the activities of soluble pyrophosphatases were shown to be essential for a number of prokaryotes and eukaryotes, plants were assumed to lack cytoplasmic soluble pyrophosphatase activity and vacuolar membrane bound proton-pumping pyrophosphatase was accepted as the only 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 (ASP1, ASP2A, ASP2B, ASP3, ASP4 and ASP5), which were shown to be highly conserved both in nucleic and in amino acid sequences. Microscopic analysis of leaves transiently transformed with C-terminal GFP tagged *A. thaliana* sPPase proteins revealed localization in the cytoplasm and/or nucleus for five isoforms (ASP1 to ASP4) and the *in vivo* plastidial localization of ASP5 could be shown.

The analysis of stably transformed plants with promoter driven GUS expression revealed both tissue-specificity and developmental stage dependency of transcription for each *A. thaliana* soluble pyrophosphatase isoform. The promoter data were further confirmed by real time PCR analysis through which the active transcription of several *A. thaliana* soluble pyrophosphatase isoforms could be shown in all plant tissues, including later stages of development.

The regulation of the expression of *A. thaliana* soluble pyrophosphatases by soluble sugars was analyzed using *A. thaliana* cell cultures and an *in planta* approach. A specific induction of the expression of ASP2B in response to sugar starvation was observed. Furthermore, the results indicate the possibility of sucrose regulation of the expression of ASP3, while the transcription of plastidial isoform (ASP5) was induced in response to 100 mM glucose *in planta*.

The changes in the expression of *A. thaliana* soluble pyrophosphatases in response to ABA and different environmental stresses were analyzed using real time PCR. The data revealed an isoform- and/or tissue-specific and time dependent stress response suggesting specific roles for each isoform *in vivo*. Based on the data, the possible role of ASP3 in regulation of UGPase activity could be proposed.

The wounding experiments using sugar beet (*Beta* vulgaris) plants overexpressing either Bsp1 (*Beta vulgaris* soluble pyrophosphatase isoform 1) or Bvp1 (*Beta vulgaris* vacuolar pyro-

phosphatase isoform 1) revealed the possibility of post-transcriptional regulation of mRNA levels of both soluble and vacuolar pyrophosphatases. Using recombinant Bsp1 protein expressed in *Escherichia coli*, the *in vitro* phosphorylation by protein kinase C could be shown and the possibility of post-translational regulation of plant soluble pyrophosphatase activity by phosphorylation was discussed.

The overexpression of neither Bsp1 nor Bvp1 caused a significant effect on the sucrose accumulation of sugar beet taproots. On the other hand, the heterologous overexpression of not only Bvp1 but also Bsp1 revealed an impaired root growth and a possible contribution to the salt tolerance of *A. thaliana*.

In summary, the results suggest that the plant soluble pyrophosphatases can perform multiple and vital functions during plant development and stress responses. In addition, the expression patterns of plant soluble pyrophosphatases indicate a strong link to the plant carbohydrate metabolism.

ZUSAMMENFASSUNG

Obwohl die Aktivität löslicher Pyrophosphatasen (sPPasen) essentiell für eine Reihe von Pro- und Eukaryonten ist, wurde für Pflanzen lange Zeit angenommen, dass sie keine im Zytosol löslichen Pyrophosphatasen besitzen und die membran-gebundene Protonen-transportierende vakuoläre Pyrophosphatase das einzige Enzym ist, welches das als Nebenprodukt verschiedener Biosynthesewege anfallende Pyrophosphat im Zytosol beseitigen könnte. Allerdings finden sich im Genom von *Arabidopsis thaliana* sechs lösliche Pyrophosphatase-Isoformen (ASP1, ASP2A, ASP2B, ASP3, ASP4 und ASP5), welche sowohl auf Nukleinsäure- als auch auf Aminosäure-Ebene hoch konserviert sind. Die mikroskopische Analyse von Blätten, welche transient mit C-terminalem GFP-Fusionsprotein der Arabidopsis sPPasen transformiert wurden, zeigt dass fünf Isoformen (ASP1-ASP4) im Zytoplasma und/oder dem Kern und eine Isoform (ASP5) in den Plastiden vorkommen.

Durch Analyse von stabil mit Promotor:GUS-Konstrukten transformierten Pflanzen lässt sich die Gewebs-spezifische und entwicklungsabhängige Transkription für jede sPPase aus *A. thaliana* zeigen. Diese Daten werden unterstützt durch eine quantitative "real time PCR"-Analyse, über die Transkripte der verschiedenen sPPase-Isoformen in allen untersuchten Pflanzenorganen (auch in späten Entwicklungsstadien) nachgewiesen werden konnten. Die Regulation der Expression von sPPasen aus *A. thaliana* durch lösliche Zucker wurde mit Hilfe von Arabidopsis-Zellkulturen und einem *in planta*-Ansatz untersucht. Dabei zeigte sich, dass ASP2B spezifisch durch Zucker-Mangel induziert wird. Außerdem deuten die Ergebnisse auf eine mögliche Saccharose-Regulation der Expression von ASP3, während die Transkription der plastidären Isoform (ASP5) *in planta* durch 100mM Glukose induziert wird.

Mittels "real time PCR" wurden auch Änderungen in der Expression der *A. thaliana* sPPasen in Antwort auf ABA und unterschiedliche Stresssituationen analysiert. Die Ergebnisse zeigen sowohl eine Isoform- und Gewebe-spezifische als auch eine zeitabhängige Stress-Antwort, was eine spezifische Rolle jeder einzelnen Isoform *in vivo* nahe legt. Anhand der Daten lässt sich eine mögliche Rolle der ASP3-Isoform, reguliert durch die Aktivität der UGPase, vorschlagen.

Verwundungsexperimente mit Zuckerrüben-Pflanzen (*Beta vulgaris*) die entweder Bsp1 (Beta vulgaris soluble pyrophosphatase isoform 1) oder Bvp1 (Beta vulgaris vacuolar pyrophosphatase isoform 1) überexprimieren zeigen die Möglichkeit der post-transkriptionalen Regulation der mRNA-Mengen von beiden Genen.

Rekombinant in *Escherichia coli* hergestelltes BSP1-Protein wird *in vitro* durch Protein Kinase C phosphoryliert und die mögliche post-translationale Regulation löslicher Pyrophosphatase-Aktivität durch Phosphosylierung wird diskutiert.

Weder die Überexpression von Bsp1 noch von Bvp1 führte zu einem signifikanten Effekt auf die Saccharose-Akkumulation in Zuckerrüben-Speicherwurzeln. Auf der anderen Seite führte die heterologe Expression von Bvp1 und auch von Bsp1 zu einem veränderten Wurzelwachstum und möglicherweise zu veränderter Salztoleranz in *A. thaliana*.

Zusammenfassend lässt sich sagen, dass die Ergebnisse vielfältige und lebenswichtige Funktionen pflanzlicher, löslicher Pyrophosphatasen während der Entwicklung und während Stressantworten nahe legen. Außerdem deuten die Expressionsmuster der löslichen Pyrophosphatasen auf eine starke Vernetzung mit dem pflanzlichen Kohlenhydrat-Metabolismus.

2 INTRODUCTION

Plants are unique organisms; they are photoautotroph and immobile. Thus, they evolved special mechanisms enabling the fine regulation of common metabolic pathways also present in other eukaryotes or increasing their chance of survival during changes in their environment.

Although the significance of inorganic pyrophosphate and inorganic pyrophosphatases are well studied in prokaryotes and animals, recent data indicate that what is known is mostly not applicable for plants. Thus, this study mainly focuses on the understanding of plant inorganic pyrophosphatases and the importance of inorganic pyrophosphate in plant metabolism and in response to environmental responses.

2.1 Inorganic Pyrophosphate

Pyrophosphate is a simple chemical structure composed of two metaphosphate groups linked by an oxygen anion (P-O-P structure) which is called a pyrophosphate bond. Thereby, in addition to the universal energy currency ATP, pyrophosphate also stores readily usable energy for biochemical reactions.

Inorganic pyrophosphate is a by-product of the activation or polymerization steps of several vital anabolic reactions in the plant cell like the synthesis of nucleic acids (DNA and RNA), carbohydrates, proteins, carotenoids, amino acids, and fatty acids (Geigenberger et al., 1998; Stitt, 1998; Rojas-Beltran et al., 1999; Farre et al., 2001a; Sonnewald, 2001; Lopez-Marques et al., 2004). Its removal is essential to make these reactions thermodynamically irreversible in the direction of biosynthesis, since the steps leading to the formation of inorganic pyrophosphate are reversible in nature and operate at near-equilibrium (Geigenberger et al., 1998; Lopez-Marques et al., 2004). In addition, the hydrolysis of PP_i is required to renew inorganic phosphate (P_i) concentration in the cell which is used for ATP replenishing (Perez-Castineira et al., 2001b).

The PP_i metabolism is a central and distinctive feature of plants, however, the role and significance of PP_i in plant growth and development and why plants differ from other organisms with respect to tight control of cytosolic PP_i concentration is still unknown (Jelitto et al., 1992).

Two main characteristics of PP_i-utilizing reactions complicate investigations of the role of PP_i in the cytosol of plant cells. First, each PP_i-utilizing reaction operates in parallel with an ATP-utilizing reaction. Since ATP-utilizing enzymes are present in the same tissues with PP_i-utilizing enzymes, it is difficult to distinguish the contribution of the PP_i and the ATP-dependent reactions (Geigenberger et al., 1998; Stitt, 1998). Secondly, the PP_i-utilizing reactions are close to or at their thermodynamic equilibrium *in vivo*, which means it is not clear if they are operating in parallel with ATP-utilizing reactions, or in the opposite direction to generate PP_i (Jelitto et al., 1992; Geigenberger et al., 1998; Stitt, 1998).

2.1.1 Inorganic Pyrophosphate as an Energy Source

The PP_i can be an important molecule with respect to cellular bioenergetics. There is even a hypothesis which suggests that PP_i is the predecessor of ATP as the "energy currency" during the early stages of biochemical evolution (Perez-Castineira et al., 2001b; Perez-Castineira et al., 2001a).

The hydrolysis of PP_i releases a free energy of about half of that of ATP which can allow PP_i to act as a source of energy in cellular processes ($\Delta G^{\circ'} = -33.5$ kJmol^{-1}). There are at least three enzymes in the plant cell that use PP_i as an energy source: (i) a pyrophosphate-dependent proton pump functioning in the tonoplast energization, (ii) UDP-glucose pyrophosphorylase (UGPase) during sucrose degradation via sucrose synthase (UDPGlu + $PP_i \leftrightarrow$ G6P + UTP), and (iii) pyrophosphate:fructose 6-phosphate phosphotransferase (PFP) in glycolysis (Weiner et al., 1987; du Jardin et al., 1995; Geigenberger et al., 1998; Stitt, 1998; Rojas-Beltran et al., 1999). The reactions mentioned are ubiquitous in plants and known to be strongly regulated.

For a long time pyrophosphate was considered to be only a waste product of anabolism. However, it is highly possible that PP_i contributes to the flexibility of plant metabolism to respond to a changing environment. Provided that adequate PP_i supply is present, substitution of ATP-utilizing reactions with PP_i-utilizing reactions under energy limiting conditions may allow ATP to be conserved and might improve plant cell performance (Stitt, 1998).

2.1.2 Inorganic Pyrophosphate in Plant Carbohydrate Metabolism

2.1.2.1 Carbohydrate Metabolism in Plants

Plants are photoautotrophic organisms. They have a complex carbon metabolism since they must feed heterotrophic tissues (like roots, flowers, seeds and developing leaves) with the organic carbon reduced from inorganic compounds using light energy in phototrophic tissues.

In the presence of light, carbon is fixed via photosynthesis in the leaves and more than half of it is stored as starch whereas the rest is converted to cell wall components, protein and lipids or accumulated as cationic or anionic compounds. The photosynthate in the form of sucrose is then remobilized and translocated to sink organs via phloem (Sonnewald, 1992; Lerchl et al., 1995a; Chiou and Bush, 1996).

Photosynthesis and many of the biosynthetic reactions of plants including starch and lipid biosynthesis take place in plastids. However, some biosynthetic pathways of plant metabolism occur both in cytosol and plastids, and this requires the fine regulation of pathway flux in response to changes in light, carbon dioxide (CO_2) supply or sucrose export and presence of several unique transporters between different compartments (Dennis and Blakeley, 2000). Starch synthesis in parenchyma cells is a good example for a pathway occurring in two different compartments (plastids and the cytosol) and thereby requiring fine regulation (Figure 2.1). Regulation mainly occurs via regulatory circuits acting on key enzymes of photosynthetic metabolism in response to changes in metabolite levels (Geigenberger, 2003).

2.1.2.1.1 Sucrose Metabolism

Sucrose is the major product of CO_2 fixation during photosynthesis and the main form of carbon in long-distance transport between plant tissues. It can be stored for either a short- or long-term period depending on the requirement of the plant. Short-term storage of sucrose occurs in vacuoles of photosynthetically active cells at times of high photosynthetic activity and saturated phloem capacity (Echeverria, 1998). Long-term storage refers to storage of sucrose before prolonged dormant periods as massive accumulation to specific plant organs like taproot of sugar beets (Echeverria, 1998; Dennis and Blakeley, 2000).

Since sucrose is the main end product of photosynthesis, it was assumed that sucrose synthesis takes place in the chloroplasts (the green photosynthetic plastids) (Dennis and Blakeley, 2000). However, it was proved that triose phosphates are transported from chloroplasts to the cytosol for hexose production and sucrose synthesis in this compartment (Dennis and Blakeley, 2000).

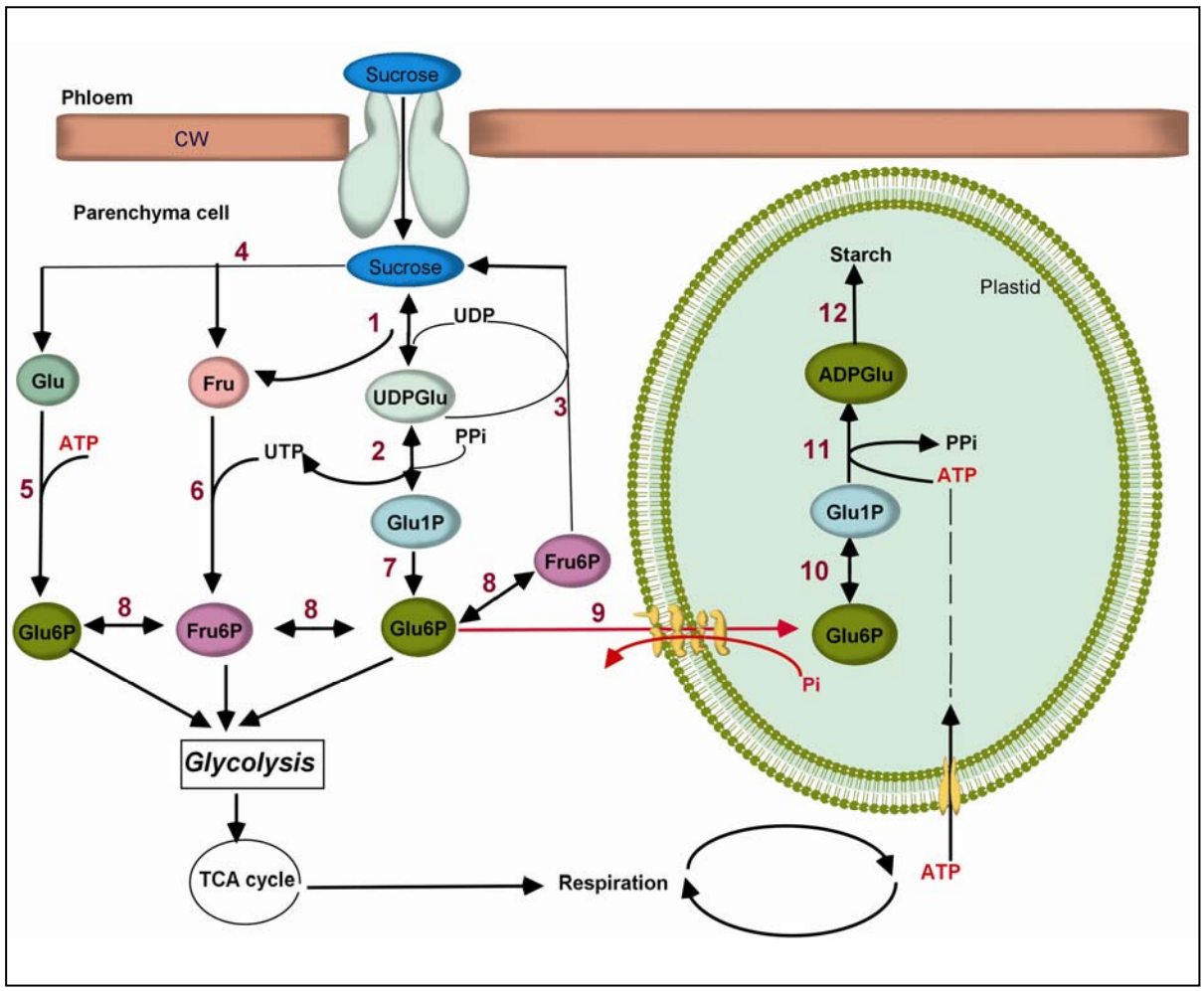

Figure 2.1 Schematic represantation of sucrose translocation and sucrose and starch metabolism in parenchyma cells. CW; Cell wall, (1) Sucrose synthase, (2) UDP-glucose pyrophosphorylase, (3) Sucrose phosphate synthase, (4) Invertase, (5) Hexokinase, (6) Phosphofructokinase, (7) Phosphoglucomutase, (8) Cytosolic phosphoglucose isomerase, (9) Hexose phosphate translocator, (10) Plastidial phosphoglucomutase, (11) ADP-glucose pyrophosphorylase, (12) Starch synthase and starch branching enzymes (Geigenberger, 2003).

Sucrose synthase (SuSy) is one of the key enzymes involved in sucrose synthesis/metabolism (Figure 2.1). It catalyzes a readily reversible reaction *in vivo*; i.e., it is capable of synthesis and degradation of sucrose (Geigenberger and Stitt, 1993). Most plant species have been shown to contain at least two isoforms of SuSy with distinct developmental- and organ-

specific expression patterns (Dennis and Blakeley, 2000). It was shown that the expression of sucrose synthase is increased by sucrose, anaerobiosis and wounding (Sturm and Tang, 1999). In general, cleavage of sucrose by SuSy activity is associated with anabolic processes, however, it appears that the main action of sucrose synthase is degradation rather than synthesis of sucrose (Geigenberger and Stitt, 1993; Sturm and Tang, 1999; Dennis and Blakeley, 2000).

The flow of sucrose into a tissue (sink strength) is regulated by the rate of sucrose utilization. The fate of sucrose from thereon is determined by the three enzymes known to be capable of metabolizing the sucrose transported from source organs to sink tissues: cell wall invertase (a hydrolase), cytosolic invertase and sucrose synthase (a glycosyl transferase) (Dennis and Blakeley, 2000). The sucrose can enter the cell in two forms; either transported as it is or converted to glucose and fructose by the action of cell wall invertase. Since the gene regulation by carbohydrates depends on the form of carbohydrate, the means of sucrose transported into the cell is particularly important (Fernie et al., 2002). The precise roles of invertases and sucrose synthase in the degradation of sucrose are not clear since both occur in the same tissue, however, the dominance of invertases during the early stages of tuber initiation, and sucrose synthase in the developing tuber of potato was shown (Ap Rees, 1992; Dennis and Blakeley, 2000; Fernie et al., 2002).

The main differences in the sucrose cleavage via sucrose synthase- or invertase-dependent pathways are the energy donor dependence of the pathway and the end products of degradation. The action of invertase is irreversible and degradation of sucrose to hexoses (glucose and fructose) via invertase-dependent pathway requires ATP. On the other hand, sucrose synthase is a reversible enzyme and SuSy-dependent sucrose cleavage pathway expenses pyrophosphate (PP_i). Degradation of sucrose via invertase leads to the glucose and fructose which require phosphorylation to enter hexose phosphate pool. The action of sucrose synthase ends up with fructose and UDP-glucose (UDPGlu) which is the form of carbon entering to the cell wall polysaccharide synthesis (Dennis and Blakeley, 2000).

The presence of two separate sucrose degradation pathways in plants adds another level of metabolic control. The carbohydrates are known to function in gene regulation (Fernie et al., 2002) and therefore the action of different sucrose cleavage enzymes may directly or indirectly affect different processes, such as carbohydrate metabolism, assimilate partitioning, osmoregulation, adaptation to cold and low oxygen levels, response to wounding and infection and development.

2.1.2.1.2 Starch Metabolism

Starch is the polymer of glucose and the main storage form of carbohydrate in plants. It is synthesized and stored in plastids; temporarily in chloroplasts and for longer periods in amyloplasts of storage tissues such as tubers and seeds. Transitory starch is a reserve for reduced carbon and it is degraded to supply energy and carbon for metabolism during dark periods. Stored starch usually accumulates in one developmental phase to be used during a subsequent one (Caspar, 1992; Dennis and Blakeley, 2000).

The major regulatory enzyme of the starch biosynthesis pathway is the ADP-glucose pyrophosphorylase (AGPase) (Geigenberger, 2003). The enzyme is sophistically regulated at three different levels; at the level of gene expression, post-translationally and by allosteric regulation (Tiessen et al., 2002). In photosynthetic tissues, AGPase is activated by 3-phosphoglycerate and inhibited by inorganic phosphate (P_i). Thus, it is the P_i/3-phosphoglycerate ratio that determines the flux of starch synthesis in green tissues (Geigenberger, 2003). This allosteric regulation operates in a time frame of seconds to adjust the rate of starch synthesis to the balance between sucrose breakdown and respiration (Tiessen et al., 2002). The expression of AGPase was shown to be regulated positively by increasing amounts of sucrose and negatively by increasing amounts of nitrate and phosphate (Dennis and Blakeley, 2000; Geigenberger, 2003). This transcriptional regulation allows more gradual changes in AGPase activity, which may require days to develop (Tiessen et al., 2002). The last mechanism is the redox regulation which changes AGPase activity in a time frame of 30 to 60 minutes by oxidative inactivation or reductive activation. The activation of AGPase was shown to occur in response to factors directly or indirectly related to increased sucrose availability, which leads to stimulation of starch synthesis and decreased glycolytic metabolite levels (Tiessen et al., 2002).

Although starch synthesis is well studied, there is not much known about how starch is remobilized, and how both synthetic and degradative pathways are controlled and integrated with other pathways of metabolism, especially with sucrose metabolism (Zeeman et al., 2004).

2.1.2.2 Function of Inorganic Pyrophosphate in Carbohydrate Metabolism

There are four fundamental steps in plant carbohydrate metabolism with the participation of inorganic pyrophosphate: (i) sucrose breakdown via sucrose synthase requires pyrophosphate as a co-substrate, (ii) it is produced during sucrose synthesis by the action of UGPase (G1P +

UTP ↔ UDPGlu + PP_i), (iii) PP_i is produced during starch biosynthesis by the action of AGPase, and (iv) it participates in glycolysis during the reaction catalyzed by PFP (Farre et al., 2001a; Sonnewald, 2001). The reaction catalyzed by PFP is interesting as it depends on the ratio of P_i to PP_i, PP_i being a powerful inhibitor of the reverse reaction and P_i being a strong non-competitive inhibitor of the forward reaction (Stitt, 1998; Sonnewald, 2001).

As indicated in Chapter 2.1.2.1, sucrose can be cleaved into its components by the action of invertase or sucrose synthase. The most striking feature of sucrose degradation via invertase-dependent or sucrose synthase-dependent pathways is the difference in the dependence of an energy donor; invertase-dependent cleavage requires the expense of ATP for phosphorylation of degradation products of sucrose (glucose and fructose), whereas degradation via SuSy saves ATP and uses PP_i instead, leading to fructose and UDPGlu. Currently, there are not enough data on the mechanism that coordinates the shift in metabolism between invertase and SuSy-dependent sucrose degradation pathways, however, the potential involvement of PP_i cannot be ruled out (Farre et al., 2001a).

At least in potato tubers, there are clear data indicating the involvement of PP_i in integrating the pathways of cytosolic sucrose breakdown and plastidial starch biosynthesis (Farre et al., 2000). Transgenic potato tubers overexpressing yeast invertase in their cytosol alone or in combination with a bacterial glucokinase have increased levels of PP_i (Lerchl et al., 1995a). The inhibition of AGPase activity, thereby starch biosynthesis, by an antisense RNA approach led to lower levels of PP_i in potato tuber extracts (Farre et al., 2000). Transgenic potato lines displaying an inhibition of the AGPase activity in combination with the expression of an invertase in the cytosol contained intermediate levels of PP_i (Farre et al., 2000). The data from these three transgenic approaches suggest the integrating role of PP_i between SuSy-dependent breakdown of sucrose, starch synthesis and glycolysis in a starch storing tissues like potato tuber. In summary, during the early phases of development invertases are active, resulting in the accumulation of PP_i. The high levels of PP_i with a yet unknown mechanism inhibit starch synthesis and activate glycolysis via PFP. This switch from invertase to SuSy-dependent sucrose mobilization results in decreased levels of PP_i due to increased usage by the SuSy pathway. However, the conclusion is based on potato which has a strong sink strength and thereby is not easy to generalize (Stitt, 1998; Farre et al., 2000).

The production of PP_i can be a crucial regulatory and controlling step in coordinating the shift from SuSy to invertase dependent sucrose degradation in the course of shifting from sink to source tissue (Farre et al., 2001a). After the discovery, in conjunction with phosphofructokinase (PFK), PFP was considered as generating PP_i. However, the data obtained through transgenic

approaches using PFP-antisense potato plants indicated that PFP is more likely to consume PP_i rather than generate it (Rojas-Beltran et al., 1999). Another study with transgenic potato tubers points out the possibility of the function of AGPase in the generation of PP_i (Farre et al., 2000). The most likely candidate for the source of PP_i required for sucrose breakdown is the PP_i released during the synthesis of proteins and nucleic acids. Although all these mechanisms can contribute to PP_i production in cytosol, it is clear that none of them is a universally essential source (Ap Rees, 1992).

2.2 Inorganic Pyrophosphatases

Inorganic pyrophosphatases (pyrophosphate phosphohydrolase) (EC 3.6.1.1) are ubiquitous enzymes hydrolyzing inorganic pyrophosphate into two inorganic phosphates (Geigenberger et al., 1998; Stitt, 1998). The activity of pyrophosphatases is essential for life since their role is to remove the by-product PP_i. This provides a thermodynamic pull for biosynthetic reactions by making them essentially irreversible (Sivula et al., 1999; Schulze et al., 2004).

The cytosol of higher plant cells contains a considerably high concentration of pyrophosphate (200 – 300 μM) with little or no inorganic pyrophosphatase activity (du Jardin et al., 1995; Stitt, 1998; Farre et al., 2001b). The importance of the cytosolic pyrophosphate pool is further proven by the observation that overexpression of a pyrophosphatase of bacterial origin in the cytosol of tobacco and potato plants lead to significant alterations in metabolism, growth and development (Sonnewald, 1992).

2.2.1 Types of Inorganic Pyrophosphatases

Two main types of inorganic pyrophosphatases (PPases) which are structurally and functionally different from each other have been characterized to date: (i) soluble (sPPases) and (ii) membrane-bound proton-translocating inorganic pyrophosphatases (H^+-PPases) (Rojas-Beltran et al., 1999; Perez-Castineira et al., 2001b; Lopez-Marques et al., 2004). Both types of inorganic pyrophosphatases show a strict requirement for magnesium ions (Mg^{2+}) and are known to be reversibly inhibited by calcium ions (Ca^{2+}) (Rojas-Beltran et al., 1999). Magnesium ions are required as a cofactor for the stability of the holoenzyme composed of six (for prokaryotes) or two (for eukaryotes) identical monomers. Magnesium is also required as an activator and because the

substrate is an Mg^{2+}-PPi complex (Perez-Castineira et al., 2001a). However, it is not clear if changes in the concentration of Mg^{2+} and Ca^{2+} have a regulatory function with respect to enzymatic activity *in vivo* (Jelitto et al., 1992; Stitt, 1998).

2.2.2 Soluble Pyrophosphatases

Soluble pyrophosphatases are ubiquitous enzymes that hydrolyze pyrophosphate resulting in the loss of the energy of pyrophosphate bond as heat, however, their activity is essential for the fate of anabolism (Perez-Castineira et al., 2001b; Lopez-Marques et al., 2004). With respect to localization, soluble pyrophosphatases can be found in the cytosol, plastids or mitochondria depending on the organism.

There are two families of soluble pyrophosphatases. Family I includes most of the known sPPases, whereas family II includes sPPase of *Bacillus subtilis* as well as putative sPPases of four other bacterial species. The two families do not show any sequence similarity with each other and family II has a distinct metal ion dependence (Young et al., 1998; Sivula et al., 1999). Figure 2.2 gives the phylogenetic tree of family I soluble pyrophosphatases based on residues at the active site and subunit interfaces. The family I soluble pyrophosphatases are the focus of the following chapters.

The eukaryotic and prokaryotic soluble pyrophosphatases differ from each other in several ways. Both enzymes do function as homooligomers; however, the subunits of prokaryotic sPPases are significantly smaller (approximately 20 kDa) than those of higher organisms (ca. 32 kDa) (Sivula et al., 1999). Furthermore, prokaryotic sPPases are functional as homohexamers arranged as a dimer of trimers, whereas eukaryotic sPPases act as homodimers. Therefore, the subunit interfaces of prokaryotic and eukaryotic sPPases are completely different (Sivula et al., 1999).

The phylogenetic analysis in Figure 2.2 indicates that plant pyrophosphatases bear a close similarity to prokaryotic sPPases rather than animal and fungal sPPases with respect to residues at the active site and subunit interfaces (Sivula et al., 1999). In the plant soluble pyrophosphatase family, there are both ‘eukaryotic-like’ and ‘prokaryotic-like’ sPPases both of which are nuclear encoded. Plastidial plant sPPase is more ‘eukaryotic-like’, compared to ‘prokaryotic-like’ cytosolic soluble pyrophosphatases (Perez-Castineira et al., 2001b). The endosymbiosis hypothesis states plastids as evolutionary descendants of blue-green algae. It is reasonably clear that the original chloroplast endosymbiont was some kind of oxygenic photosynthetic prokaryote.

Through the evolution, much of the original endosymbiont genomes has been confiscated by the nucleus or discarded. Thus, it is possible that plant 'prokaryotic-like' sPPase genes have been introduced into the ancestral eukaryotic cell by one of the bacterial endosymbionts and transferred to the nuclear genome. This idea is further supported by the fact that animal and fungal sPPases have no 'prokaryotic-like' isoforms (Rojas-Beltran et al., 1999).

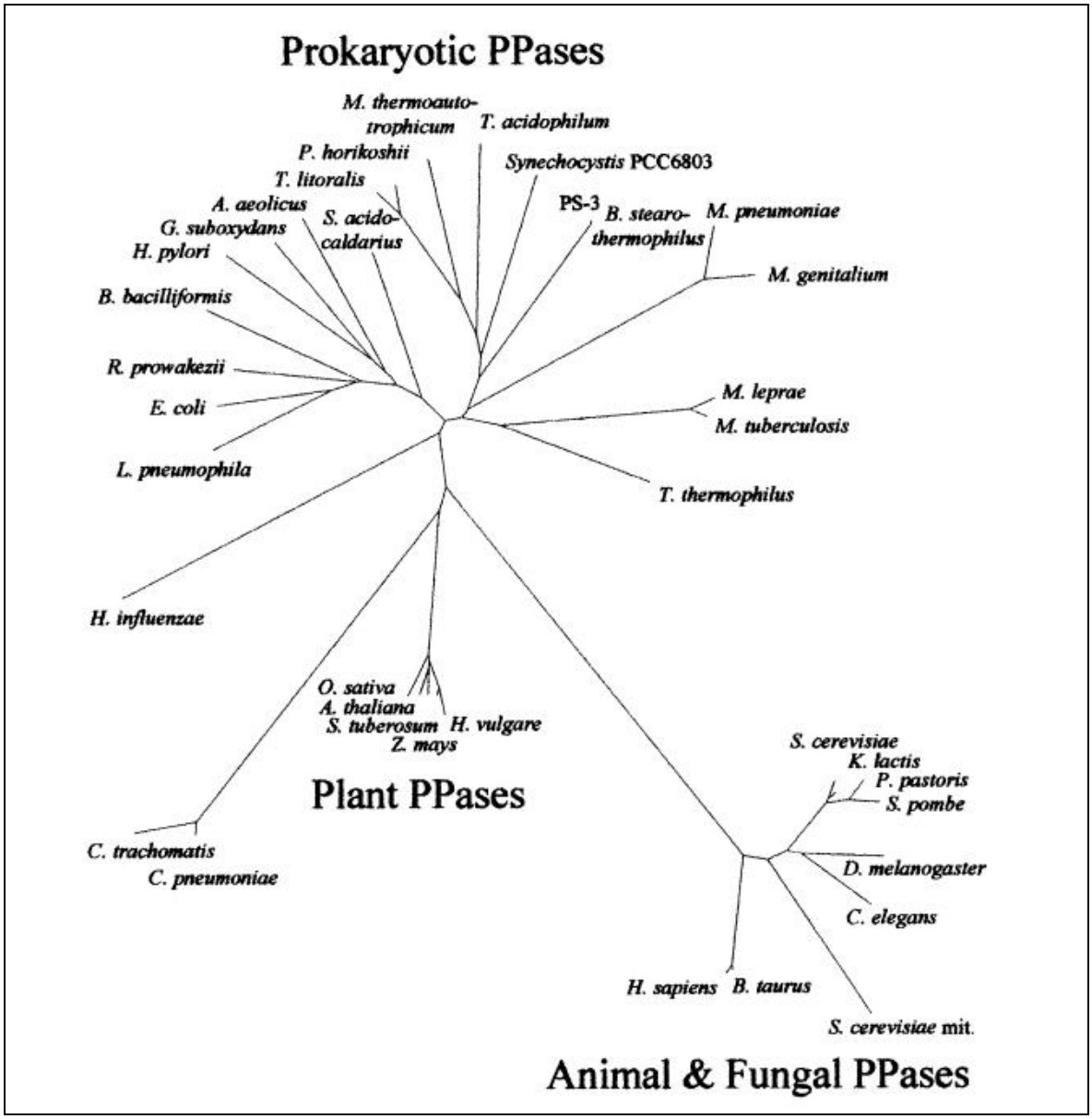

Figure 2.2 Phylogenetic tree of known family I soluble pyrophosphatases. The phylogenetic analysis was performed based on the alignment of amino acid residues at the active site and at the subunit interfaces (Sivula et al., 1999).

2.2.2.1 Plastidial Plant Soluble Pyrophosphatases

Plastidial plant soluble pyrophosphatases are found to be slightly larger than other soluble isoforms. All plant sPPases so far studied contain two stretch of amino acids missing in protein sequences in comparison with animal and fungal sPPases, however, plastidial sPPases does not have these deletions (Schulze et al., 2004).

The hypothesis that by being photosynthetic organisms, plants should have soluble pyrophosphatases in their mitochondria and plastids but not in their cytosol comes from several findings. First, as mentioned before, most of the biosynthetic reactions leading to the formation of PP_i as by-product are localized in plastids of plant cells. Since the accumulation would inhibit several vital reactions like purine, pyrimidine and starch synthesis, PP_i should be eliminated from environment. This can be achieved by two ways; either by the activity of soluble pyrophosphatase or by a translocator protein that exports PP_i out of the plastid. Measurements using fully developed spinach leaves showed that isolated plastids contain a very high PPase activity (at least 90 % of total soluble pyrophosphatase activity measured) and very low PP_i levels, but the cytosol contains relatively low PPase activity and a significant pool of PP_i (200 – 300 µM) (Weiner et al., 1987; Stitt, 1998). Therefore, it was concluded that most of the soluble pyrophosphatase activity in plants is located in plastids (Weiner et al., 1987; Geigenberger et al., 1998; Stitt, 1998; Rojas-Beltran et al., 1999; Schulze et al., 2004). However, there is currently no convincing data on the *in vivo* action of plastidial soluble pyrophosphatases. The second possibility is the rapid channeling back of PP_i produced to the cytosol. There is at least one report on a PP_i transporter located in the spinach chloroplast membrane supporting this alternative (Farre et al., 2000).

The plastidial *Arabidopsis thaliana* soluble pyrophosphatase was recently extensively studied (Schulze et al., 2004). The enzyme was predicted to be localized in plastids based on the signal peptide sequence. The localization is further proven by *in vitro* import experiments to intact plastids (Schulze et al., 2004). The optimal concentration of magnesium chloride ($MgCl_2$) which is required for activity was found to be 5 mM (Schulze et al., 2004). The pH optimum for the enzyme was measured to be 7,5 at this $MgCl_2$ concentration (Schulze et al., 2004). The K_m of plastidial *A. thaliana* sPPase was determined to be approximately 600 µM PP_i using Michaelis-Menten plot (Schulze et al., 2004). The main drawback of the study is the usage of a recombinant pyrophosphatase containing the signal peptide, which could interfere with the enzymatic properties of the enzyme (Schulze et al., 2004).

The plastidial soluble pyrophosphatase should be present and active in all plant tissues, as metabolic pathways producing PP_i are also present in all tissues. This is confirmed for *A. thaliana* plastidial sPPase isoform (Schulze et al., 2004). By using full clone as a probe, Northern blotting analysis indicated that the gene expression was highest in flowers, leaves and roots, and present to a lesser extent in siliques and stems in *A. thaliana* (Schulze et al., 2004). This is in contrast to an earlier study in which the expression of plastidial sPPase was not detected in roots using Northern blot analysis (Perez-Castineira et al., 2001b).

The response of plastidial sPPase to soluble sugars is important since plastidial sPPase is important for the fate of carbohydrate metabolism. Using the *A. thaliana* plastidial sPPase, the response to several metabolizable sugars (glucose, sucrose, fructose and sorbitol) was analyzed and it was stated that the expression of the plastidial sPPase was only responsive to 300 mM metabolizable sugar treatment but does not change in 100 mM sugar concentration (Schulze et al., 2004).

2.2.2.2 Mitochondrial Plant Soluble Pyrophosphatases

In bacteria, fungi and animal cells, mitochondrial sPPases were extensively studied and are clearly essential for the function of this organelle during respiratory growth (Vianello and Macri, 1999; Perez-Castineira et al., 2001b). The identification of mitochondrial soluble pyrophosphatases of animal and fungal cells is based on the presence of an extra four amino acid residues present in the active site of the enzyme. These extra residues are suggested to play a role in loosely linking the enzyme to the membrane. The mitochondrial sPPase is functional as a H^+-translocator in a protein complex attached to the inner mitochondrial membrane (Sivula et al., 1999). Therefore, mitochondrial sPPases are functionally related to membrane-bound pyrophosphatases whereas they are structurally similar to soluble pyrophosphatases. Thus, they are sometimes considered as a third group of pyrophosphatases (Lundin et al., 1991; Rojas-Beltran et al., 1999).

There is not much information available about the presence and function of mitochondrial soluble pyrophosphatases in plants. The first evidence for the presence of a sPPase in plant mitochondria came from to the isolation of submitochondrial particles from pea stem which are able to couple PP_i hydrolysis with the generation of an inside-positive proton gradient (Zancani et al., 1995). The mitochondrial sPPase isolated this way was shown to be associated with a protein complex at the inner mitochondrial membrane and to have a MW of about 35 kDa, a K_m value of

200 μM PP_i and an apparent H^+/PP_i stoichiometry of 2 (Zancani et al., 1995; Vianello et al., 1997; Casolo et al., 2002).

The presence of a sPPase in plant mitochondria and the physiological role of this protein is still a matter of speculation. There are PP_i-generating biosynthetic pathways that are active in plant mitochondria such as DNA synthesis and butyrate activation. This necessitates the removal of PP_i to prevent accumulation which otherwise can inhibit the metabolism. This can be achieved by either with an action of a soluble pyrophosphatase or by a transporter protein channeling PP_i to cytosol. There are no available data on the presence of a PP_i translocator protein in the mitochondrial membrane, thereby it is largely accepted that plant mitochondria should possess soluble pyrophosphatase activity (Perez-Castineira et al., 2001b).

2.2.2.3 Cytosolic Plant Soluble Pyrophosphatases

There is not much known about the soluble pyrophosphatases localized in plant cytosol. Since the cytosolic activity of sPPases has been shown to be low in fully developed spinach leaves (Weiner et al., 1987), they are accepted as not present or not functional in the cytosol (Stitt, 1998). Cytosolic sPPases might be important in young growing tissues where most of the PP_i generating biosynthetic reactions function at higher rates. However, the presence of membrane bound inorganic pyrophosphatases in plants contradicts with this idea. In general, vacuolar membrane bound H^+-translocating inorganic pyrophosphatase is accepted as the sole enzyme responsible for the removal of cytosolic PP_i in plants (Weiner et al., 1987; Farre et al., 2000).

du Jardin et al. (1995) isolated and characterized a soluble inorganic pyrophosphatase from potato that did not have any transit peptide and therefore predicted to be localized in cytosol. The *Arabiopsis thaliana* genome encodes six soluble inorganic pyrophosphatase (sPPase) isoforms (http://www.tair.org), one of which was recently shown to be localized in plastids (Schulze et al., 2004). The localization of other isoforms is currently unknown; however, they exhibit a very high homology to cytoplasmic potato soluble pyrophosphatase (du Jardin et al., 1995) and therefore predicted to be localized in the cytosol (Perez-Castineria et al., 2001b).

2.2.2.4 Effect of Soluble Pyrophosphatase Overexpression in Plant Metabolism

The first study on the effects of overexpression of a soluble pyrophosphatase in plant growth and development was performed by a reverse genetic approach through ectopic expression of *Escherichia coli* soluble pyrophosphatase in tobacco and potato cytosol where the main aim was to increase the partition of carbohydrate fixation during photosynthesis by decreasing the PP_i pool in the cytosol. The approach was based on the hypothesis that the main pyrophosphatase activity in plants is localized in plastids (Jelitto et al., 1992; Sonnewald, 1992).

Tobacco and potato plants overexpressing *E. coli* soluble pyrophosphatase driven by cauliflower mosaic virus (CaMV) 35S promoter revealed a decreased PP_i content in their cytosol which was in proportion with the amount of pyrophosphatase activity measured (Jelitto et al., 1992; Sonnewald, 1992).

Analysis of source leaves of transgenic tobacco plants showed an increase of free hexoses which was explained by altering the balance of reactants in the equilibrium reaction catalyzed by UGPase (Stitt, 1998). The degree of increase was found to be dependent on the developmental stage. Thus, in sink leaves only a small increase was detected, whereas in source leaves the increase was more dramatic. Source leaves, accumulating almost 100 times more soluble sugars than the wild type, were thicker than wild type leaves and characterized by bleaching. In potato leaves, no significant increase of soluble sugars was observed (Jelitto et al., 1992; Sonnewald, 1992).

Expression of a bacterial soluble pyrophosphatase in the cytosol led to dramatic changes in growth and development of transgenic tobacco plants (Jelitto et al., 1992; Sonnewald, 1992). They showed a phenotype with a stunted growth caused by reduced internode length. On the other hand, the growth of transgenic potato plants was found to be almost not affected. The only changes observed were the increased number of stolons and tubers with a reduced fresh weight obtained from transgenic potato plants (Jelitto et al., 1992; Sonnewald, 1992). Tubers from the transgenic potato plants contained more sucrose and less starch than wild type, therefore the decreased tuber size was explained as the consequence of a decreased use of sucrose (Jelitto et al., 1992; Sonnewald, 1992). The differences found between transgenic tobacco and potato plants were explained by the presence of strong sink organs (tubers and stolons) in potato plants. In addition, the transgenic potato tubers contained slightly lower ATP and higher ADP levels. This was indicated as a rather unexpected finding by the authors and left unexplained (Jelitto et al., 1992; Sonnewald, 1992).

It was concluded that introducing a cytosolic soluble pyrophosphatase into plants results in an increased sucrose/starch ratio, accumulation of carbohydrates in the leaf, perturbed sink strength, increased side-shoot development and large numbers of small low-starch tubers in potato, proving that PP_i plays an important role in plant metabolism (Sonnewald, 1992). However, it is not clear if the observed changes are direct or indirect effects of low cytosolic PP_i. Changes in the PP_i pool can affect several metabolic pathways, and therefore pleiotropic effects are expected (Jelitto et al., 1992; Sonnewald, 1992).

The results of these first studies (Jelitto et al., 1992; Sonnewald, 1992) indicated that the export of photoassimilates was also affected by a decreased cytosolic PP_i pool and led to the hypothesis that PP_i might also be essential for sucrose transport. In order to investigate the putative role of PP_i in phloem transport, *E. coli* soluble pyrophosphatase driven by the phloem specific rolC promoter from *Agrobacterium rhizogenes* was introduced to tobacco plants (Lerchl et al., 1995a).

The analysis of transgenic tobacco plants indicated that the removal of PP_i in phloem cells led to the accumulation of starch in source leaves (decreased sucrose/starch ratio), accumulation of soluble sugars and a lower ATP/ADP ratio (Lerchl et al., 1995a). The removal of PP_i by CaMV35S promoter driven overexpression of *E. coli* soluble pyrophosphatase in mesophyll cells resulted in a higher sucrose/starch ratio by directing photoassimilate flow towards sucrose synthesis (Jelitto et al., 1992; Sonnewald, 1992). On the other hand, the removal of PP_i in phloem cells inhibited the export of assimilates leading to a strong accumulation of starch in photosynthetically active cells (Lerchl et al., 1995a). Therefore, it was concluded that cytosolic PP_i is essential for long-distance sucrose transport and the authors claimed that to maintain a proton gradient across the membrane of companion cells which is required for proton sucrose co-transport, a small proportion of the incoming sucrose has to be hydrolyzed via sucrose synthase in the phloem tissue (Lerchl et al., 1995a; Stitt, 1998).

A detailed study to understand the effect of ectopic expression of *E. coli* soluble pyrophosphatase was performed using transgenic potato plants (Jelitto et al., 1992; Sonnewald, 1992). The initial analysis of potato plants showed that removal of PP_i led to a complex phenotype with changes of metabolites in primary metabolism, in carbohydrate levels, in growth rates and in sink-source relations (Jelitto et al., 1992; Sonnewald, 1992). This was fitting well to the model that change in cytosolic PP_i pool alters the balance of reactants in the equilibrium reaction catalyzed by UGPase. However, the measurement of sucrose mobilization in transgenic potato tubers indicated that low PP_i did not restrict UGPase activity (Geigenberger et al., 1998). Further contradicting with the hypothesis was the observations that there was no change in the concentra-

tions of key substrates and products of SuSy, UGPase and AGPase in growing potato tubers (Geigenberger et al., 1998). The activity of AGPase and SuSy, surprisingly, proved to be stimulated rather than inhibited in transgenic plants (Geigenberger et al., 1998). Many of the enzymes that show increased activity in transgenic potato plants are known to be induced by sugars, therefore, the increased levels of sugars could lead to induction of enzymes involved in sucrose-starch conversions and carbohydrate breakdown (Geigenberger et al., 1998).

The removal of PP_i was expected to decrease glycolysis due to the restriction of sucrose mobilization, caused by decreased levels of phosphorylated intermediates and the lower levels of P_i which acts as a substrate for PFP. On the contrary, the measurements on the transgenic potato plants indicated a higher glycolysis rate and ATP/ADP ratio (Geigenberger et al., 1998). The increase in ATP/ADP ratio was explained by the outcome of increased P_i due to higher sPPase activity in transgenic potato tubers (Geigenberger et al., 1998).

In another study (Farre et al., 2001a), the role of PP_i in sprouting behavior of potato plants were investigated by overexpression of *E. coli* soluble pyrophosphatase driven by tuber specific patatin promoter. The transgenic lines showing an increased soluble pyrophosphatase activity and a lower PP_i content were found to sprout much earlier than wild type potato plants (Farre et al., 2001a). The comparison of transgenic tubers with wild type proved that transgenic potato plants produce less biomass due to significantly reduced starch contents (Farre et al., 2001a). The authors stated that the mechanism responsible for the accelerated sprouting is not clear but can be explained by an enhanced conversion of starch to sucrose and/or cell wall precursors needed for the rapidly growing tissue (Farre et al., 2001a).

In a recent study, the ectopic expression of *E. coli* soluble pyrophosphatase in *Arabidopsis thaliana* was revealed to cause similar effects (Lee et al., 2005). The transgenic *A. thaliana* plants were shown to accumulate higher levels of glucose and fructose but have no change in sucrose or starch accumulation (Lee et al., 2005). The overexpression of bacterial pyrophosphatase in *A. thaliana* caused no distinctive growth phenotype as in the case of overexpression in potato (Jelitto et al., 1992; Sonnewald, 1992; Lee et al., 2005). They were further characterized with lower levels of photosynthetic activity as compared to wild type plants (Lee et al., 2005), however, the transgenic *Arabidopsis* plants did not show an early germination phenotype as expected from the early sprouting behavior of transgenic potato plants (Jelitto et al., 1992; Sonnewald, 1992; Lee et al., 2005). The authors, thereby, claimed that the induction of cytosolic pyrophosphatase activity affects carbon partitioning between source and sink organs and the concomitant increase in P_i causes the accumulation of carbon metabolites and reduces photosynthetic activity (Lee et al., 2005).

The result of overexpression studies therefore strengthens the hypothesis that PP_i metabolism has a wider significance for plant metabolism and is probably much more complex than previously stated.

2.2.3 Membrane Bound Inorganic Pyrophosphatases

Membrane bound inorganic pyrophosphatases that are present in photosynthetic organisms including many higher plants, protists and certain prokaryotes, use the energy released by the hydrolysis of the phosphoanhydride bond of PP_i to pump a proton across biological membranes. They are present on cellular membrane of prokaryotes, the Golgi membrane and the tonoplast of plants. On the other hand, they seem to be absent from the membranes of animal, fungi and several types of bacteria (Jelitto et al., 1992; du Jardin et al., 1995; Perez-Castineira et al., 2001b).

The membrane bound H^+-PPase in prokaryotes is able to both synthesize and hydrolyze PP_i, thereby functioning as reversible H^+-PP_i synthase (Perez-Castineira et al., 2001b). The plant membrane bound inorganic pyrophosphatases were shown to function only as a H^+-PPase with a stochiometric ratio of one (du Jardin et al., 1995; Lopez-Marques et al., 2004).

The plant membrane bound inorganic pyrophosphatases can be classified into two groups according to their sensitivity to potassium ions (K^+): (i) Type I, K^+-stimulated H^+-PPases, reported to occur in the tonoplast of higher plants, and (ii) Type II, K^+-insensitive H^+-PPases, found in Golgi membranes of plants (Figure 2.3) (Ros et al., 1995; Drozdowicz and Rea, 2001; Perez-Castineira et al., 2001b; Lopez-Marques et al., 2004).

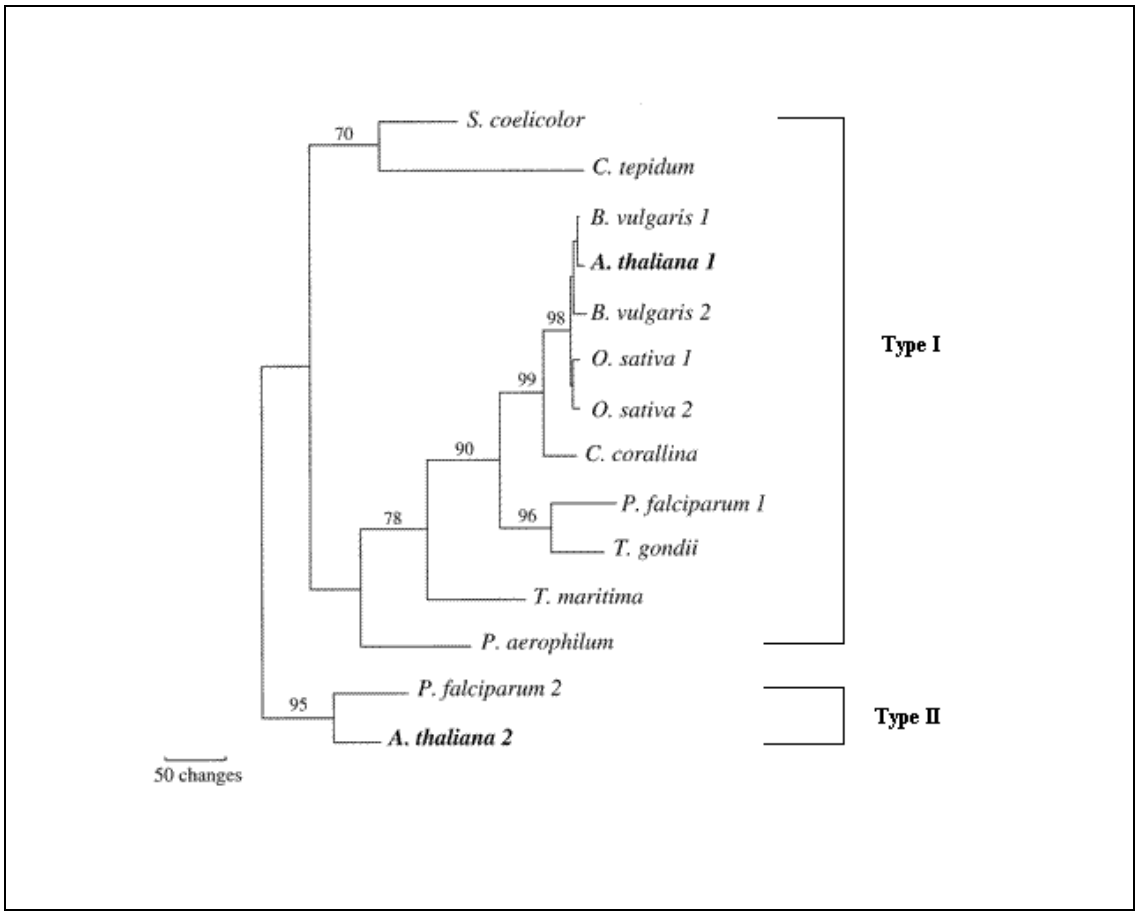

Figure 2.3 Phylogenetic analysis of vPPase sequences using Clustal W (Drozdowicz et al., 2000).

2.2.3.1 Vacuolar Membrane Bound Pyrophosphatases

The expansion of plant cells accomplished by the enlargement of the vacuole is the key process in the growth of plants. The increase of the vacuolar volume is achieved by accumulating solutes and protons to maintain the turgor pressure and acidity of inside environment (Maeshima et al., 1996; Maeshima, 2000). Therefore, the vacuolar membrane is implicated in a broad spectrum of physiological processes, such as cytosolic pH stasis, compartmentation of Ca^{2+}, sequestration of toxic ions (e.g. Na^{+}) and xenobiotics, cytosolic elimination of alkaloids, turgor regulation, and storage of amino acids and sugars; all of which depend on the maintenance of a transtonoplast H^{+}-gradient (Kim et al., 1994b; Barkla and Pantoja, 1996). Vacuolar membrane bound proton-translocating pyrophosphatases (vPPases) catalyze electrogenic H^{+}-translocation from the cytosol to the vacuole lumen, thus generating an electrochemical gradient with a similar or

greater magnitude than that created by V-ATPase localized on the same membrane (Rea and Poole, 1993; Kim et al., 1994a).

The vPPases are known to be unique to the plant kingdom (Maeshima et al., 1994). They show no close homology to other classes of proton pumps (F-, P- and V-ATPases) with respect to amino acid sequence, subunit composition, substrate-specificity and inhibitor-sensitivity, and are thereby categorized as a new class of ion translocases (Kim et al., 1995; Perez-Castineira et al., 2001b). The functional and catalytic properties (such as requirement of Mg^{2+} and sensitivity to Ca^{2+}) of the vPPases are similar to soluble pyrophosphatases but they differ in subunit size, K_m values (200 μM) and dependence on K^+ (Maeshima, 1991; Maeshima et al., 1996). The vPPases are highly hydrophobic proteins with 13 predicted transmembrane domains (Figure 2.4) (Zhen et al., 1994). A single polypeptide of vPPase (about 80 kDa) was shown to be sufficient for membrane localization and PP_i hydrolysis, whereas, coupling of PP_i hydrolysis with H^+-translocation requires a dimer formation (Maeshima et al., 1996; Baltscheffsky et al., 1999; Perez-Castineira et al., 2002).

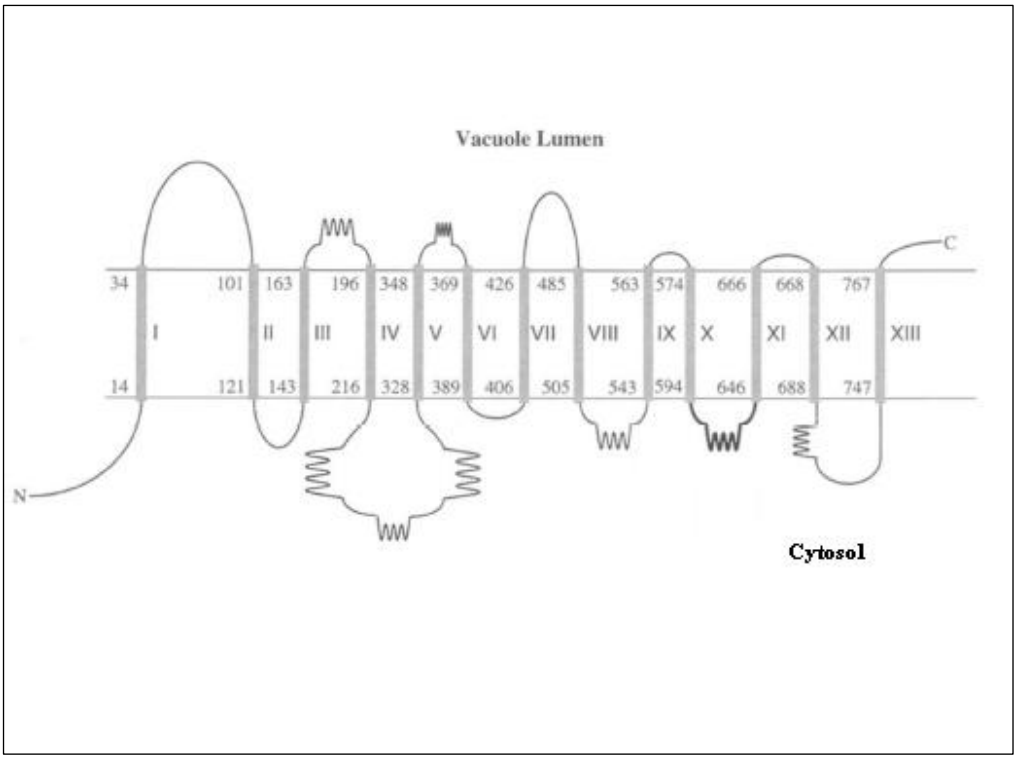

Figure 2.4 Predicted topological model of vPPases of *A. thaliana*, *Beta vulgaris* and *Hordeum vulgare* analyzed by PC/GENE program. The wavy lines indicates the α-helix and nonwavy lines random coils (Zhen et al., 1994).

The evolutionary conserved properties shared with bacterial H^+-PP_i synthase can explain the origins of vPPases. The H^+-PP_i synthase bearing bacteria belong to the same group of eubacteria which was shown to be the origin of plant mitochondria (Drozdowicz et al., 1999). Thus, it is possible that plant vPPase genes have arisen by lateral gene transfer from one of the eubacteria endosymbionts (Rea and Poole, 1993; Drozdowicz et al., 1999).

A study which aimed at cloning vacuolar pyrophosphatases from *Nicotiana tobacum* revealed that tobacco vPPase is encoded by at least three genes in the plant nuclear genome (Lerchl et al., 1995b). The appearance of several vPPase isoforms which are highly conserved with respect to coding sequence (above 80 % similarity) and different in 5' and 3'-UTRs, was further proved true for other higher plants (e.g. rice two isoforms (Sakakibara et al., 1996), and sugar beet two isoforms (Kim et al., 1994b)). Expression analysis of tobacco vPPase isoforms by Northern blotting showed that there is a differential tissue specific expression (Lerchl et al., 1995b). This indicates that vPPase isoforms are differentially and possibly individually regulated in plants (Lerchl et al., 1995b; Nakanishi and Maeshima, 1998).

Because sPPase activity is shown to be largely absent from the cytosol of photosynthetic tissues, and almost exclusively located in the chloroplast (Weiner et al., 1987), the vPPase is accepted as the sole enzyme responsible from the hydrolysis of PP_i in the cytosol (Perez-Castineira et al., 2002). In addition, the function of vPPase was hypothesized to be critical under conditions of stress by providing a back up system for metabolism since PP_i would be a more favorable energy donor under ATP-limiting conditions (Rea and Poole, 1993).

The expression analysis of vPPase in elongating and mature plant cells provided insight into dependence of vPPase activity on plant age; the expression and activity of vPPase was shown to decrease with developmental stage of the plant tissue (Maeshima et al., 1996). The explanation was based on the role of vPPase to prevent PP_i accumulation in the cytosol. In growing tissue, there is a high accumulation of PP_i that needs to be eliminated due to high metabolic activity, whereas, in mature cells, metabolic activity decreases and PP_i hydrolysis is no more required at a large extent. Interestingly, V-ATPase activity was found to be increasing with ageing. Thus it was concluded that the vPPase is the predominant pump in developing tissue and at maturity the V-ATPase is the main H^+-translocating pump in tonoplast (Maeshima et al., 1996; Davies, 1997; Nakanishi and Maeshima, 1998).

The importance of vPPase during anoxia was investigated in rice (Carystinos et al., 1995). In anoxic conditions, cessation of oxidative phosphorylation results in decrease in ATP levels whereas cellular PP_i levels tend to remain stable. The relative vPPase transcript amount was reported to be very low during aerobic conditions, while, the transcript level was shown to be rap-

idly increased when rice seedlings were exposed to anoxia (Carystinos et al., 1995). It was concluded that under energy stress conditions, plants can maintain the tonoplast energization and cytoplasmic pH homeostasis by increasing the vPPase specific activity (Carystinos et al., 1995).

Using the *Brassica napus* suspension cells as an experimental model, it was shown that P_i starvation induces vPPase protein amount, probably for conservation of limited ATP pools, and enabling P_i recycling during P_i stress (Palma et al., 2000). Later it was proven that both PP_i hydrolysis and PP_i-dependent H^+-pumping activities in plants under mineral deficient conditions (mainly deficiency of K^+, NO^{3-} and Ca^{2+}) are higher than those grown under normal conditions, indicating a role of vPPases during mineral stress (Maeshima, 2000).

The reports on salt stress response of vPPase are quite confusing and non-conclusive. It seems that salt responses of vPPase are dependent on the plant species and cannot be generalized. The main problem was that no clear correlation between activity measurements and vPPase protein amount could be detected. The results suggest a possible post-translational regulation of vPPase activity in plant cells depending on the species (Otoch et al., 2001; Wang et al., 2001).

The stress related changes in the expression of vPPases proved the expression level of the vPPase to be precisely controlled at the transcriptional level in response to various environmental conditions or developmental stages. The activity of vPPases are shown to be especially important in constantly growing tissues and under ATP-limiting conditions (Mitsuda et al., 2001a).

2.2.3.2 Golgi Membrane Bound Pyrophosphatases

The identification of a sequence-divergent, K^+-insensitive and Ca^{2+}-hypersensitive vPPase from *A. thaliana* (AVP2) led to the hypothesis that plant vacuoles possess two distinct types of H^+-PPases (Drozdowicz et al., 2000). However, a K^+-independent H^+-translocation activity was not detected in plant vacuoles, and therefore, the AVP2 was supposed to be localized to other membranes like the Golgi apparatus, plasma membrane, or mitochondria (Drozdowicz et al., 2000). A relatively new study proved that GFP-tagged AVP2 proteins are targeted mainly to the Golgi apparatus rather than to the vacuolar membrane (Mitsuda et al., 2001b). It is likely that the type II H^+-PPase functions as an H^+-pump for the Golgi apparatus in combination with Golgi apparatus resident V-ATPase for generating the acidic environment (Mitsuda et al., 2001b).

2.2.3.3 Plasma Membrane and Endoplasmic Reticulum Associated Pyrophosphatases

The occurrence of H^+-PP_i synthases in plasma membranes of photosynthetic bacteria was reported in several cases. With respect to plants, there were only a couple reports indicating the possible presence of H^+-PPase on plasma membranes based on activity measurements (Maeshima, 2000). In one study, the presence of a plasma membrane H^+-PPase in sieve elements of the stem tissue was shown with immunolocalization techniques (Langhans et al., 2001). It was speculated that the function of H^+-PPase on the plasma membrane might be related to phloem sucrose metabolism to support ATP-requiring processes (Langhans et al., 2001). During the overexpression studies concerning the function of vPPases in plant metabolism, a part of the vPPase enzyme was shown to be localized in plasma membrane which can be an unspecific localization due to bulk flow of overexpressed protein or vacuolar H^+-PPase can also be localized to the plasma membrane under yet unknown conditions (Li et al., 2005). However, the presence and physiological significance of an H^+-PPase localized in plasma membrane of plants is still unclear.

A recent study questions the possibility of presence of a proton pumping inorganic pyrophosphatase in endoplasmic reticulum of plants (Kuo et al., 2005). The isolated endoplasmic reticulum enriched vesicles from etiolated mung bean seedlings was shown to function in inorganic pyrophosphate hydrolysis and associated proton translocating activity (Kuo et al., 2005). The authors claimed that H^+-PPase may be more widely distributed than previously believed in subcellular membranes of plants. However, the endoplasmic reticulum enrichment of vesicles might be contaminated with other subcellular membranes and thereby vacuolar or Golgi membrane associated pyrophosphatases, or the enrichment from etiolated seedlings might indicate the possibility of mislocalization of Golgi membrane associated pyrophosphatase to ER membrane under these specific conditions. Therefore, the presence of an endoplasmic reticulum membrane associated pyrophosphatase is not comprehensive.

2.2.3.4 Effect of Overexpression of Vacuolar Membrane Bound Pyrophosphatases

There a number of recent studies on the effects of H^+-PPases on plant metabolism, using either homologous or heterologous ectopic expression.

The first *A. thaliana* vPPase (AVP1) gene was cloned in 1992, with a deduced protein of pI of 4,95 and MW of 81 kDa (Sarafian et al., 1992). The protein was proven to function as PP_i-

energized H^+-pump by heterologous expression in yeast (Kim et al., 1994a). Further analysis by Western blotting indicated the expression of AVP1 in vascular cylinders of roots and stems, siliques, flowers, and veins of rosette leaves. The promoter activity of AVP1 gene was found to be induced by low light similar to genes involved in sugar starvation, whereas was shown to be increasing during flower development which starts with the developmental stage when the degradation of carbohydrates stored in vacuoles occurs (Mitsuda et al., 2001a). Thus, it was concluded that the AVP1 might supply a driving force for loading sugars from the cytosol into the vacuole by generating an electrochemical proton gradient across the vacuolar membrane (Mitsuda et al., 2001a).

Salt- and drought tolerance of plants is accomplished by increasing solute accumulation in the vacuoles and, therefore, an increase in water retention. In theory, increasing the electrochemical gradient across tonoplast should enhance the tolerance of plant to salt and drought stresses. The homologous overexpression of AVP1 in *A. thaliana* proved that transgenic lines are more resistant to salt and drought than the wild type plants (Gaxiola et al., 2001). The resistance was shown to be associated with increased internal stores of solutes in vacuoles (Gaxiola et al., 2001). The phenotype of AVP1 overexpressing transgenic lines was found to be similar to plants with morphological variations typical of hormonal defects, such as increase in rosette leaf number, greater leaf area and enhanced root growth compared to wild-type plants (Gaxiola et al., 2001; Gaxiola et al., 2002).

The transporters responsible for setting cytoplasmic and apoplastic pH are reported to be likely candidates for driving polar flux of auxin required for organ development and directional growth. The further analysis of the possible function of AVP1 was performed by a study comparing the overexpression and loss-of-function mutants of AVP1 in *A. thaliana* (Li et al., 2005). The phenotypes obtained showed altered root and shoot development similar to alterations in auxin-mediated development due to altered uptake and distribution of auxin (Li et al., 2005). The authors claimed that AVP1 not only functions as a proton pump responsible for vacuolar acidification but also contributes to the regulation of apoplastic pH and to auxin transport (Li et al., 2005).

The data was further supported by heterologous overexpression of AVP1 in tomato plants (Park et al., 2005). The transgenic tomato plants were characterized with extensive root growth but no deleterious phenotypes like reduced vegetative growth, flower set or fruit yield (Park et al., 2005). The extensive root system development in transgenic tomato was proved to cause a quick recovery after relief from severe drought conditions (Park et al., 2005).

In a heterologous approach, the vacuolar pyrophosphatase of a halophyte *Suaeda salsa* was overexpressed in *A. thaliana* (Guo et al., 2006). The enzyme is 84 % homolog to AVP1 and

shown to be induced under saline conditions by Northern blot analysis (Guo et al., 2006). The overexpression in *A. thaliana* proved enhanced salt and drought resistance in transgenic plants compared to the wild type (Guo et al., 2006).

The overall results suggest that vacuolar pyrophosphatases can be a potential target for genetic engineering of root systems and increasing environmental stress resistance of important crop plants.

2.3 Model Organisms Used

2.3.1 *Arabidopsis thaliana*

Arabidopsis thaliana is a small, dicotyledonous plant and a member of the mustard (Brassicaceae) family. It has no economic value and agronomical significance, however, it is widely used for basic research in genetics and molecular biology as a model organism due to several advantages it offers; (i) a small genome size which has been completely sequenced in 2000 (The Arabidopsis genome initiative 2000), (ii) a fast life cycle (about 6 weeks from germination to mature seed), (iii) production of a lot of self progeny with almost 100 % self-pollination, (iv) very limited space requirements, (v) availability of numerous efficient methods for stable or transient transformation of the gene of interest and (vi) ease of growing in a greenhouse or growth chamber with simple requirements like light, air, water and a few minerals (Koornneef, 1994).

2.3.2 *Beta vulgaris* L.

Beta vulgaris L. is a member of the Chenopodiaceae family. It is a biennial plant, producing leaves and the taproot in the first year and using the nutrients stored in the taproot produces a seed stalk during the second one. A typical sugar beet contains about 10 % sugar (Rosenkranz et al., 2001).

B. vulgaris L. is commercially grown and it is the second most important plant in sugar production after sugarcane. Beets are harvested for processing at the end of the first year. During harvesting, the head of the taproot is removed causing a wound. Usually, plant metabolism responds to wounding by increasing respiration, by synthesizing wound-sealing compounds, by

strengthening the cell wall and by producing defense compounds. This increased metabolic activity is fed by the accumulated sucrose in the taproot tissue. Therefore, sucrose is lost during post harvest storage and processing. Post harvest sucrose metabolism is costly for the sugar beet industry. It has been postulated that 100 to 250 g of sucrose is lost per ton of roots per day during storage (Leigh et al., 1979).

2.4 Aim of the Project

The plant cytosol was shown to contain a significant amount of PP_i with little soluble pyrophosphatase activity. The plastids, however, were shown to have a negligible amount of PP_i with high soluble pyrophosphatase activity. Therefore, it was generally accepted that, in plant cells, cytosolic soluble pyrophosphatases are not significant or present and the removal of cytosolic PP_i is mainly accomplished by tonoplast H^+-PPase as a sole enzyme.

The sequencing projects of several plant species revealed that the plant genomes encode several soluble pyrophosphatase isoforms. The *in silico* prediction tools localize soluble pyrophosphatases not only to plastids but also to the cytosol. However, there are almost no data available on the presence, regulation, function and importance of cytosolically localized soluble pyrophosphatases.

Arabidopsis thaliana was selected as the model organism to understand the plant soluble pyrophosphatase family. The *in vivo* subcellular localization of each sPPase isoform was studied to find out if all nuclear encoded soluble pyrophosphatases are localized in plastids or if there are some isoforms present in cytosol or in other organelles. The tissue-specific expression and environmental stress responses of *A. thaliana* sPPase isoforms were analyzed to figure out the possible isoform specific regulations with respect to tissue, developmental age and environmental conditions. The effect of knockdown of soluble pyrophosphatases on plant growth and development was observed by RNAi approach to selected isoforms. Finally, the effect of overexpression of heterologous sPPase and vPPase isoforms on salt stress response was analyzed to understand the effect of changes in cytosolic PP_i pool on stress tolerance.

The plant metabolism is unique for its capability for using the energy generated during inorganic pyrophosphate cleavage by H^+-PPase to create and electron motive force through the vacuolar membrane for secondary active transport. Therefore, it is possible to increase the concentration of storage compounds in the plant vacuoles by overexpressing H^+-PPase or by chang-

ing the cytosolic PP_i pool. The homologous overexpression of vPPase in *Arabidopsis thaliana*, indeed, proved to enhance the salt stress tolerance.

Inorganic pyrophosphate is also known to function as an energy donor for reactions in plant carbohydrate metabolism. However, there are only non-conclusive data on the importance of PP_i in the regulation of sucrose/starch metabolism. *Beta vulgaris* was selected as the model organism to understand the effect of homologous overexpression of sPPase and vPPase isoforms on the plant carbohydrate metabolism, by mainly focusing on the sucrose loading during development and sucrose loss after harvest.

The enzymes that are important in the plant carbohydrate metabolism were shown to be regulated not only transcriptionally but also post-translationally. Therefore, the recombinant *Beta vulgaris* soluble pyrophosphatase isoform 1 was used to reveal the possible post-translational regulation mechanisms of inorganic pyrophosphatases.

3 RESULTS

3.1 The *Arabidopsis thaliana* Soluble Pyrophosphatase Family

The *Arabidopsis thaliana* genome sequencing was completed in the year 2000 (The Arabidopsis genome initiative, 2000) and the results are available to academic research on the TAIR (The Arabidopsis Information Resource) 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 recently shown to be localized in plastids (Schulze et al., 2004).

Based on the chromosome their genes are located, the ***A****. thaliana* **s**oluble **p**yrophosphatase isoforms are abbreviated as ASP1, ASP2A, ASP2B, ASP3, ASP4 and ASP5 (the plastidial isoform). The basic properties of *A. thaliana* sPPase family are summarized in Table 3.1.

Table 3.1 Some properties of *Arabidopsis thaliana* sPPase family. SP; signal peptide. Prediction of molecular weight and isoelectric point was performed using EXPASY pI/MW tool (http://us.expasy.org).

Abbreviation	Accession Number	Length (amino acid)	Predicted MW (kDa)	Predicted pI
ASP1	At1g01050	212	24,5	5,97
ASP2A	At2g18230	218	24,6	5,97
ASP2B	At2g46860	216	24,9	5,63
ASP3	At3g53620	216	24,5	5,15
ASP4	At4g01480	216	24,7	6,09
ASP5	At5g09650	300 (with SP) 244 (without SP)	26,4 (without SP)	4,75 (without SP)

The alignment of *A. thaliana* sPPase sequences downloaded from TAIR database (http://www.tair.org) indicates the high homology with respect to both amino acid (Table 3.2, Figure 3.1) and coding DNA sequences (Figure 3.2). The main divergence of nucleotide se-

quences is observed at 5'- and 3'-UTRs (Chapter 7.2). The amino acid sequence alignment indicates the low homology of plastidial isoform (Schulze et al., 2004) to other *A. thaliana* sPPase isoforms (Table 3.2).

```
                        *.... . +.....  ...*.+.**+*+++.++.++.++******+***.+* ***.***
ASP1          (     1) MSEE----TKDNQR--LQRPAPRLNERILSSLSRRSVAAHPWHDLEIGPGAPQIFNVVVE
ASP2A         (     1) MAEIKDEGSAKGYAfPLRNPNVTLNERNFAAFTHRSAAAHPWHDLEIGPEAPTVFNCVVE
ASP2B         (     1) MSEEAYEETQESSQ--SPRPVPKLNERILSTLSRRSVAAHPWHDLEIGPEAPLVFNVVVE
ASP3          (     1) MAPP-IEVSTKSYV-EKHVSLPTLNERILSSMSHRSVAAHPWHDLEIGPEAPIIFNCVVE
ASP4          (     1) MNGEEVKTSQPQKK--LQNPTPRLNERILSSLSKRSVAAHPWHDLEIGPGAPVIFNVVIE
ASP5          (     1) VQEE--------------GPAESLDYRVF-FLDGSGKKVSPWHDIPLTLG-DGVFNFIVE

                        *.**+*+***+  **.+*+**+*    *++*++++***    ****+***
ASP1          (    55) ITKGSKVKYEL--DKKTGLIKVD----RILYSSVVYPH----NYGFVPRT----------
ASP2A         (    61) ISKGGKVKYEL--DKNSGLIKVD----RVLYSSIVYPH----NYGFIPRT----------
ASP2B         (    59) ITKGSKVKYEL--DKKTGLIKVD----RILYSSVVYPH----NYGFIPRT----------
ASP3          (    59) IGKGSKVKYEL--DKTTGLIKVD----RILYSSVVYPH----NYGFIPRT----------
ASP4          (    59) ISKGSKVKYEL--DKKTGLIKVD----RILYSSVVYPH----NYGFVPRT----------
ASP5          (    45) IPKESKAKMEVatDEDFTPIKQDtkkgKLRY----YPYninwNYGLLPQTwedpshanse

                          .* +*+**+**++++*+*+++*+***+*+**+********+*+******.****++*+
ASP1          (    95) --LC-EDNDPIDVLVIMQEPVLPGCFLRARAIGLMPMIDQGEKDDKIIAVCVDDPEYKHY
ASP2A         (   101) --IC-EDSDPMDVLVLMQEPVLTGSFLRARAIGLMPMIDQGEKDDKIIAVCADDPEFRHY
ASP2B         (    99) --LC-EDNDPLDVLVLMQEPVLPGCFLRARAIGLMPMIDQGEKDDKIIAVCADDPEYKHF
ASP3          (    99) --LC-EDSDPIDVLVIMQEPVIPGCFLRAKAIGLMPMIDQGEKDDKIIAVCADDPEYRHY
ASP4          (    99) --LC-EDNDPIDVLVIMQEPVLPGCFLRARAIGLMPMIDQGEKDDKIIAVCVDDPEYKHI
ASP5          (   101) veGCfGDNDPVDVVEIGETQRKIGDILKIKPLAALAMIDEGELDWKIVAISLDDPKAHLV

                        +**++++**   +*.+**+******+****++.+*+* *  .*+.+  +.++**.++*+*
ASP1          (   152) TDIKELPPH---RLSEIRRFFEDYKKNENKEVAVNDF-L---PSES--AVEAIQYSMDLY
ASP2A         (   158) RDIKELPPH---RLAEIRRFFEDYKKNENKKVDVEAF-L---PAQA--AIDAIKDSMDLY
ASP2B         (   156) TDIKQLAPH---RLQEIRRFFEDYKKNENKKVAVNDF-L---PSES--AHEAIQYSMDLY
ASP3          (   156) NDISELPPH---RMAEIRRFFEDYKKNENKEVAVNDF-L---PATA--AYDAVQHSMDLY
ASP4          (   156) TNINELPPH---RLSEIRRFFEDYKKNENKEVAVNDF-L--QPGPA---IEAIQYSMDLY
ASP5          (   161) NDVEDVEKHfpgTLTAIRDWFRDYKIPDGK--PANRFgLgdKPANKdyALKIIQETNESW

                        *+**...+++
ASP1          (   203) AEYILHTLRR------
ASP2A         (   209) AAYIKAGLQR------
ASP2B         (   207) AEYILHTLRR------
ASP3          (   207) ADYVVENLRR------
ASP4          (   207) AEYILHTLRR------
ASP5          (   219) AKLVKRSVDAgdlsly
```

Figure 3.1 Multiple alignment (Nikolaev et al., 1997) of amino acid sequences of *A. thaliana* sPPase isoforms. (*); conserved residues, (+); highly conserved residues. The sequence highlighted with red is the pyrophosphatase signature referring to the conserved active site motif ($EX_{7\text{-}8}KXE$) of sPPases (Baltscheffsky et al., 1999). The deletions observed in sPPases but not in plastidial sPPase isoforms (Schulze et al., 2004) are indicated with bold letters on ASP5 sequence. Note that the plastidial isoform (ASP5) sequence used in the alignment does not include the signal peptide.

Table 3.2 The percent homology of *A. thaliana* sPPase isoforms based on amino acid sequence alignments.

	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**

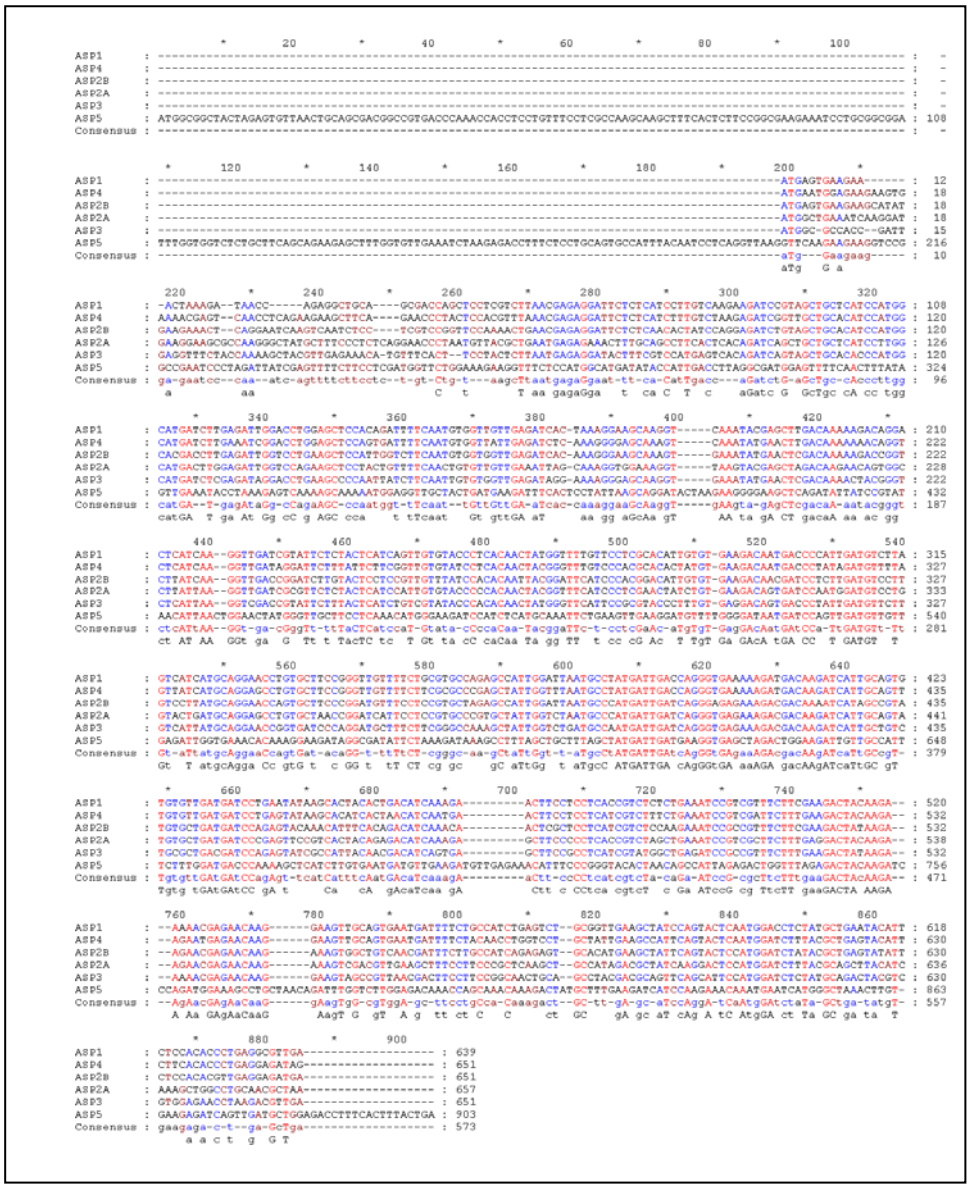

Figure 3.2 Alignment of coding sequences of *A. thaliana* sPPase isoforms according to Corpet (1998). The conserved bases are highlighted with red, and highly conserved bases with blue. Consensus sequence based on homology is also given. Note that ASP5 (plastidial isofom) sequence used in the alignment includes the leader peptide.

3.1.1 Subcellular Localization of *A. thaliana* Soluble Pyrophosphatases

3.1.1.1 *In Silico* Analysis of Subcellular Localization of *A. thaliana* sPPases

In fully developed plant leaves, the cytosol was shown to contain 200-300 μM inorganic pyrophosphate (PP_i) with almost non-detectable soluble pyrophosphatase (sPPase) activity. On the contrary, isolated plastids were demonstrated to have negligible amount of PP_i with high sPPase activity (Weiner et al., 1987; Farre et al., 2001). Therefore, as explained in detail in Chapter 2.2.2.1, it was hypothesized and generally accepted that plant soluble inorganic pyrophosphatases should localize in plastids and/or mitochondria, but not in the cytosol (Stitt, 1998; Perez-Castineira et al., 2001).

In order to test the reliability of this hypothesis, the subcellular localization prediction of *Arabidopsis thaliana* sPPase isoforms was performed using web-based programs PSORT (plant specific) (Nakai and Horton, 1999), TargetP (for eukaryotic organisms, not plant specific) (Emanuelsson et al., 2000), LOCTree (plant specific) (Nair and Rost, 2005) and PredictNLS (detection of eukaryotic nuclear localization signals) (Cokol et al., 2000). The results of *in silico* localization prediction performed are summarized in Table 3.3.

Table 3.3 Prediction of *in silico* localization of *A. thaliana* sPPase isoforms. NLS; Nuclear localization signal. TargetP program only searches for the N-terminal signal peptides for chloroplast, mitochondria and secretory pathway localization and refers the proteins that do not contain a known signal sequence as in "other compartments".

	PSORT (Nakai and Horton, 1999)	**TargetP** (Emanuelsson et al., 2000)	**LOCTree** (Nair and Rost, 2005)	**PredictNLS** (Cokol et al., 2000)
ASP1	Cytoplasm	Mitochondria	Cytoplasm	No NLS
ASP2A	Cytoplasm	Other compartments	Cytoplasm	No NLS
ASP2B	Cytoplasm	Other compartments	Cytoplasm	No NLS
ASP3	Cytoplasm	Other compartments	Cytoplasm	No NLS
ASP4	Cytoplasm	Other compartments	Cytoplasm	No NLS
ASP5	Vacuole	Chloroplast	Chloroplast	No NLS

The programs used for *in silico* localization prediction, namely PSORT (Nakai and Horton, 1999), TargetP (Emanuelsson et al., 2000), LOCTree (Nair and Rost, 2005) and PredictNLS

(Cokol et al., 2000) are basically searching for the presence of a known and/or functionally verified sequence motif for specific localization of a queried amino acid sequence. Among *A. thaliana* sPPase isoforms, as summarized in Table 3.3, ASP5 and ASP1 are predicted to be localized in chloroplasts or the vacuole and mitochondria or cytoplasm, respectively, whereas, the other isoforms are predicted to be localized in the cytosol. None of the isoforms contains a known nuclear localization motif.

The results of *in silico* localization prediction analysis of *A. thaliana* sPPases were in contrast to what was expected based on the hypothesis on the localization of plant sPPases on plastids and/or mitochondria but not in cytosol (Perez-Castineira et al., 2001). Therefore, it was decided to confirm the subcellular localization of *A. thaliana* sPPases *in vivo* (Chapter 3.1.1.2).

3.1.1.2 Subcellular Localization of *Arabidopsis thaliana* Soluble Pyrophosphatases *in vivo*

The *A. thaliana* soluble pyrophosphatase isoform-specific full length coding sequences were amplified from leaf (ASP1 and ASP2A), etiolated seedling (ASP2B), flower (ASP3), root (ASP4) or seedling cDNAs (ASP5). The amplified fragments were introduced in Gateway entry vector pDONR201 (Invitrogen). After confirmation of the sequences, they were cloned in frame with GFP in Gateway destination vectors pK7FWG2 (N-terminal GFP construct driven by CaMV 35S promoter, Invitrogen) and pK7WGF2 (C-terminal GFP construct driven by CaMV 35S promoter, Invitrogen) (Karimi et al., 2004). The constructs were mobilized in *Agrobacterium tumefaciens* strain C58C51 containing Ti plasmid pGV2260 by electroporation. The overnight grown *A. tumefaciens* cells were collected by centrifugation and infiltrated to tobacco leaves as described (Wroblewski et al., 2005). 48 hours after infiltration, the epidermal cells in the infiltrated regions of tobacco leaves were analyzed by Confocal Laser Scanning Microscopy (CLSM).

The CLSM analysis indicated aggregate formation in N-terminal GFP constructs for all *A. thaliana* sPPases (Figure 7.1) except ASP5 which did not yield any green fluorescence as expected; since after the cleavage of N-terminal signal peptide, GFP signal should disappear due to degradation of the peptide. Figure 3.3 shows the subcellular localizations of ASP-GFP fusion protein observed with transient expression in epidermal cells of tobacco leaves.

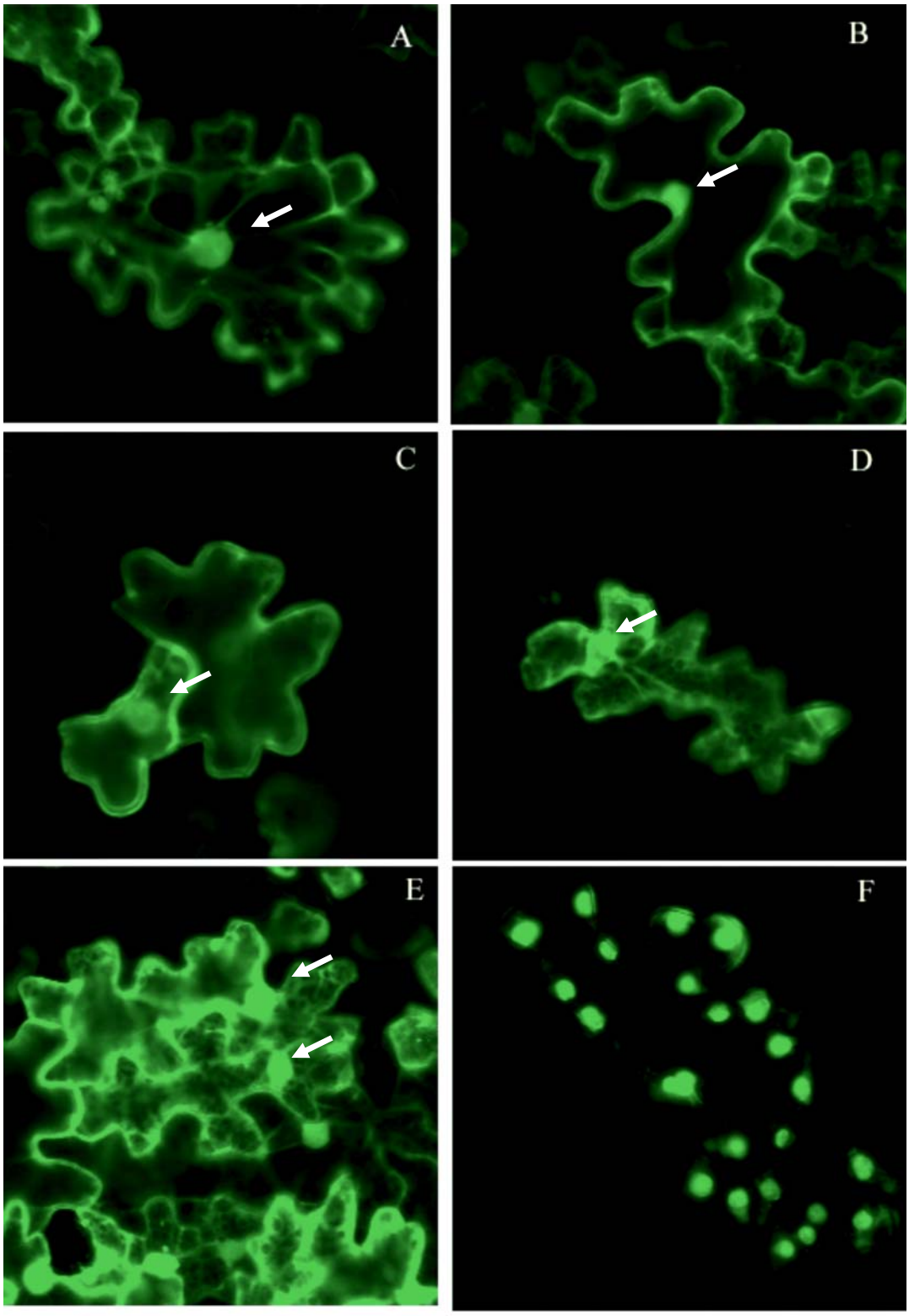

Figure 3.3 CLSM images of ASP:GFP chimeras visualized 48 hours after infiltration to tobacco leaves. A) ASP1-GFP, B) ASP2A-GFP, C) ASP2B-GFP, D) ASP3-GFP, E) ASP4-GFP and F) ASP5-GFP fusion proteins. The arrows point the nucleus. Accordingly, all *A. thaliana* sPPases except ASP5 are localized in the cytosol and the nucleus. In the case of ASP5, the green fluorescence is observed only in the plastids.

Figure 3.3.A shows the CLSM result of a single epidermal tobacco cell expressing ASP1-GFP fusion protein. The cytoplasmic strands are clearly visible in picture indicating cytosolic localization for ASP1. The fusion protein is also observed in the nucleus, however, as the size exclusion limit of the nuclear pore complex has been estimated to be 60 kDa (Haasen et al., 1999; Koroleva et al., 2005) and since the chimera protein has the approximate size of 55 kDa, it is not clear if the nuclear localization is specific or just the consequence of free diffusion of fusion protein between the cytosol and the nucleus. The absence of nuclear localization signal in ASP1 amino acid sequence (Table 3.3) further supports the possibility of mislocalization to the nucleus.

The subcellular localization results of transient expression of ASP2A-GFP (Figure 3.3.B), ASP2B-GFP (Figure 3.3.C), ASP3-GFP (Figure 3.3.D) and ASP4-GFP (Figure 3.3.E) fusion proteins are similar to ASP1:GFP chimera (Figure 3.3.A), i.e., all these isoforms are localized in the cytosol and the nucleus. In the case of ASP3-GFP (Figure 3.3.C) and ASP4-GFP (Figure 3.3.D) chimeras, not only the diffuse distribution of green fluorescence in the cytoplasm but also the association of fusion proteins with so-called 'restricted sub-cytoplasmic domains' are visible (Koroleva et al., 2005). The nuclear localization of ASP2A, ASP2B, ASP3 and ASP4 are also non-conclusive as in the case of ASP1, since the fusion proteins have an approximate size of 55 kDa, apparently smaller than 60 kDa.

Figure 3.3.F shows the localization of ASP5-GFP fusion protein in a single epidermal tobacco cell. Overlay of GFP fluorescence with the chlorophyll autofluoresence indicates the exclusive localization in plastids (data not shown).

In summary, the results of *in vivo* subcellular localization studies showed that *A. thaliana* sPPase isoforms ASP1, ASP2A, ASP2B, ASP3 and ASP4 are localized in the cytosol and/or the nucleus, whereas ASP5 is localized exclusively in the plastids, confirming the expectations of *in silico* subcellular localization predictions (Chapter 3.1.1.1). It is interesting to observe that in contrast to the literature, none of the isoforms shows mitochondrial localization under these conditions (Perez-Castineira et al., 2001).

3.1.2 Differential Expression of *A. thaliana* Soluble Pyrophosphatases during Plant Development

The subcellular localization analysis of *Arabidopsis thaliana* soluble pyrophosphatase isoforms indicated that ASP1, ASP2A, ASP2B, ASP3 and ASP4 are present in the same organ-

elles, namely the cytosol and/or the nucleus (Figure 3.3.A to E) and ASP5 is exclusively found in the plastids confirming the recent *in vitro* localization studies (Schulze et al., 2004) (Figure 3.3.F). Therefore, it is very likely that *A. thaliana* sPPase isoforms localized to the same compartments (cytoplasm and/or nucleus) are different from each other in tissue specificity of gene expression.

In order to analyze the tissue specific expression of *A. thaliana* sPPase isoforms two main approaches were used. In the first approach, the promoter regions of *A. thaliana* sPPases were used to drive the expression of β-glucuronidase (GUS) to analyze the specificity of promoter activity in different tissues. In the second approach, the quantification of the transcript amount of *A. thaliana* sPPase isoforms in different tissues were performed by real-time PCR.

3.1.2.1 Analysis of Plants with *A. thaliana* sPPase Promoter Driven GUS Expression

The aim of this approach was the analysis of tissue specificity of promoter activity of *A. thaliana* soluble pyrophosphatase isoforms. For this purpose, the 1 kb region upstream of the start codon of each *A. thaliana* sPPase isoform was amplified from genomic DNA and cloned to the Gateway entry vector pDONR201 (Karimi et al., 2002) for sequencing. The sequences were confirmed by aligning to the promoter region sequence downloaded from TAIR database (http://www.tair.org). Afterwards, the 1 kb promoter region of each isoform was introduced in Gateway destination vector pBGWFS7 (Karimi et al., 2002) carrying the GUS reporter gene. The constructs were mobilized in *A. tumefaciens* strain C58C51 containing Ti plasmid pGV2260 and *A. thaliana* were stably transformed by the flower dip method (Clough and Bent, 1998). The transgenic plants were selected by spraying 0,02 % (v/v) BASTA™ solution to rosette leaves and positive plants were analyzed by histochemical GUS activity for the appearance of blue color confirming promoter activity.

Figures 3.4 to 3.9 summarize the results of GUS staining of different tissues of stably transformed *A. thaliana* after BASTA™ selection. In each case, the tissues were removed from five independent plants at different developmental stages (sink leaves; 4 weeks after germination; source leaves; 4 weeks after germination; flowers and siliques; 6 to 7 weeks after germination depending on the development) and analyzed with histochemical GUS activity assay. The figures are selected as the representatives of similar results.

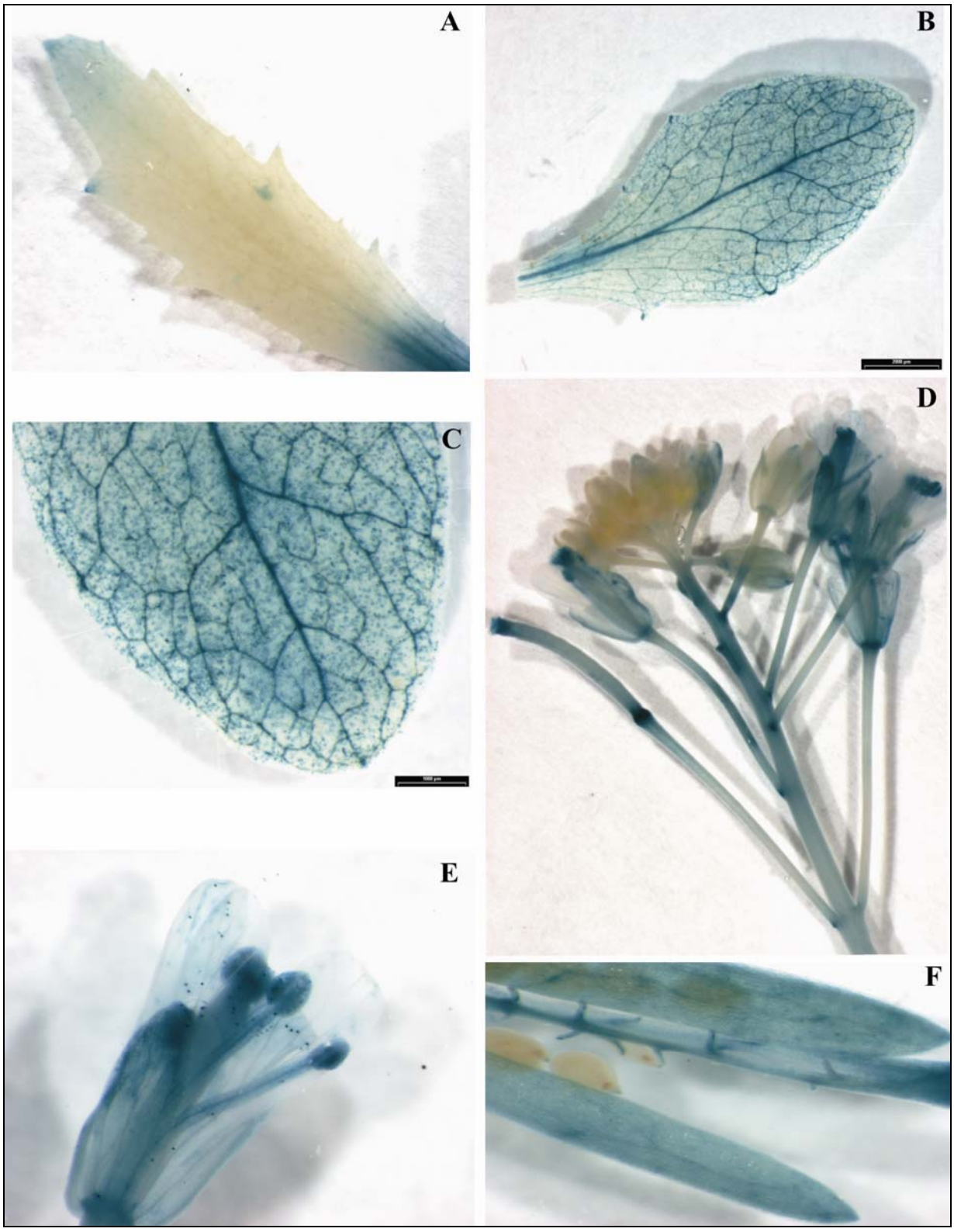

Figure 3.4. Promoter activity of ASP1 in stably transformed *A. thaliana* plants. A) Sink leaf, B-C) Source leaf, D-E) Flower, and F) Silique and seeds.

Figure 3.4 shows GUS activity driven by the 1 kb region upstream of the start codon of the ASP1 promoter. In sink leaves, the blue staining confirming GUS activity is detected in hydathodes and petioles (Figure 3.4.A). In source leaves, the promoter activity is mainly on vascular bundles (Figure 3.4.B). The dot-shaped staining patterns in the blades of source leaves are commonly observed, exclusively for ASP1 (Figure 3.4.C). A detailed analysis of these regions proved that the GUS staining is restricted to the spongy mesophyll cells. It is clear that GUS staining of some but not all mesopyhll cells is related with the specific activity of the ASP1 promoter in these cells.

The blue color indicating the ASP1 promoter activity is strongly dependent on developmental stage of flowers (Figure 3.4.D and E). In fully developed flowers, the promoter activity is detected on sepals, stigma, stamen and receptacle. The GUS staining confirms the ASP1 promoter activity in pollen (Figure 3.4.E). The ASP1 promoter drives the expression of GUS in siliques and funiculus but not in seeds or seed coat (Figure 3.4.F).

The GUS activity of 1 kb region of ASP2A promoter detected by histochemical assay in stably transformed *A. thaliana* plants indicates the overall staining of blade of sink leaves (Figure 3.5.A). However, the promoter activity in adult leaves is exclusively restricted to vascular bundle (Figure 3.5.B). The change in the activity of ASP2A promoter during flower growth is very similar to that of ASP1, showing a rising activity by increasing developmental stage (Figure 3.5.C and D). The staining of stem indicates promoter activity in vascular tissue (Figure 3.5.E).

The developing siliques reveal a growth dependent ASP2A promoter activity, very high in young siliques, which becomes restricted to stigma region during growth and not detectable in fully developed siliques (Figure 3.5.C and F). There is no detectable promoter activity in seed coat or funiculus (Figure 3.5.C and F).

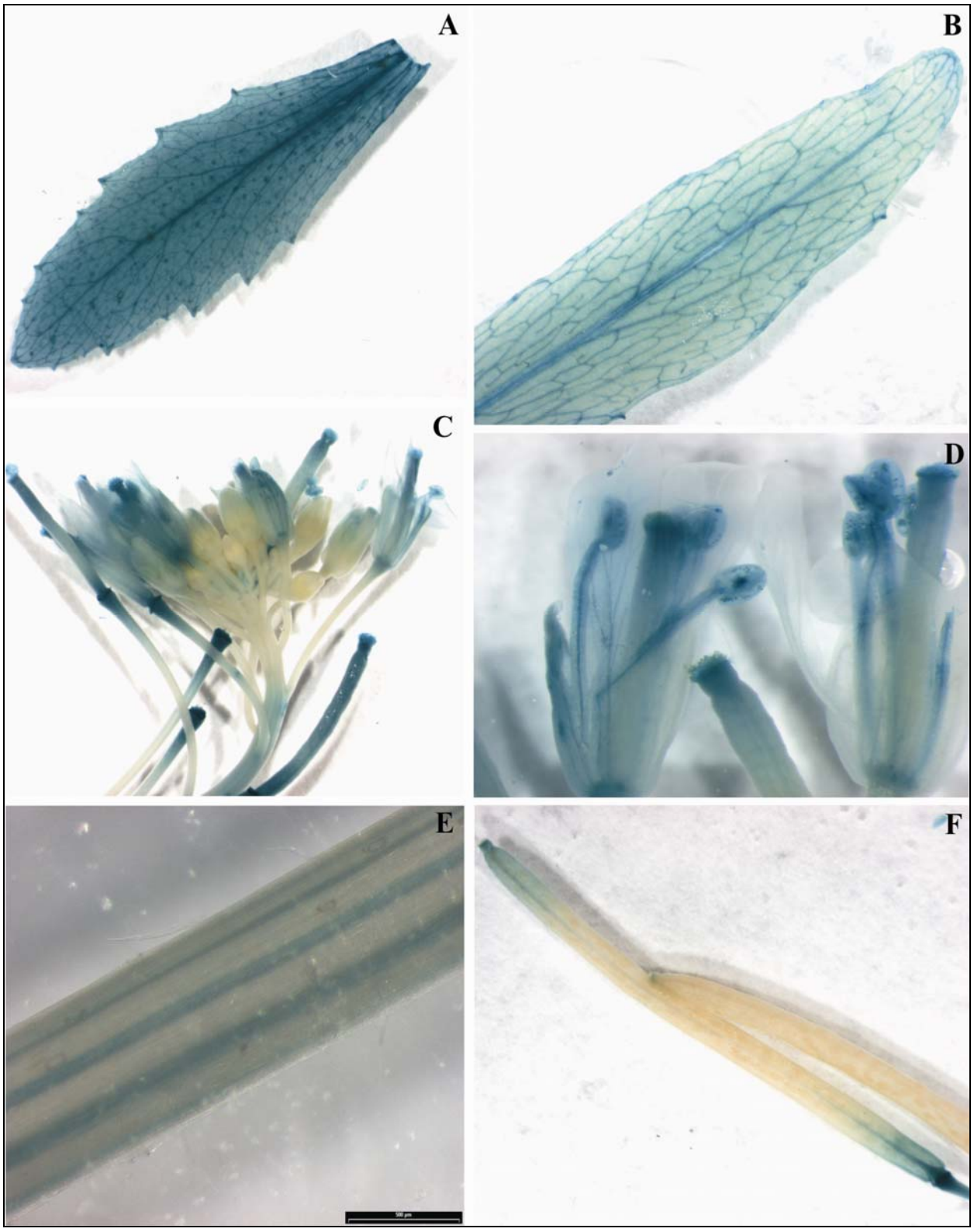

Figure 3.5. ASP2A promoter driven GUS activity in stably transformed *A. thaliana*. A) Sink leaf, B) Source leaf, C-D) Flower, E) Stem, and F) Siliques.

The activity of the 1 kb region of the ASP2B promoter in sink leaves is found to be exclusively dependent on the age of the leaf, i.e. very young rosette leaves are stained only in the vascular bundle and the promoter activity spreads to mesophyll cells as the leaf grows (Figure 3.6.A). The staining pattern stays the same during later stages of leaf development (Figure 3.6.B).

The comparison of staining patterns of flowers from *A. thaliana* plants expressing ASP1 promoter:GUS and ASP2A promoter:GUS constructs with the plants expressing ASP2B promoter:GUS construct reveals that ASP2B promoter activity is similarly dependent on developmental stage of flowers (Figure 3.6.C and D). The ASP2B promoter activity in siliques shows a close similarity to the staining pattern of ASP2A promoter activity, i.e., the promoter activity decreases as the silique grows (Figure 3.6.E and F).

The ASP3 promoter (1 kb region upstream of the start codon) driven GUS activity in stably transformed *A. thaliana* plants shows a high staining both in very young (Figure 3.7.A) and fully developed rosette leaves (Figure 3.7.B). In addition, the ASP3 promoter activity in flowers (Figure 3.7.C and D) is detectable starting from very early stages of flower development compared to that of ASP1, ASP2A and ASP2B promoters. The GUS activity driven by the 1 kb region of ASP3 promoter is restricted to receptacle and sepals in early stages and it spreads to pistils at later stages of development. The ASP3 promoter activity decreases with development of siliques and no activity is detected in seed coat and funiculus (Figure 3.7.E and F).

The 1 kb region of the ASP4 promoter is solely active in hydathodes in sink rosette leaves (Figure 3.8.A). At the later stages of leaf development, the histochemical GUS assay indicates the ASP4 promoter activity in the whole leaf blade with still a relatively higher activity in hydathodes (Figure 3.8.B). The GUS staining pattern of flowers and siliques shows a clear developmental stage dependence of promoter activity (Figure 3.8.C-E) as in the case of ASP2A and ASP2B promoters summarized before with the exception that the ASP4 promoter activity is also found in the funiculus (Figure 3.8.F).

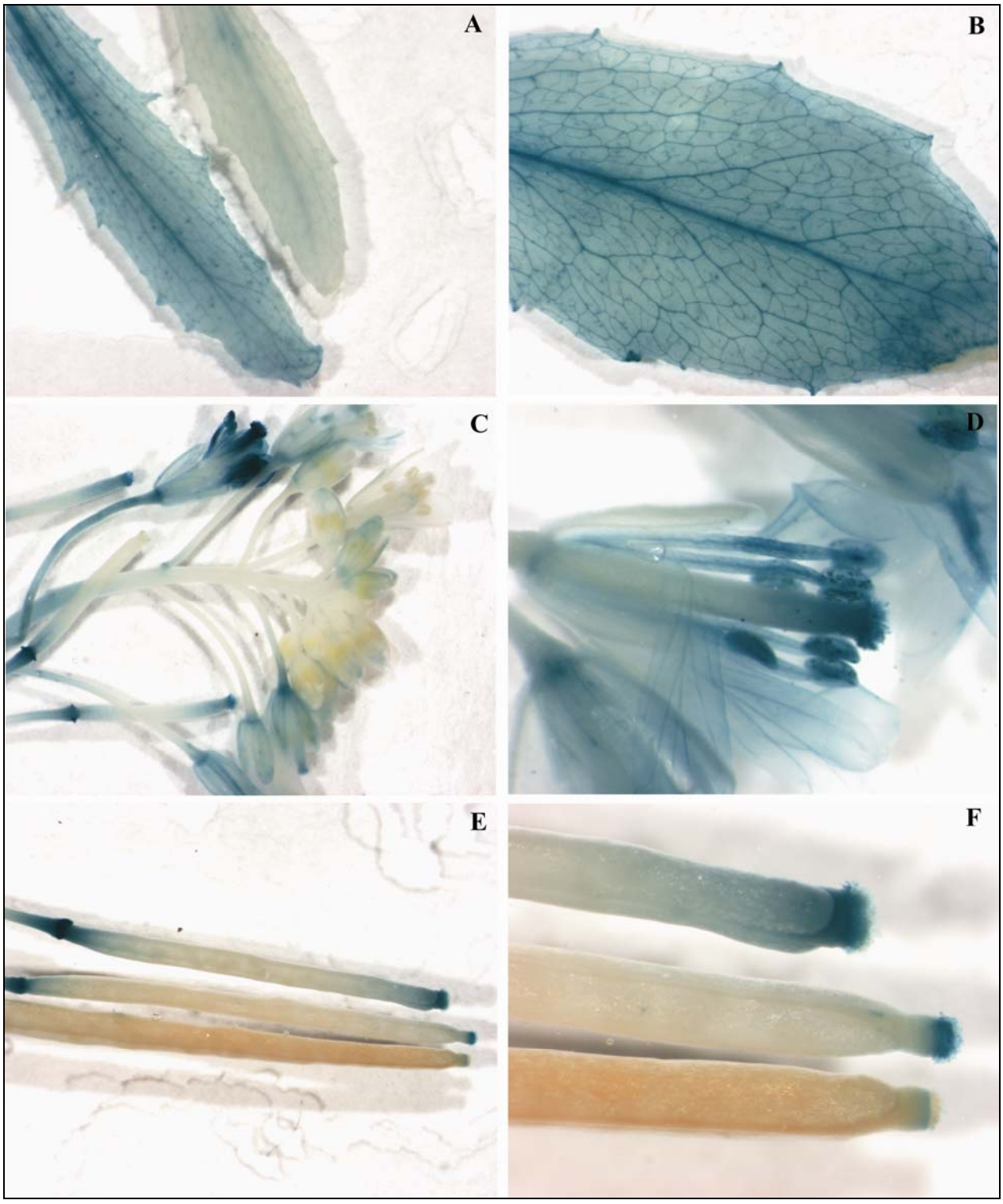

Figure 3.6 Histochemical analysis of *A. thaliana* plants with ectopic expression of ASP2B:GUS. A) Sink leaves, B) Source leaf, C-D) Flower, and E-F) Siliques.

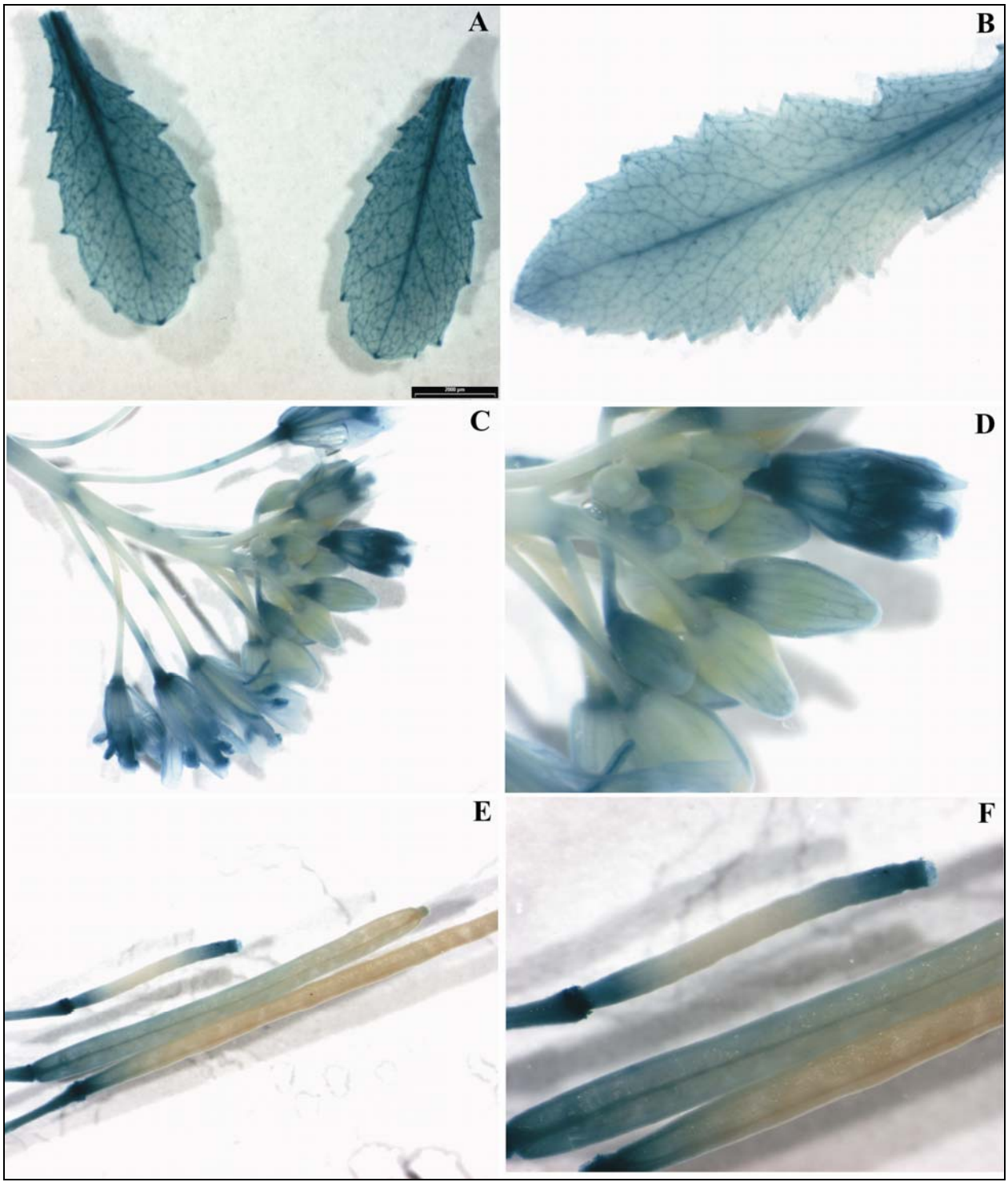

Figure 3.7 Histochemical analysis of ASP3 promoter driven GUS activity in stably transformed *A. thaliana* plants. A) Sink leaves, B) Source leaf, C-D) Flowers, and E-F) Siliques.

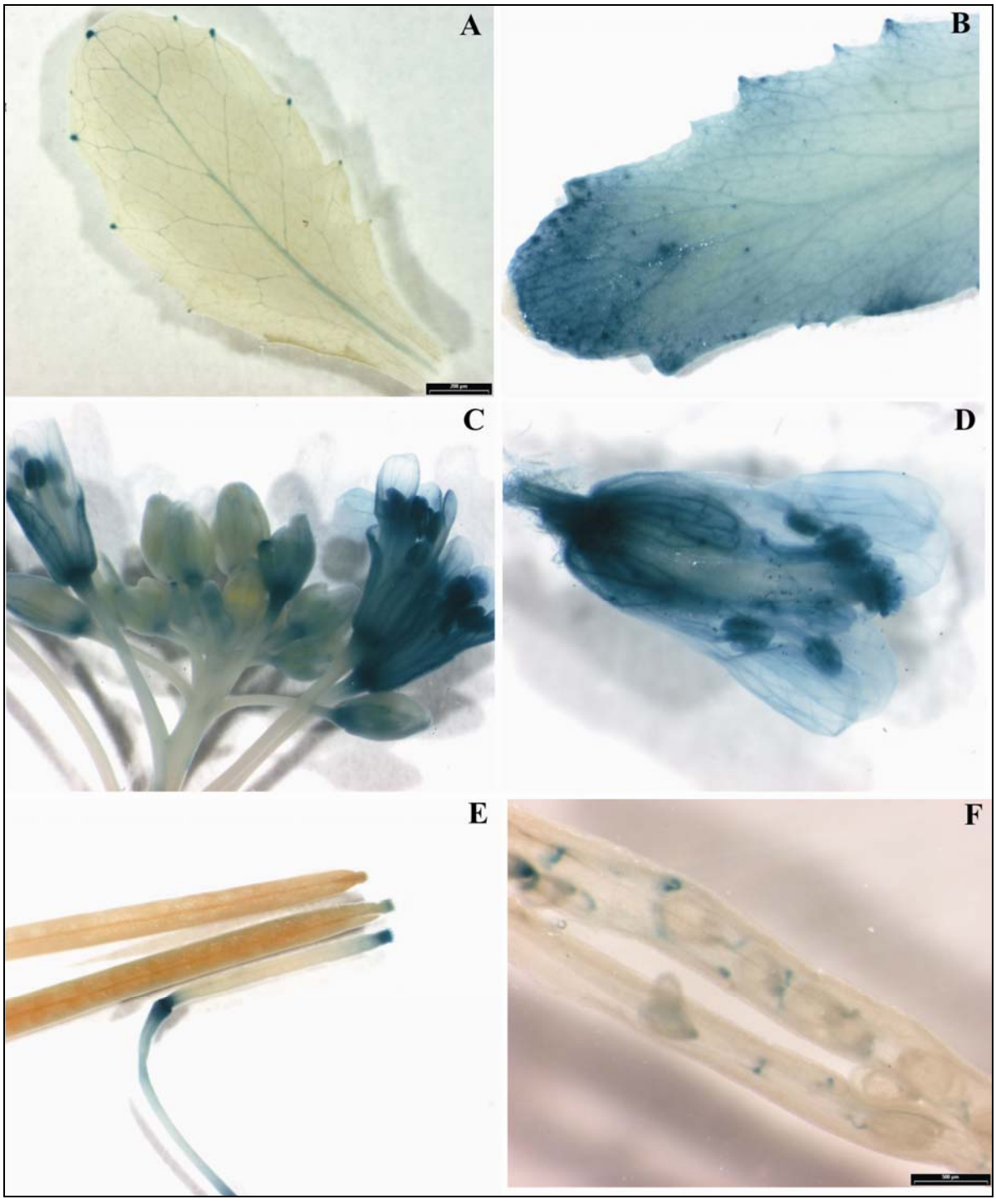

Figure 3.8 ASP4 promoter driven GUS activity in *A. thaliana* detected by histochemical analysis. A) Sink leaf, B) Source leaf, C-D) Flowers, E) Siliques, and F) Seeds.

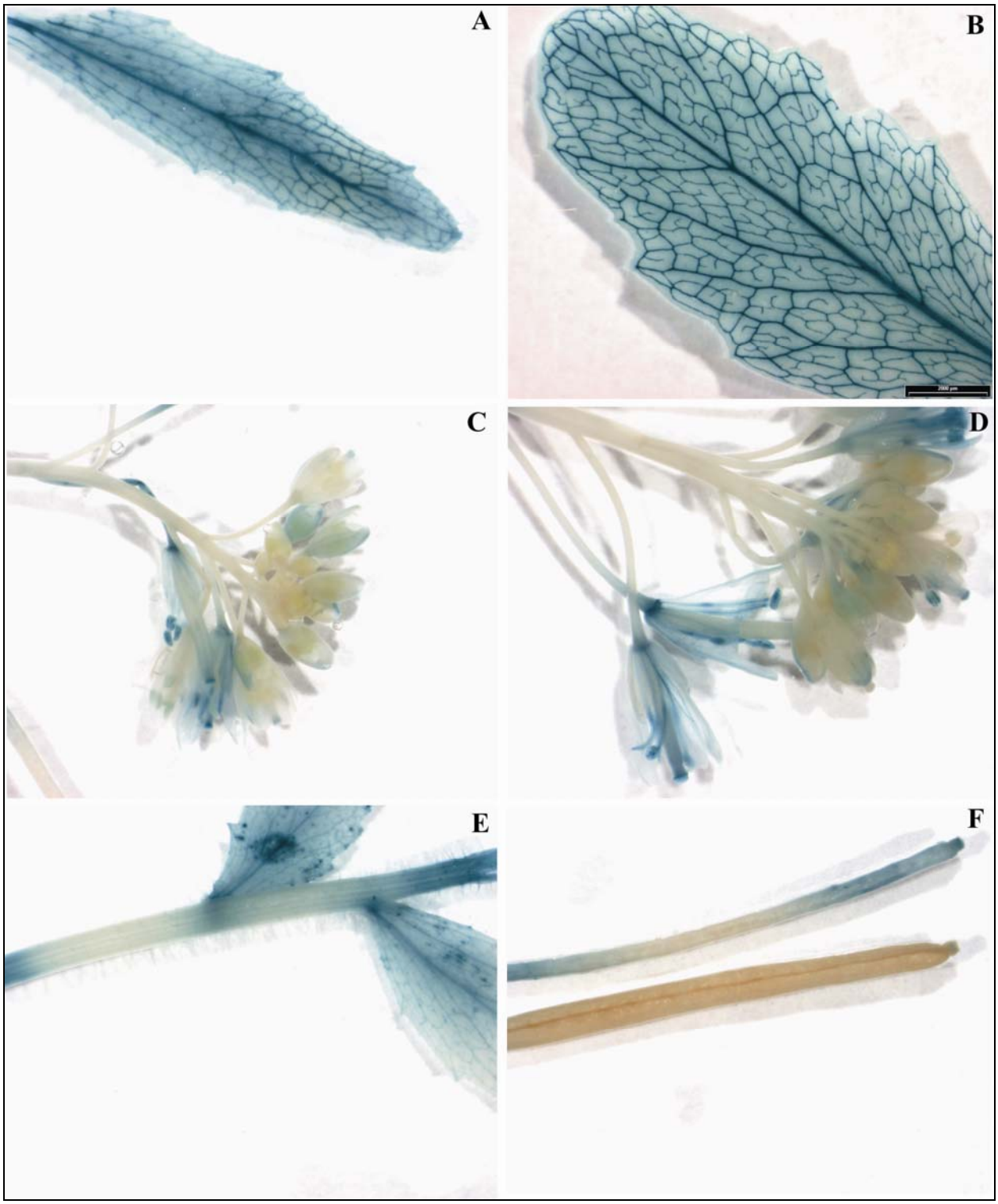

Figure 3.9 Promoter activity of ASP5 (the plastidial isoform) in stably transformed *A. thaliana* plants. A) Sink leaf, B) Source leaf, C-D) Flowers, E) Internode, and F) Siliques.

The histochemical assay of rosette leaves of stably transformed *A. thaliana* plants with ASP5 promoter driven GUS activity shows a high vascular bundle and relatively low blade staining independent of developmental stage (Figure 3.9.A and B). This is in contrast to what was expected based on the data showing the high activity of plastidial sPPases in fully developed spinach leaves (Weiner et al., 1987; Stitt, 1998). The promoter region of ASP5 is relatively short (385 base pairs; http://www.tair.org). It is relatively well known that the promoter activities of eukaryotic genes are not only controlled by cis-acting elements but also by trans-acting elements. The effect of trans-acting elements can be not functional after re-insertion of the promoter element by transformation and, therefore, this may cause differences in observed and real promoter activities. It is important to note that, the data indicating the high activity of plastidial enzyme is obtained from only one study; therefore, it may not be comprehensive for all plants or for all developmental stages.

The promoter activity of ASP5 in flowers is considerably low compared to the promoter activities of other *A. thaliana* sPPase isoforms (Figure 3.9.C and D). The main GUS staining is observed in stigma, stamen and petals of fully developed flowers (Figure 3.9.D). The histochemical GUS activity assays of shoots show the promoter activity in node regions (Figure 3.9.E). The ASP5 promoter activity is observed to be decreasing with increasing developmental stage of siliques (Figure 3.9.F).

In the case of GUS activity driven by the 1 kb regions of ASP2B and ASP3 promoters, blue staining is also observed in trichomes (Figure 3.10) which are single epidermal cells differentiated to giant unicellular structures (Marks, 1997).

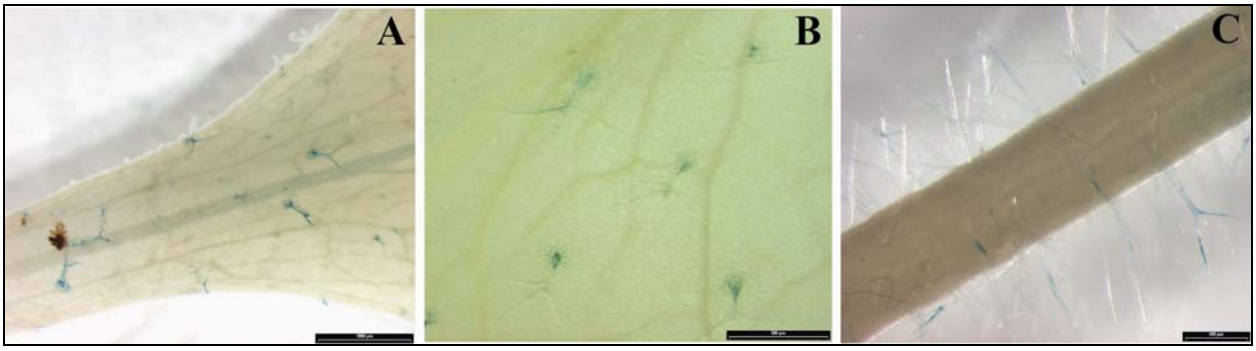

Figure 3.10. Blue staining observed in trichomes of stably transformed *A. thaliana* plants expressing A-B) ASP2B promoter:GUS and C) ASP3 promoter:GUS constructs.

The *A. thaliana* soluble pyrophosphatase promoter driven GUS staining shows an isoform specific activity distribution in root tips of stably transformed *A. thaliana* plants (Figure 3.11).

Accordingly, the 1 kb region of promoters of ASP1 and ASP5 drives the GUS expression in apical meristem and root cap, but no activity is observed in elongation and differentiation regions of the roots. On the other hand, the promoters of ASP2A, ASP3 and ASP4 show GUS activity in all defined root tip regions (root cap, apical meristem, zone of elongation and zone of differentiation). ASP2B promoter driven GUS activity has a very distinct pattern when compared to those of other ASP promoter regions by not having any activity in either apical meristem or root cap. The GUS staining of roots of *A. thaliana* plants transformed with ASP2B promoter:GUS construct shows an activity after the zone of cell differentiation. Among all ASP isoforms, only ASP4 promoter driven GUS activity yields an overall staining of roots, whereas the blue staining derived from other isoforms diminishes as root length increases.

From the results shown in Figures 3.4 to 3.11, it may be concluded that as expected from isoforms of enzyme families localized to the same subcellular compartments, the analysis of stably transformed plants with GUS activity driven by the 1 kb promoter region of each *A. thaliana* sPPase isoform shows tissue and/or developmental stage specificity of gene expression. For example, the expression profiles of 1 kb promoter regions of ASP2A, ASP2B and ASP4 are very similar during flower development, on the other hand, that of ASP3 promoter shows activity in very early stages and ASP5 shows an activity at the later stages of flower development. During silique development, all isoforms except ASP1 shows the same change in the promoter activities. In fully developed siliques, only the promoter activity of ASP1 is observed and both ASP1 and ASP4 promoter regions drive expression of GUS in seed funiculus. The blue staining of leaf vascular bundles are observed in all isoforms and leaf blade staining shows both isoform- and developmental stage-specific changes in expression.

The promoter regions of *A. thaliana* sPPase isoforms are found to activate transcription not only in very young and growing, but also in fully developed tissues. Therefore, it seems that the cytosolic sPPases are also functional at later stages of development. However, it is important to note that in the analysis the full promoter regions of only ASP2B, ASP4 and ASP5 were used, whereas only a 1 kb region upstream of the start codon of ASP1, ASP2A and ASP3 was used to enable the comparison of histochemical GUS assay results of *A. thaliana* sPPases with each other.

The promoter:GUS fusions are not sufficient to understand the *in vivo* regulation of gene expression, since it only reflects the promoter activity in different tissues but does not give any data on post-transcriptional regulation like mRNA stability and regulation associated with UTRs (Taylor, 1997). Therefore, the quantitative determination of the amount of transcript in different tissues is important.

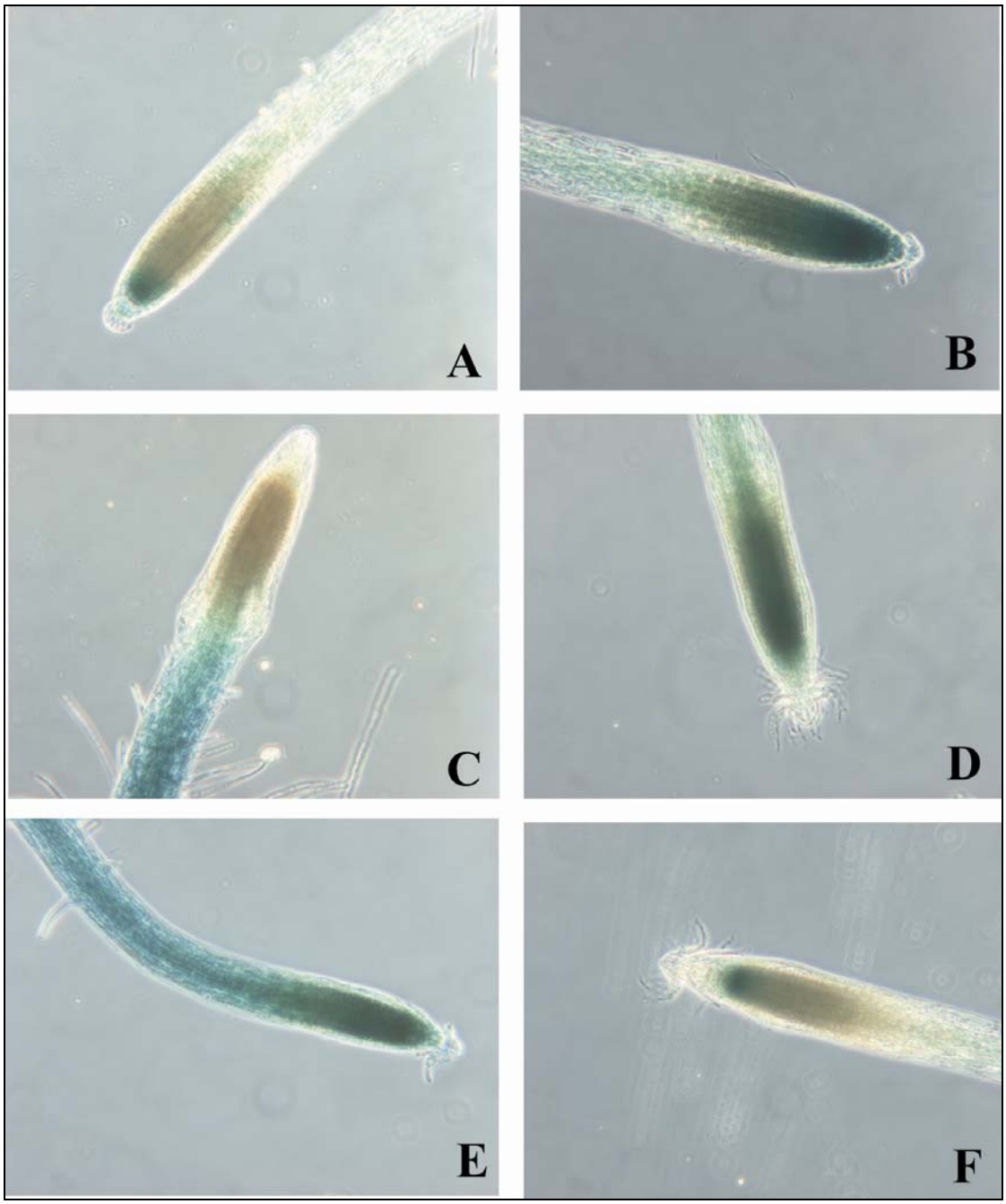

Figure 3.11. GUS activity of 1 kb region of ASP observed in root tips of stably transformed *A. thaliana* plants. A) ASP1 promoter:GUS, B) ASP2A promoter:GUS, C) ASP2B promoter:GUS, D) ASP3 promoter:GUS, E) ASP4 promoter:GUS, and F) ASP5 promoter:GUS.

3.1.2.2 Quantitative Expression Analysis of ASP Isoforms by Real Time PCR

The quantification of the transcript amount of each *A. thaliana* sPPase isoform was performed by real time PCR analysis using cDNAs amplified from different tissues; seedling (2 weeks after germination), sink leaf (4 weeks after germination), source leaf top (where cells are fully expended), source leaf bottom (where cells are still expanding) (both 4 weeks after germination), flower (6 weeks after germination), root (4 weeks after germination), siliques (green siliques containing seeds; 6 weeks after germination) and stem (4 weeks after germination).

As indicated in Chapter 3.1, the coding sequences of *A. thaliana* sPPase isoforms are highly conserved (Figure 3.2). Therefore, in order to amplify isoform-specific transcripts, the forward primers used in real time PCR amplification were designed according to 5'-UTR sequences. The expression data was normalized using actin as the reference gene, whose expression is relatively constant in different tissues and at different developmental stages (Muller et al., 2002). The results of mean normalized expression of each ASP isoform at different tissues with respect to actin expression are given in Figure 3.11.

The expression of the plastidial isoform (ASP5) has the highest expression compared to the other *A. thaliana* soluble pyrophosphatase isoforms especially in green tissues like seedling, sink and source leaves (Figure 3.11). The expression of ASP5 in source leaf top and source leaf bottom tissue regions are similar as can be seen with the comparison of mean normalized expression in these regions (Figure 3.12). It is important to note that the expression of the plastidial isoform is also observed in heterotrophic tissues like root and stem.

Although Figure 3.12 emphasizes the detection of gene expression of all *A. thaliana* sPPases, Figure 3.13 shows the mean normalized expression values of ASP isoforms localized in cytosol and/or nucleus (ASP1, ASP2A, ASP2B, ASP3 and ASP4) more clearly.

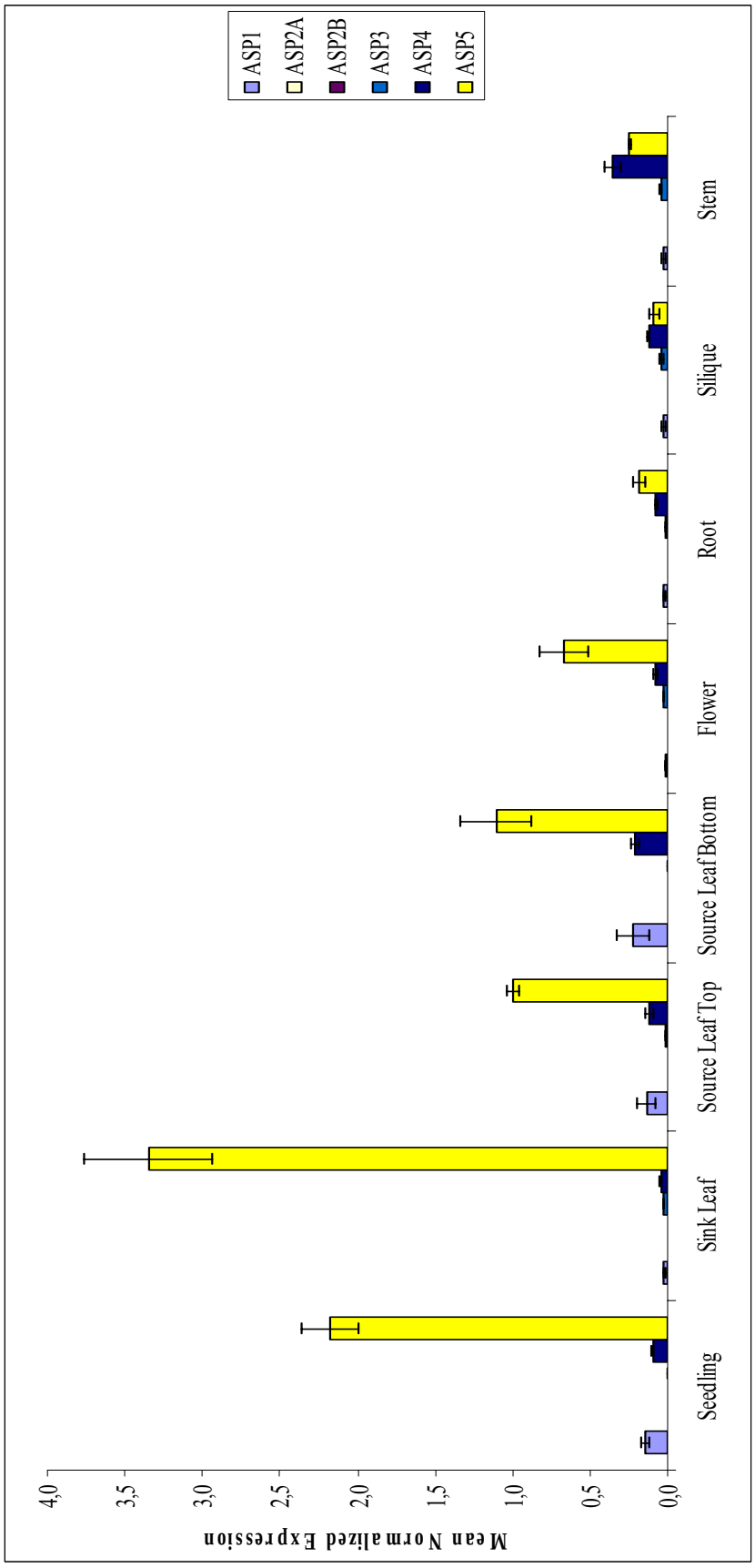

Figure 3.12. Real time PCR analysis of *A. thaliana* sPPase isoforms at different tissues. The gene expression was normalized using actin as the reference gene (Muller et al., 2002). The data was averaged using three independent experiments and error bars indicates the ± SD. The source leaves were cut into half and the side close to node was represented as "source leaf bottom" whereas the other half as "source leaf top" in order to compare the expression of sPPases in fully expended cells with still expanding ones. The siliques were collected at the stage where seeds are formed but siliques are still green (6 weeks after germination).

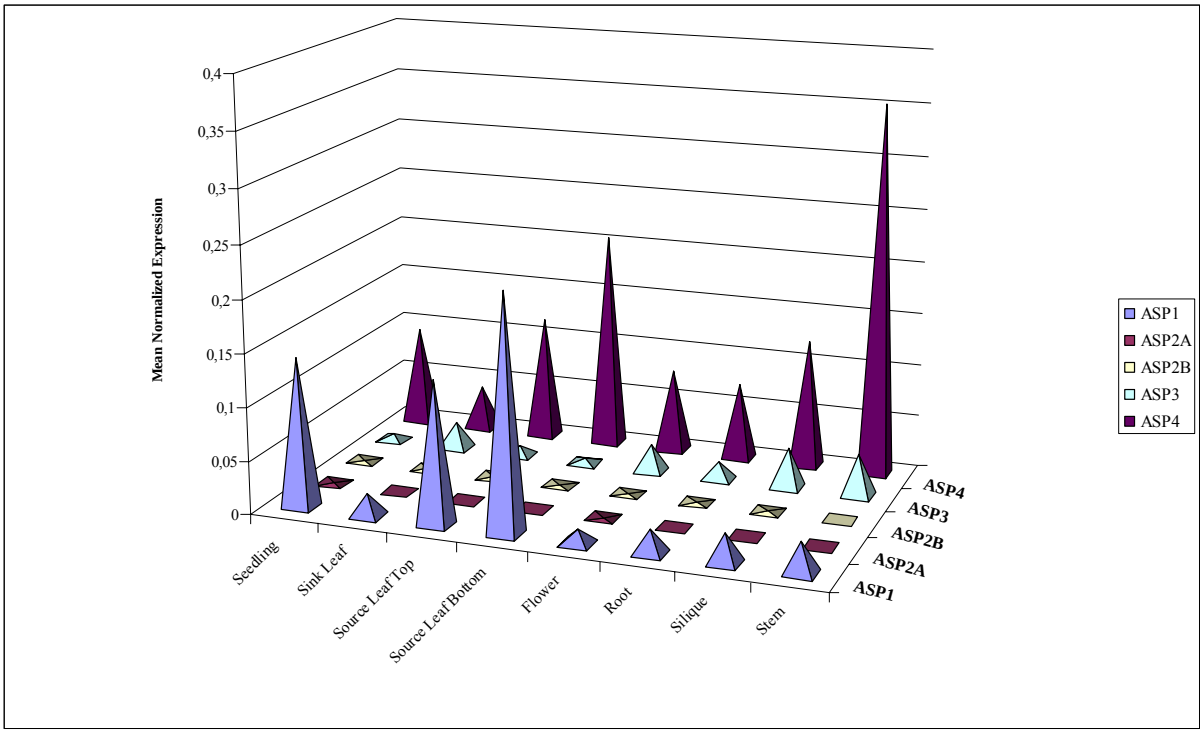

Figure 3.13. Real time PCR analysis of *A. thaliana* sPPase isoforms localized in cytoplasm and/or nucleus (excluding the plastidial isoform). The expression was normalized using actin as the reference gene (Muller et al., 2002). The data was averaged using three independent experiments and error bars indicate ± SD. The source leaves were cut into half and the side close to node was represented as "source leaf bottom" whereas the other half as "source leaf top" in order to compare the expression of sPPases in fully expended cells with still expanding ones. The siliques were collected at the stage where seeds are formed but siliques are still green (6 weeks after germination).

The real time PCR analysis for the quantification of the expression of *A. thaliana* sPPase isoforms which are shown to localize in cytosol and/or nucleus in subcellular localization studies (Chapter 3.1.1.2) reveals very low transcript amounts in comparison to both actin and ASP5 (the plastidial isoform) (Figure 3.13). However, the gene expression is still detectable and shows clear tissue specificity depending on the isoform.

ASP1 and ASP4 have the highest transcript amount among *A. thaliana* sPPase isoforms localized in the same compartment and are expressed in all tissues used for real time PCR analysis. On the other hand, although the histochemical analysis of the stably transformed *A. thaliana* plants with 1 kb promoter region driven GUS expression showed a promoter activity in several tissues like leaves and flowers, the transcript amount of ASP2A and ASP2B are almost not detectable in these tissues used for quantitative real time PCR analysis. The reasons of inconsis-

tency between histochemical promoter analysis and transcript quantification can be related with factors like post-transcriptional regulation or mRNA stability.

The comparison of mean normalized expression values of ASP1, ASP3 and ASP4 in source leaf top and bottom regions indicates that the transcription of these sPPases are not significantly regulated with the cell expansion.

The results obtained from stable transformation of promoter:GUS constructs and quantitative real time PCR analysis of soluble pyrophosphatase isoforms indicated that in *A. thaliana*, not only the plastidial isoform but also some cytoplasmic and/or nuclear localized sPPases have active transcription in all tissues used in the analysis. This is the first indication that cytoplasmic plant pyrophosphatases are actively transcribed in several tissues during development; and therefore, they might be more important in the regulation of plant metabolism than hypothesized before (Chapter 2.2.2.3).

3.1.3 Response of *A. thaliana* sPPases to Different Stresses

There are no data available in the literature with respect to the changes in expression of soluble plant pyrophosphatases under stress conditions. Therefore, a detailed analysis of the response of *A. thaliana* sPPases to different environmental conditions was performed by quantitative real time PCR. In order to reveal which possible environmental conditions may lead to changes in ASP expression, the promoter region of each isoform was searched for the presence of known transcriptional regulatory motifs using several web-based programs (Chapter 3.1.3.1).

3.1.3.1 Transcriptional Factor Binding Motifs in Promoter Regions of ASPases

The full promoter regions of *A. thaliana* soluble pyrophosphatases were downloaded from TAIR database (http://www.tair.org) and analyzed for the presence of known cis-acting elements related with the response to changes in environmental conditions using web-based programs PLACE (plant specific) (Higo et al., 1999), TFSearch (plant specific) (Heinmeyer et al., 1998), and Softberry NSITE-PL (plant specific) TSSP (for eukaryotes, not specific to plants) (http://www.softberry.com). The transcriptional factor binding motifs most commonly found in the promoter regions of ASP are summarized in Table 3.4.

Table 3.4. Environmental stress responsive transcriptional factor binding motifs found in the promoter regions of *A. thaliana* soluble pyrophosphatases. Web-based tools PLACE (Higo et al., 1999), TFSearch (Heinmeyer et al., 1998), and Softberry NSITE-PL and TSSP programs (http://www.softberry.com) were used for the detection of possible regulatory motifs.

Regulatory Element	Motif	Responsive to	ASP	Reference
ABRELATERD1	ACGTG	Etiolation	2B, 4, 5	(Simpson et al., 2003)
AGCBOXNPGLB	AGCCGCC	Wounding	2B	(Rushton et al., 2002)
CCAATBOX1	CCAAT	Heat	All	(Haralampidis et al., 2002)
CDA1ATCAB2	CAAAACGC	Dark	2B	(Maxwell et al., 2003)
CGACGOSAMY3	CGACG	Sugar starvation	2A, 3	(Hwang et al., 1998)
DRE1COREZMRAB17	ACCGAGA	Drought	1	(Kizis and Pages, 2002)
ELRECOREPCRP1	TTGACC	Wounding	2B, 4, 5	(Rushton et al., 2002)
GARE1OSREP1	CAACTC	Gibberellin and ABA	2B	(Sutoh and Yamauchi, 2003)
GATABOX	GATA	Light	All	(Gilmartin et al., 1990)
GCCCORE	GCCGCC	Wounding	2B	(Rushton et al., 2002)
GT1CORE	GGTTAA	Light	2A, 5	(Gilmartin et al., 1990)
MYCATERD1	CATGTG	Drought	3, 4, 5	(Simpson et al., 2003)
TATCCAOSAMY	TATCCA	Sugar and hormone	1, 2B, 4, 5	(Lu et al., 2002)
TATCCAYMOTIFOSRAMY3D	TATCCAY	Sugar	1, 2B	(Toyofuku et al., 1998)
TCA1MOTIF	TCATCTTCTT	Salicyclic acid	2A	(Urao et al., 1993)

Based on the transcriptional regulatory motifs found in the promoter regions (Table 3.4), the changes in the expression of ASP isoforms were examined in response to wounding, etiolation, hormone treatment (ABA), salt treatment, different temperatures (heat and cold treatments), feeding with different sugars and phosphate starvation by quantitative real time PCR analysis.

3.1.3.2 Response of *A. thaliana* sPPase Expression to Etiolation

The response of ASP isoforms to etiolation was determined by using *A. thaliana* seedlings grown in ½ MS medium under either dark (for etiolation) or light conditions for 7 days after germination. The real time PCR analysis was performed to detect the changes in gene expression of each *A. thaliana* soluble pyrophosphatase isoforms (Figure 3.14).

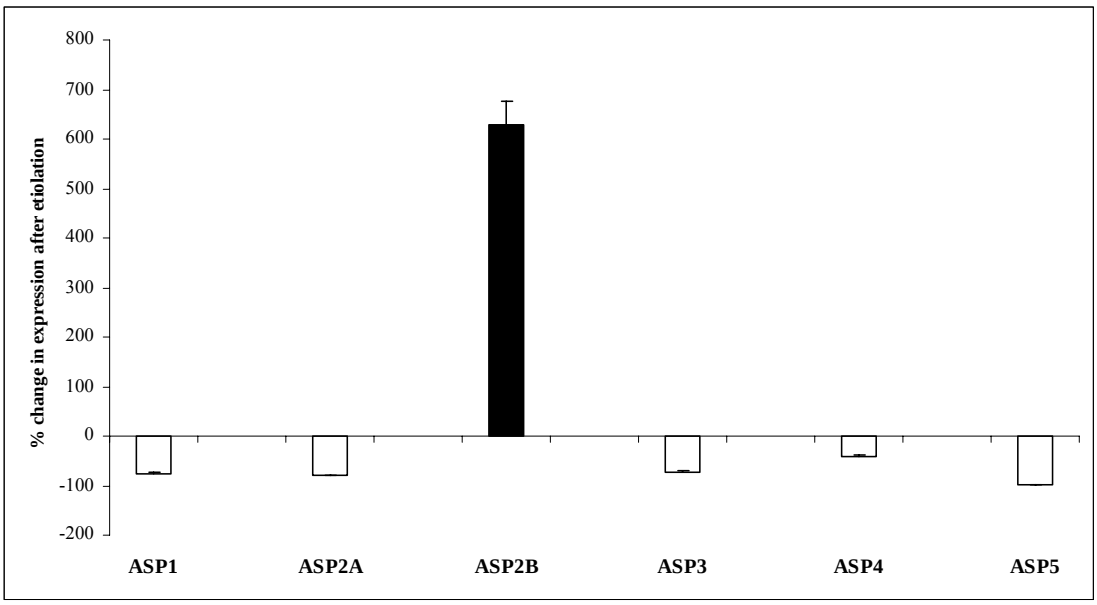

Figure 3.14. Percent change of the ASP expression in dark grown *A. thaliana* seedlings compared to light grown seedlings. The data were normalized (Muller et al., 2002) using actin gene expression as the reference. The percent change in expression is the average of two independent experiments and ± SD values are given as error bars.

The expression of ASP5 (the plastidial isoform) which has the highest expression in seedlings (Figure 3.12) is completely repressed (~100 % decrease in gene expression) after etiolation as expected. Among the other *A. thaliana* sPPases, only ASP2B is strongly induced, whereas the expression of ASP1, ASP2A, ASP3 and ASP4 are significantly down-regulated. It is important to note that ASP2B is expressed in very low levels in seedling (Figure 3.13), and therefore, the induction can simply be considered as the starting of gene expression of ASP2B after etiolation.

3.1.3.3 Expression of ASP Isoforms in Response to Different Sugars Analyzed in *A. thaliana* Cell Culture

The enzymes of plant carbohydrate metabolism are known to be transcriptionally regulated by soluble sugars. In order to determine the effect of soluble sugars to the gene expression of ASP family, *Arabidopsis thaliana* cell culture in exponential growth phase was inoculated to fresh medium containing different feeding sugars after successive washings with sugar-free medium. The expressions of *A. thaliana* sPPase isoforms in cell cultures grown in the presence of either glucose or sucrose was quantified using real time PCR analysis (Figure 3.15).

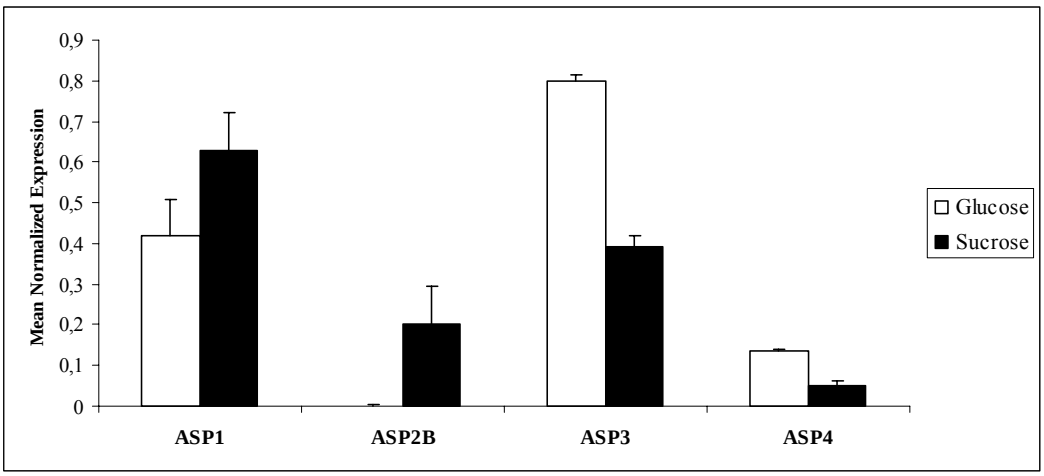

Figure 3.15. Mean normalized expression levels of ASP1, ASP2B, ASP3 and ASP4 in cell culture medium containing either glucose or sucrose quantified by real time PCR analysis. The data normalized (Muller et al., 2002) using actin as the reference gene and averaged from two independent experiments. Error bars indicate ± SD values.

The expressions of ASP2A and ASP5 are below the detection limits of real time PCR analysis, therefore, can be considered as not expressed in *A. thaliana* cell cultures. The expressions of other ASP isoforms that are shown to be localized in cytoplasm and/or nucleus (Chapter 3.1.1.2), are dependent on the form of feeding sugar (Figure 3.15). Accordingly, ASP1, ASP2B, ASP3 and ASP4 have expression in growth medium containing sucrose, whereas, although the expression of ASP1, ASP3 and ASP4 are still detected in growth medium containing glucose, ASP2B is not expressed under these conditions.

For analysis of the effect of sugar starvation to the gene expression of ASP isoforms, the glucose in the growth medium was replaced with 3-*O*-methylglucose (Cortes et al., 2003) and the cells were collected after 3 hours, 1 day and 3 days of inoculation to the new medium. Expression of isoforms ASP2A and ASP5 were not detected in *A. thaliana* cell culture grown in medium containing 3-*O*-methylglucose as carbohydrate source, either. The percent changes in the expression of other ASP isoforms are shown in Figure 3.16.

As can be seen from Figure 3.16, ASP2B, which has no expression in growth medium supplied with glucose, is the only isoform that is induced under sugar starvation conditions mimicked by growing *A. thaliana* cell cultures in a medium containing 3-*O*-methylglucose as feeding sugar. All other isoforms having expression in growth medium containing glucose have repressed after replacing sugar source with 3-*O*-methylglucose. It is noteworthy that ASP2B has no detect-

able expression under normal growth conditions (Figures 3.13), however, strongly induced by both etiolation (Figure 3.14) and sugar starvation (Figure 3.16).

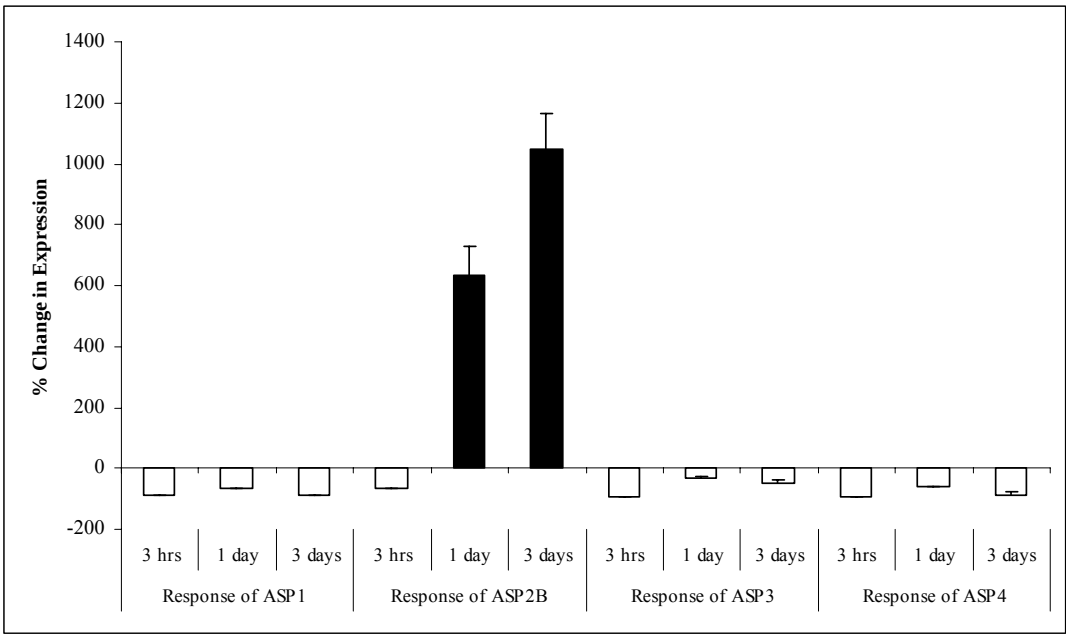

Figure 3.16. Percent change in the expression of ASP1, ASP2B, ASP3 and ASP4 after inoculating *A. thaliana* cell culture grown in glucose containing medium to growth medium containing 3-*O*-methylglucose after two successive washings with sugar-free medium. The data normalized (Muller et al., 2002) using actin as the reference gene and averaged from two independent experiments. Error bars indicate ± SD values.

3.1.3.4 Expression of ASP Isoforms in Response to Different Sugars *in planta*

The quantitative real time PCR analysis performed by feeding *A. thaliana* cell culture with different sugars indicated isoform-specific differences in expression of ASPs. However, *A. thaliana* cell cultures cannot be used as the representative of *in planta* responses, especially because the expression of plastidial isoform (ASP5) is undetectable (Chapter 3.1.3.3).

The investigation of the response of ASP isoforms to different sugars *in planta* was performed by incubating leaf disks from fully developed rosette leaves (R = 0.05 cm) in 100 or 300 mM concentrations of glucose, sucrose or 3-*O*-methylglucose overnight at dark at room temperature. The control tissue was incubated at distilled water under the same conditions. The changes in the expression of each ASP isoform were quantified using real time PCR analysis (Figure 3.17).

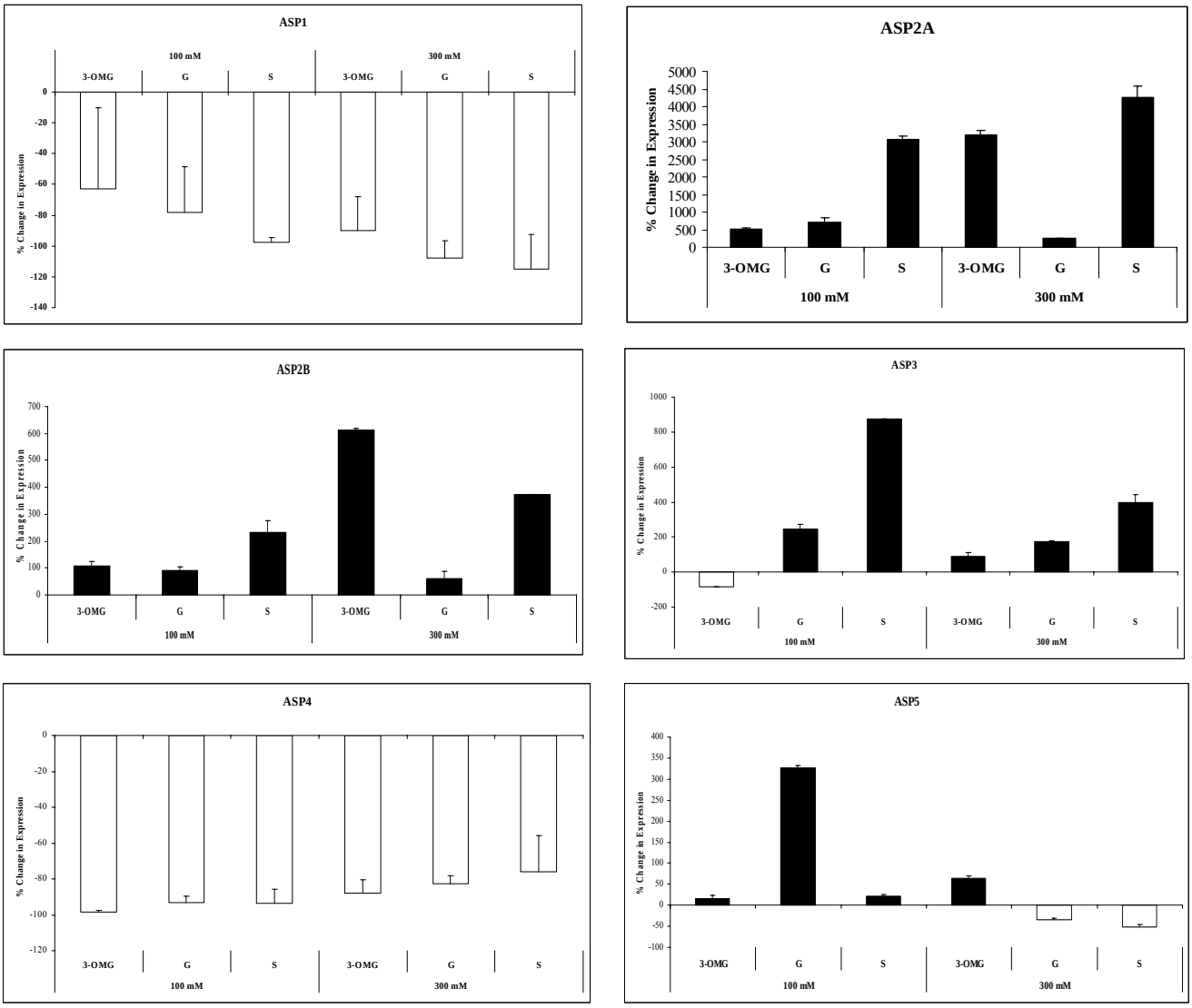

Figure 3.17. Percent change in the expressions of ASP isoforms in response to feeding with different sugars. 3-OMG; 3-*O*-methlyglucose, G; glucose and S; sucrose. The data normalized (Muller et al., 2002) using actin as the reference gene and averaged from two independent experiments. Error bars indicate ± SD.

The results of *in planta* changes in the gene expression of ASP isoforms in response to feeding different sugars indicate that all soluble pyrophosphatase isoforms of *A. thaliana* are responsive to carbohydrate source available in the medium (Figure 3.17). The responses of ASP1 and ASP4 to 100 mM or 300 mM glucose, sucrose and 3-*O*-methlyglucose indicate a complete down-regulation of gene expression (about 100 % decrease). The expressions of other isoforms (ASP2A, ASP2B and ASP3) are mainly upregulated to different degrees depending on the form of sugar. For example, ASP2A expression is induced in response to both 100 mM and 300 mM sucrose, whereas it is almost not responsive to different concentrations of glucose.

The response of gene expression of ASP2B to different sugars closely resembles that of ASP2A. Although the percent change in gene expressions are quite different from each other, in both cases the expression is not affected with glucose feeding, but significantly induced by 300 mM 3-*O*-methlyglucose and 100 or 300 mM sucrose. The induction in the gene expression of ASP2B to feeding with 3-*O*-methlyglucose is similarly induced both *in planta* and in cell culture.

The transcript amount of ASP3 shows a very clear dependence on the source of carbohydrate; it does not significantly changes in response to 100 or 300 mM 3-*O*-methlyglucose, however, induces with both 100 and 300 mM of glucose and sucrose. The induction of gene expression of ASP3 has the highest up-regulation in response to 100 mM sucrose.

The response of plastidial *A. thaliana* sPPase isoform (ASP5) to different sugar sources can be seen on Figure 3.17. The gene expression of ASP5 is significantly induced only by feeding with 100 mM glucose. The treatment with other concentrations or different sugar sources did not significantly changed the gene expression of ASP5.

3.1.3.5 Response of *A. thaliana* sPPase Expression to ABA and to Different Environmental Stresses

The responses of *A. thaliana* sPPase isoforms to the changes in environmental conditions were quantified by real time PCR analysis using six weeks old plants grown in hydroponics. The hormone treatment was performed by adding 100 µM ABA to hydroponics solution and leaf and root tissues were collected 16 hours afterwards. The salt stress was also applied for 16 hours by adding 200 mM NaCl to hydroponics solution. The response to temperature changes were analyzed by keeping the plants either at cold (4°C, dark) or heat (37°C, light) for 2 hours. The plants were later recovered for 16 hours under greenhouse conditions. The leaf and root samples were collected 2 hours after treatment (only for heat stress) and after recovery (for both cold and heat stresses). Phosphate starvation was performed by keeping plants in hydroponics solution prepared without phosphate (by replacing KH_2PO_4 with K_2SO_4 and keeping $[K^+]$ and $[SO4^{2-}]$ constant) for 5 days. The rosette leaves were wounded with a leaf-piercing device opening 100 holes in 1 cm^2 area and samples were collected 1 hour and 5 hours after wounding. The control leaf and root tissues for each treatment were collected at the same time points.

The changes in the gene expression of each *A. thaliana* sPPase isoform in response to ABA and to environmental stresses were quantified by real time PCR analysis and normalized (Muller et al., 2002) using actin expression as the reference. The percent change in expression

was calculated by averaging the data obtained from two independent experiments (Figures 3.18-23). 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 (Figure 3.13); 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.

The ASP1 gene expression is responsive to all treatments used for the analysis (Figure 3.18). The ABA treatment causes a significant induction of expression (about 175 %) in leaf tissue, whereas does not give in a significant change in expression in root tissue. After salt treatment, the expression is repressed by -78 % in leaf and about -40 % in root tissue. The cold treatment yields a 90 % increase in ASP1 expression in leaf, but causes no effect on the expression in root tissue. The induction of expression in heat-treated leaf tissue is observed only after recovery (29 % vs. about 100 % increase). The response in heat-treated root tissue is completely opposite to that of leaf tissue, where the 80 % induction in root tissue after 2 hrs at 37°C returns to initial level after recovery. The phosphate starvation causes a significant repression (by -85 %) in root tissue. There is about 80 % decrease in ASP1 expression after wounding.

Figure 3.19 shows the changes in ASP2A transcript amount after different treatments quantified by real time PCR analysis. The expression of ASP2A increases by 394 % in root tissue but significantly decreases (about -90 %) in leaf tissue after ABA treatment. The salt treatment of whole plants causes induction of expression both in leaf (about 190 %) and root (80 %) tissues. The cold treatment yields a significant repression of expression both in leaf (by -78 %) and root (by -49 %) tissues. The expression in leaf 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 phosphate starvation causes a significant repression on expression in leaf (by -84 %), whereas has no effect on that of root tissue. The wounding causes a significant induction in ASP2A expression, which is observed to be increasing by time (274 % after 1 hr and about 600 % after 5 hrs).

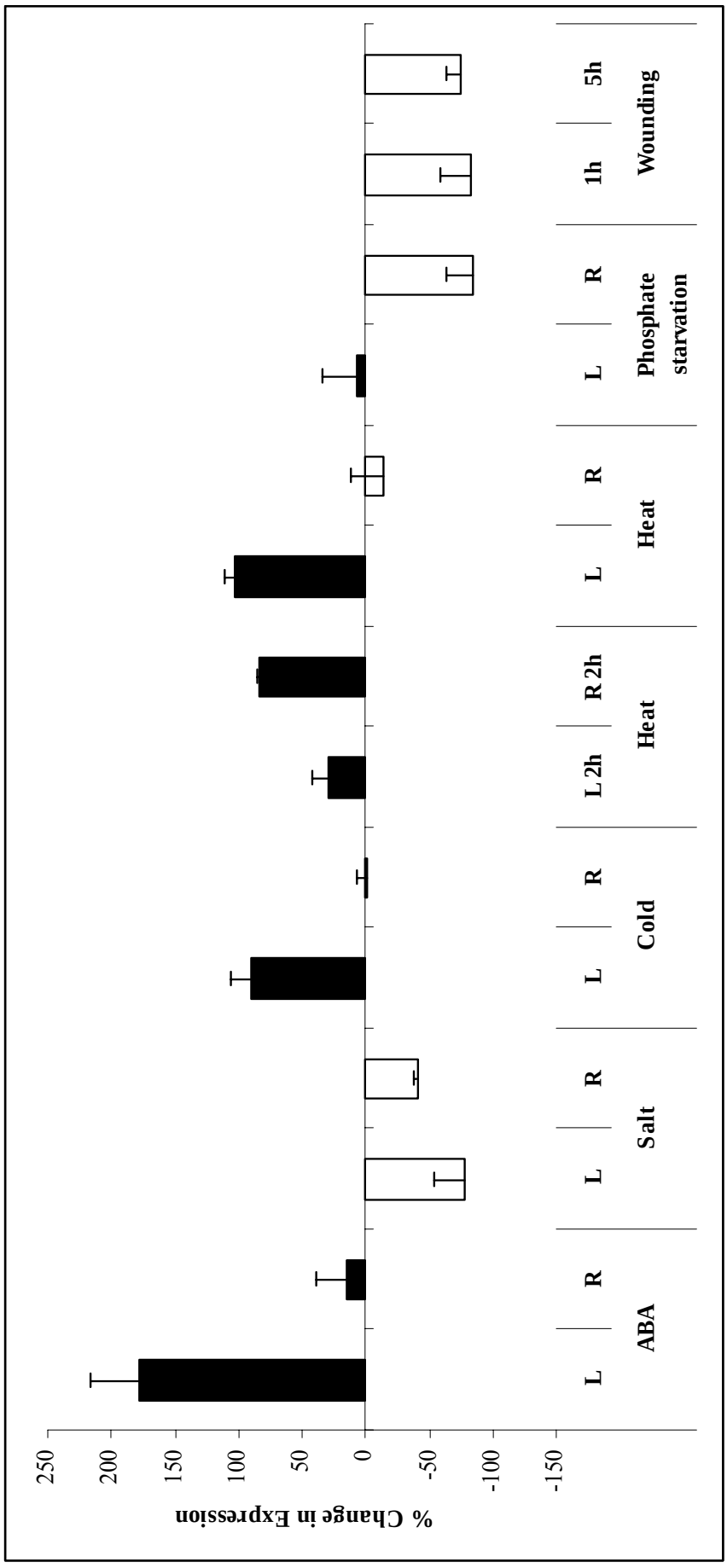

Figure 3.18. Percent change in the expression of ASP1 in response to ABA and to different environmental stresses. The data normalized (Muller et al., 2002) using actin as the reference gene and averaged from two independent experiments. Error bars indicate ± SD. L, leaf tissue; R, root tissue. Stress treatments were performed as follows: Salt 200 mM for 16 hours; ABA, 100 µM for 16 hours; Cold, 2 hours at 4°C and 16 hours recovery; Heat 2h, 2 hours at 37°C; Heat, 2 hours at 37°C and 16 hours recovery; Phosphate starvation, 5 days by replacing KH_2PO_4 in hydroponics solution with K_2SO_4, and Wounding; by wounding rosette leaves with 100 needle holes in 1 cm^2 area.

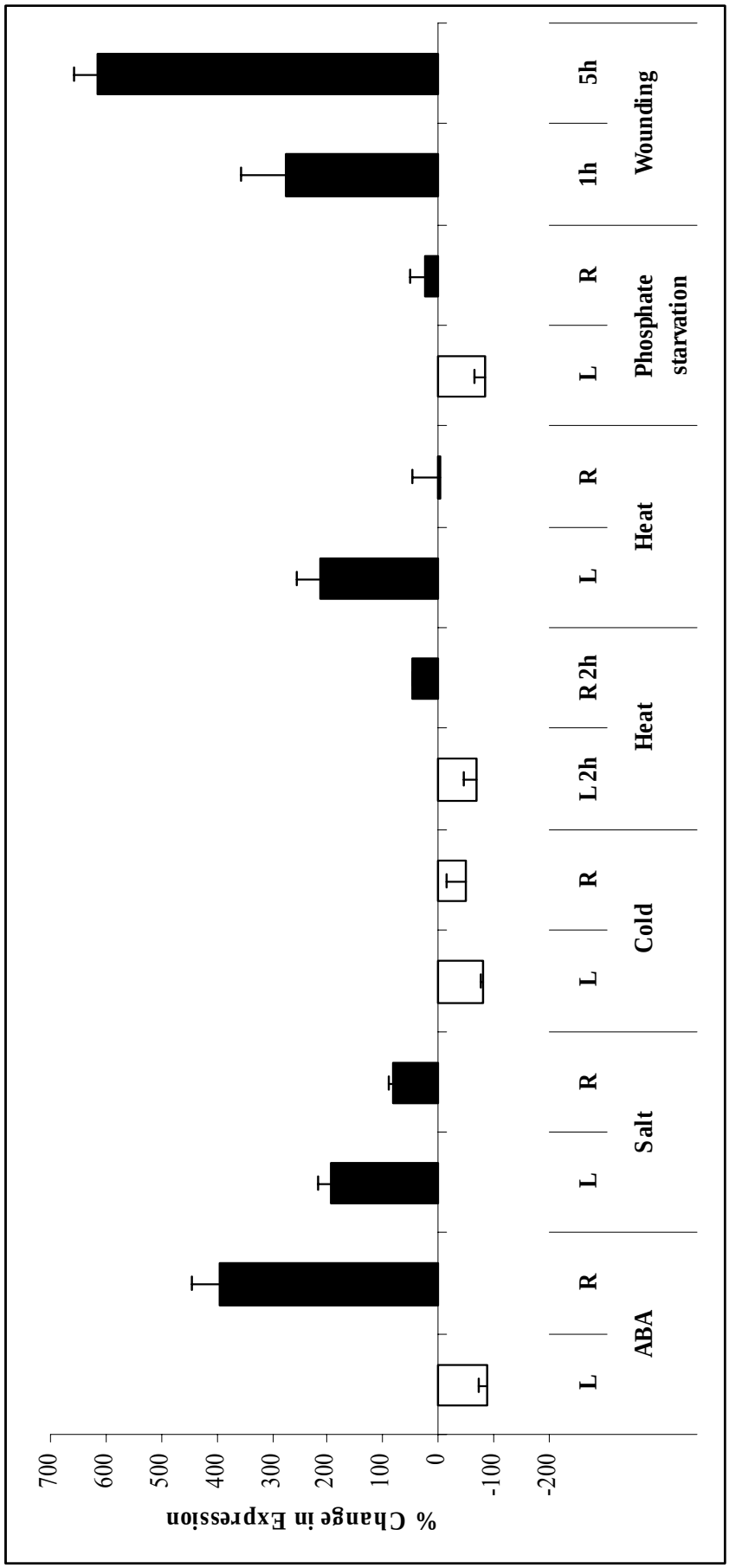

Figure 3.19. The change in the ASP2A expression in response to ABA and to environmental stresses. The data normalized (Muller et al., 2002) using actin as the reference gene and averaged from two independent experiments. Error bars indicate ± SD. L, leaf tissue; R, root tissue. Stress treatments were performed as follows: Salt 200 mM for 16 hours; ABA, 100 µM for 16 hours; Cold, 2 hours at 4°C and 16 hours recovery; Heat 2h, 2 hours at 37°C; Heat, 2 hours at 37°C and 16 hours recovery; Phosphate starvation, 5 days by replacing KH_2PO_4 in hydroponics solution with K_2SO_4, and Wounding; by wounding rosette leaves with 100 needle holes in 1 cm^2 area.

The gene expression of ASP2B changes by all stress treatments with distinct differences between leaf and root responses (Figure 3.20). The expression in leaf is increased by 83 % after 16 hours of salt treatment, whereas the expression in root is almost not affected (increased only by 17 %). The response of ASP2B gene expression to ABA, cold and heat treatments are completely the opposite in leaf and root tissues, where the expression in leaf is significantly repressed and that in root is induced. For example, ABA treatment causes 87 % decrease in leaf tissue and induces the expression by 274 % in root tissue. The response of ASP2B to cold stress is down-regulated by -88 % in leaf and upregulated by 98 % in root tissue. 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 after 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 phosphate starvation has no affect on ASP2B expression in leaf tissue but decreases the expression in root tissue by -75 %. The expression induces only 5 hours after wounding (by approximately 550 %) where no induction was observed one hour after treatment.

Figure 3.21 shows the change in gene expression of ASP3 under different environmental stresses. The highest induction in the expression of ASP3 is observed after ABA treatment both in leaf (by about 1300 %) and in root (by 632 %) tissues. The salt treatment causes a contrary change in the expression in leaf and root tissues; it is repressed by -78 % in leaf and induced by 589 % in root tissue. The change upon cold treatment is the induction of gene expression both in leaf and in root by 116 % and 175 %, respectively. 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 down regulated (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 phosphate starvation does not cause any significant change in the expression of ASP3 neither in leaf nor root 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 of wounding.

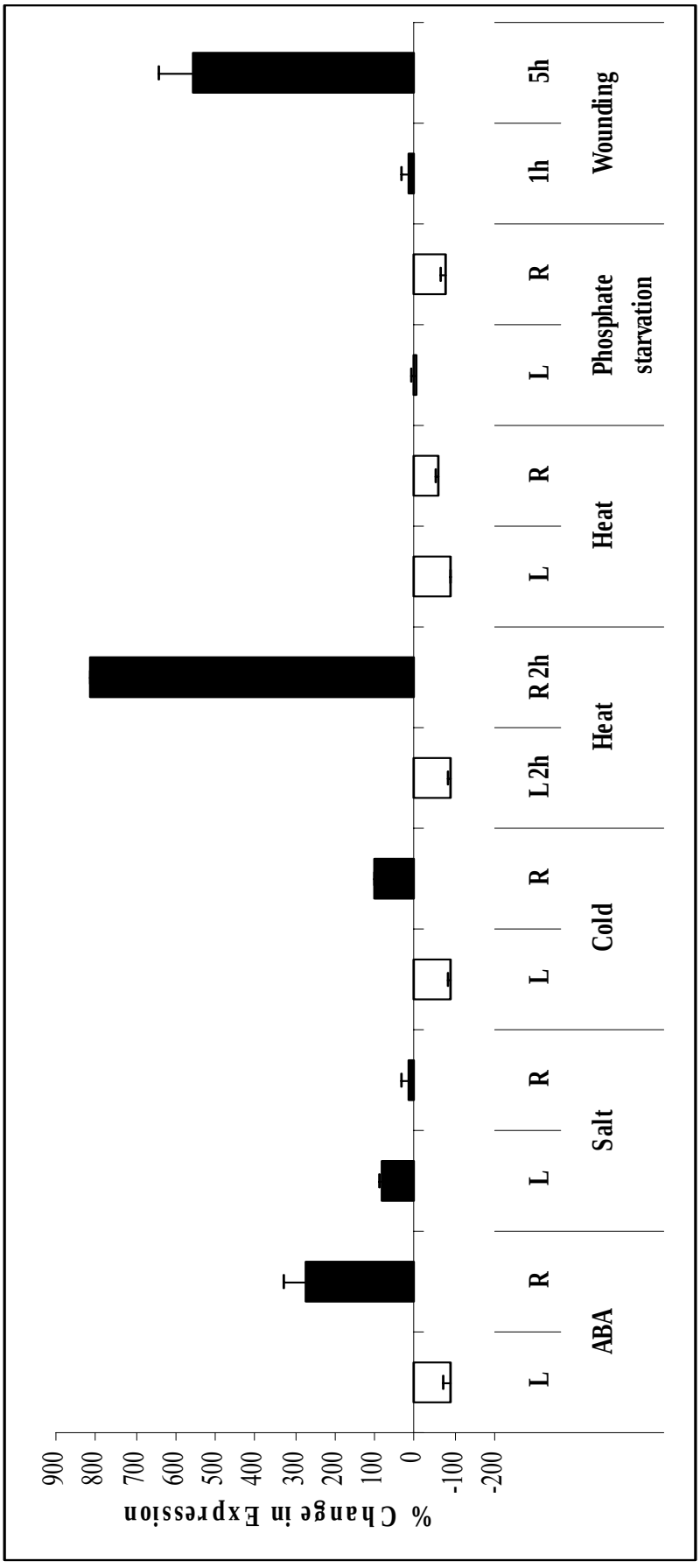

Figure 3.20. The response of ASP2B expression to different treatments. The data normalized (Muller et al., 2002) using actin as the reference gene and averaged from two independent experiments. Error bars indicate ± SD. L, leaf tissue; R, root tissue. Stress treatments were performed as follows: Salt 200 mM for 16 hours; ABA, 100 µM for 16 hours; Cold, 2 hours at 4°C and 16 hours recovery; Heat 2h, 2 hours at 37°C; Heat, 2 hours at 37°C and 16 hours recovery; Phosphate starvation, 5 days by replacing KH_2PO_4 in hydroponics solution with K_2SO_4, and Wounding; by wounding rosette leaves with 100 needle holes in 1 cm^2 area.

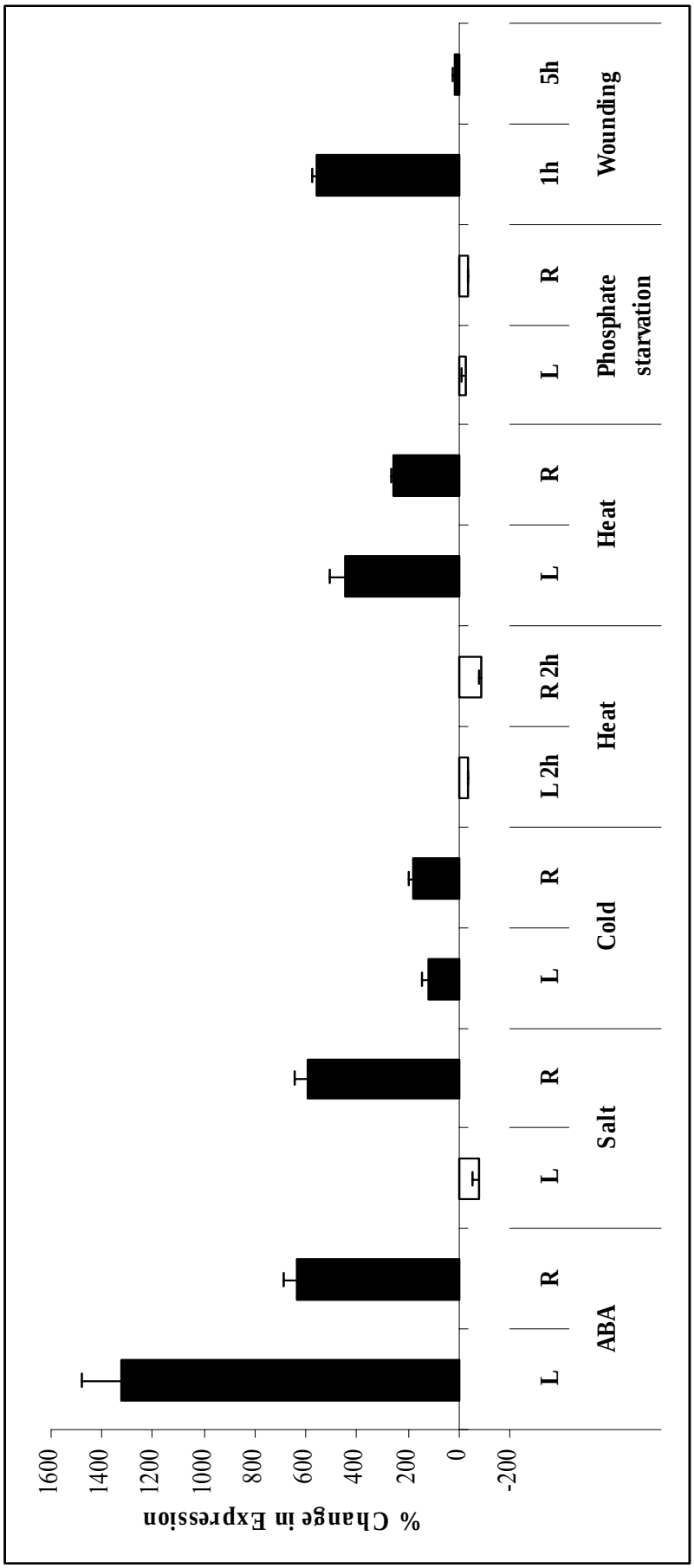

Figure 3.21. The change in expression of ASP3 upon different environmental conditions. The data normalized (Muller et al., 2002) using actin as the reference gene and averaged from two independent experiments. Error bars indicate ± SD. L, leaf tissue; R, root tissue. Stress treatments were performed as follows: Salt 200 mM for 16 hours; ABA, 100 µM for 16 hours; Cold, 2 hours at 4°C and 16 hours recovery; Heat 2h, 2 hours at 37°C; Heat, 2 hours at 37°C and 16 hours recovery; Phosphate starvation, 5 days by replacing KH_2PO_4 in hydroponics solution with K_2SO_4, and Wounding; by wounding rosette leaves with 100 needle holes in 1 cm^2 area.

The changes in the ASP4 expression in response to different environmental stresses can be seen in Figure 3.22. Accordingly, the ABA treatment induces the gene expression only in leaf tissue (by 353 %). The 200 mM salt treatment for 16 hours causes a down-regulation in gene expression with -86 % in leaf and -65 % in root tissues. The ASP4 expression is induced both in leaf and in root tissue with a considerably high up-regulation in the latter (74 % and 921 %, respectively). The heat treatment causes a repression of gene expression of ASP4 in leaf tissue after 2 hours (by -81 %) and induces that of root tissue by about 750 %. The expression changes significantly during recovery after 2 hours heat treatment and ends up with an induction in leaf tissue by approximately 420 %, whereas the root induction after 2 hours turns to normal level of gene expression during recovery. The phosphate deficiency causes a repression in expression of ASP4 both in leaf (by -58 %) and root tissues (by -94 %). Wounding transiently induces the transcript amount of ASP4, which is increased by about 120 % one hour of wounding and reduces to 43 % after 5 hours of wounding.

The response of gene expression of ASP5 to ABA and different stress treatment is given in Figure 3.23. The response of ASP5 expression to ABA is upregulation in leaf tissue (260 %) and only a 28 % increase in root tissue. The salt treatment for 16 hours increases the transcript amount in leaf tissue (by about 450 %), on the other hand slightly decreases that in root tissue (by -45 %). The cold does not cause a significant change in the transcript amount of plastidial ASP isoform. Similarly, the tissues collected 2 hours after heat treatment at 37°C shows only a slight change in expression (leaf increased by 28 %, root decreased by -45 %). Whereas, 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. Interestingly, the phosphate deficiency does not affect the expression in leaf tissue, but causes a significant decrease in root (by -92 %). 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.

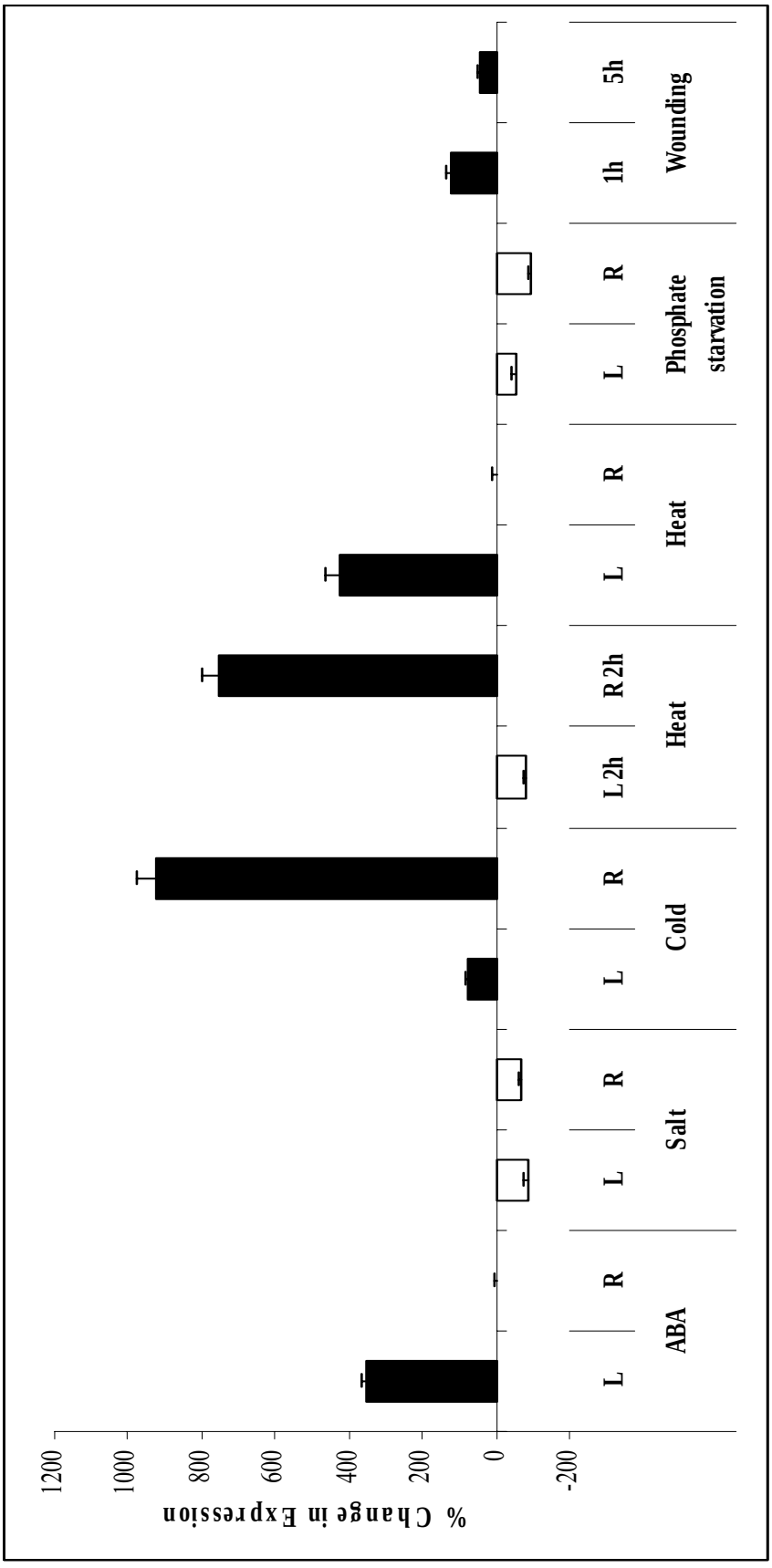

Figure 3.22. The response of ASP4 to changes in environmental conditions. The data normalized (Muller et al., 2002) using actin as the reference gene and averaged from two independent experiments. Error bars indicate ± SD. L, leaf tissue; R, root tissue. Stress treatments were performed as follows: Salt 200 mM for 16 hours; ABA, 100 µM for 16 hours; Cold, 2 hours at 4°C and 16 hours recovery; Heat 2h, 2 hours at 37°C; Heat, 2 hours at 37°C and 16 hours recovery; Phosphate starvation, 5 days by replacing KH_2PO_4 in hydroponics solution with K_2SO_4, and Wounding; by wounding rosette leaves with 100 needle holes in 1 cm^2 area.

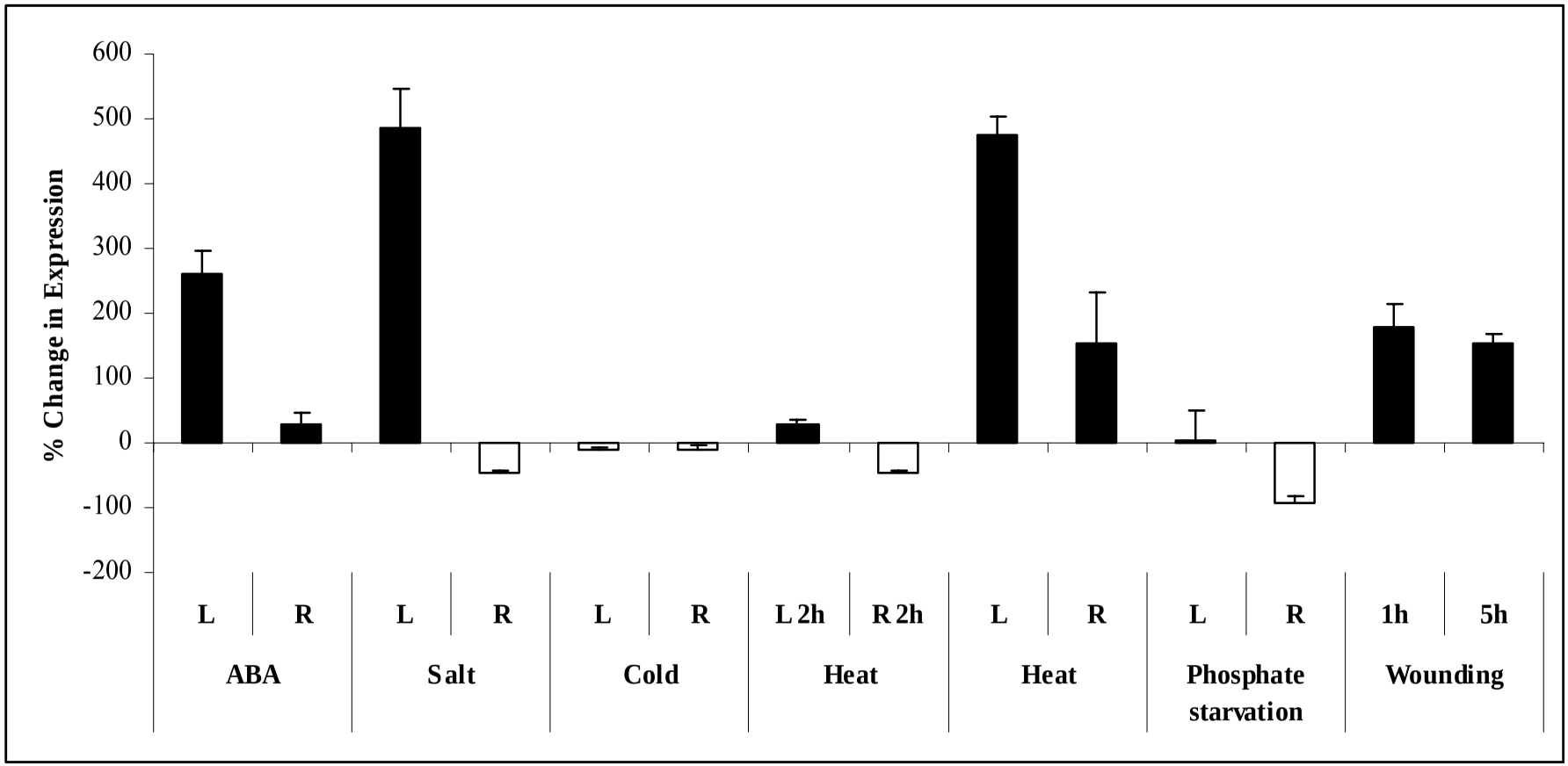

Figure 3.23. ABA and environmental stress response of gene expression of ASP5. The data normalized (Muller et al., 2002) using actin as the reference gene and averaged from two independent experiments. Error bars indicate ± SD. L, leaf tissue; R, root tissue. Stress treatments were performed as follows: Salt 200 mM for 16 hours; ABA, 100 µM for 16 hours; Cold, 2 hours at 4°C and 16 hours recovery; Heat 2h, 2 hours at 37°C; Heat, 2 hours at 37°C and 16 hours recovery; Phosphate starvation, 5 days by replacing KH_2PO_4 in hydroponics solution with K_2SO_4, and Wounding; by wounding rosette leaves with 100 needle holes in 1 cm^2 area.

The *A. thaliana* sPPase isoforms were shown to be differentially expressed in different tissues both with promoter:GUS fusion approach (Chapter 3.1.2.1) and quantitative real time PCR analyses (Chapter 3.1.2.2). The quantification of changes in the expression of ASP isoforms under different environmental conditions indicates that the isoforms not only have tissue-specificity of gene expression but also highly specific stress responses. For example, salt treatment causes a significant increase in the gene expressions of ASP2A and ASP5 in leaf tissues and only ASP4 in root tissue, whereas, ASP1, ASP3 and ASP4 expressions in leaf, and ASP4 and ASP5 expression in root tissues are significantly downregulated. The response to ABA treatment causes a significant upregulation in all *A. thaliana* sPPase isoforms in different tissues; ASP1 (leaf), ASP2A (root), ASP2B (root), ASP3 (leaf and root), ASP4 (leaf) and ASP5 (leaf). The same stress downregulates the gene expression of ASP2A and ASP2B in leaf tissue. The cold treatment of whole plants at 4°C for 2 hours followed by recovery for 16 hours affects ASP1 (leaf), ASP3 (root) and ASP4 (root) by upregulating the expression, whereas downregulates the expressions of ASP2A (leaf) and ASP2B (leaf). Heat treatment was performed as two steps, first by incubating the plants at 37°C for 2 hours and further 16 hours recovery where tissue samples were collected for both cases. The effect after 2 hours of treatment is upregulation of expression for ASP2A and ASP2B only in the root tissues. The expressions of ASP2A, ASP2B and ASP4 in leaf tissue, and ASP3 and ASP5 in root tissue are downregulated with the same treatment. The ASP1, ASP2A, ASP3 and ASP4 transcript amount significantly increases in leaf tissue during recovery from heat stress, and that of ASP5 is increased in both leaf and root tissues. The transcript amount of ASP2B in leaf tissue is the only isoform that shows a downregulation after recovery to heat treatment. Interestingly, phosphate deficiency does not cause increased expression in any of the *A. thaliana* sPPase isoforms. It effects the expression reversibly, by decreasing the transcript amounts of ASP1 (root), ASP2A (leaf), ASP2B (root) and ASP4 (root) significantly. Wounding mostly induces the expression of ASP isoforms, only ASP1 responds with a decrease in gene expression.

Although, the results are preliminary, since the different stress treatments are not comprehensive (only one selected concentration was used in treatment with ABA and salt stress) and the samples were collected only in one time point for most cases, the responses to ABA and to different environmental stresses are fairly isoform-specific (Table 2.5). Accordingly, the soluble pyrophosphatase isoforms of *A. thaliana* are seem to be sophistically regulated. Therefore, the role of plant sPPases can be important in the regulation of metabolism under stress conditions and/or each isoform might have specific function *in vivo*.

Table 2.5. Percent change in expression of ASP isoforms in response to ABA, salt and cold treatments.

	ABA		Salt		Cold	
	Leaf	Root	Leaf	Root	Leaf	Root
ASP1	177	14	-78	-40	90	0
ASP2A	-88	394	192	80	-78	-49
ASP2B	-87	274	83	17	-88	98
ASP3	1325	632	-78	589	116	175
ASP4	353	-1	-86	-65	74	921
ASP5	260	28	485	-45	-11	-10

3.2 Studies using *Beta vulgaris* Soluble and Vacuolar Pyrophosphatase Isoforms

It is known from the literature that soluble pyrophosphatases are highly phosphorylated in self-incompatibility response of *Papaver rhoeas* (Franklin-Tong and Franklin, 2003) which suggests the possibility of post-translational regulation of sPPase activity by phosphorylation in plants. In order to analyze the possible post-translational regulation of soluble pyrophosphatases localized in the cytoplasm, recombinant *Beta vulgaris* soluble pyrophosphatase isoform 1 was used.

Furthermore, as the overexpression of a bacterial origin soluble pyrophosphatase (*E. coli* sPPase) causes changes in plant carbohydrate metabolism (as discussed in Chapter 2.2.2.4), the effect of homologous overexpression of Bsp1 (*B. vulgaris* sPPase isoform 1) or Bvp1 (*B. vulgaris* vacuolar pyrophosphatase isoform 1) on sucrose loading of *Beta vulgaris* was investigated.

Finally, as the homologous overexpression of AVP1 results in salt- and drought-resistance plants (Gaxiola et al., 2001), and since is no study was available on the effect of overexpression of sPPase during environmental stress conditions, the changes in salt-stress resistance of *A. thaliana* plants with ectopic expression of Bsp1 or Bvp1 were analyzed comparatively. It is important to mention that although AVP1 overexpressing *Arabidopsis thaliana* lines were reported to show a resistance to salt and drought (Gaxiola et al., 2001), a later study indicated that the same lines have a phenotype similar to auxin-deficient plants, i.e., ectopic expression led to

changes like having more roots or larger rosette leaf area, and the loss-of-function of AVP1 caused a phenotype having no root formation (Li et al., 2005).

3.2.1 Post-translational Regulation of Soluble Pyrophosphatases Studied with Recombinant *Beta vulgaris* sPPase Isoform 1

The coding sequence of *Beta vulgaris* soluble pyrophosphatase isoform 1 was cloned in pQE30 vector (Qiagen) for overexpression in *E. coli* and further purification through Ni-NTA column (Schmitt et al., 1993) (Schirmer, 2004). The *E. coli* Origami strain was selected as the host cell, since it has loss-of-function mutations in the glutathione reductase and thioredoxin reductase genes, thus contains an oxidizing cytosol enabling the formation of possible disulfide bridges in recombinantly expressed protein.

The *E. coli* cells grown in LB medium until OD_{600} reaches 0,6 and induced with 1 mM IPTG to facilitate overexpression of recombinant Bsp1 protein. Afterwards, the recombinant protein was eluted using Ni-NTA column by washing the column with increasing concentration of imidazole buffer containing 5 mM $MgCl_2$ which is required for the stabilization of Bsp1 activity (Figure 3.24).

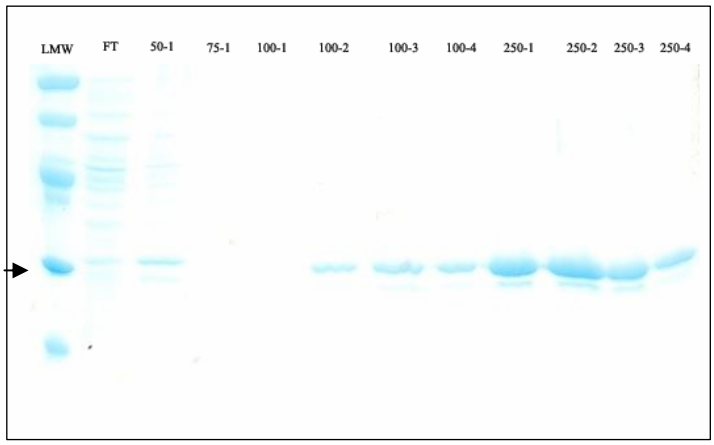

Figure 3.24. Elution profile of recombinant Bsp1 on 14 % SDS-polyacrylamide gel. LMW; Low molecular weight marker (Amersham), FT; flow through from column after incubation with bacterial lysate, 50, 75, 100 and 250; imidazole concentration of wash solution (containing 5 mM $MgCl_2$). The number after concentration of imidazole states the number of wash. The arrow indicates 30 kDa marker protein.

In the following analyses, the protein eluted from either the first or second wash with 250 mM imidazole, 5 mM $MgCl_2$ was used to ensure highest purity of recombinant Bsp1.

3.2.1.1 Redox Regulation of Recombinant *B. vulgaris* Soluble Pyrophospatase Isoform 1

The alignment of *A. thaliana* sPPases with known *B. vulgaris* soluble pyrophosphatases indicates two cysteine residues; the first one well conserved in all isoforms, whereas the other is well conserved except for the plastidial isoform (At5g09650) (Figure 3.25).

Although unlikely for a cytoplasmic protein, these two cysteine residues may form a disulfide bridge enabling the redox regulation of sPPase *in vivo*. The possible disulfide bridge formation of soluble pyrophosphatases and its effect on the activity of the enzyme was analyzed using recombinant Bsp1 protein.

As can be seen from Figure 3.26.A, the running size of recombinant Bsp1 protein in 14 % SDS-polyacrylamide gel was not changed under reducing or non-reducing conditions, indicating there is no disulfide bridge formation in native protein isolated from *E. coli* Origami host cells with an oxidizing cytosol, thus enabling the disulfide bridge formation in recombinant protein.

Figure 3.26.B further proves this result by showing that there is no effect of increasing concentrations of DTT (reducing agent) on enzyme activity (du Jardin et al., 1995) neither at room temperature nor at 55°C.

```
                        **  **.+*+**+*    *++*++++***    ****+***            +* **+*
AT1G01050   (    64) EL--DKKTGLIKVD----RILYSSVVYPH----NYGFVPRT-----------LC-EDND
AT2G18230   (    70) EL--DKNSGLIKVD----RVLYSSIVYPH----NYGFIPRT-----------IC-EDSD
AT2G46860   (    68) EL--DKKTGLIKVD----RILYSSVVYPH----NYGFIPRT-----------LC-EDND
AT3G53620   (    68) EL--DKTTGLIKVD----RILYSSVVYPH----NYGFIPRT-----------LC-EDSD
AT4G01480   (    68) EL--DKKTGLIKVD----RILYSSVVYPH----NYGFVPRT-----------LC-EDND
AT5G09650   (   120) EVatDEDFTPIKQDtkkgKLRY----YPYninwNYGLLPQTwedpshanseveGCfGDND
BSP1        (    74) EL--DKKTGLIMVD----RILYSSVVYPH----NYGFIPRT-----------LC-EDGD
BSP2        (    71) EL--DKTTGLIKVD----RVLYSSVVYPH----NYGFIPRT-----------LC-EDSD


                        *+***+++***+++*.***+*+**+******************+****++*++**++****
AT1G01050   (   101) PIDVLVIMQEPVLPGCFLRARAIGLMPMIDQGEKDDKIIAVCVDDPEYKHYTDIKELPPH
AT2G18230   (   107) PMDVLVLMQEPVLTGSFLRARAIGLMPMIDQGEKDDKIIAVCADDPEFRHYRDIKELPPH
AT2G46860   (   105) PLDVLVLMQEPVLPGCFLRARAIGLMPMIDQGEKDDKIIAVCADDPEYKHFTDIKQLAPH
AT3G53620   (   105) PIDVLVIMQEPVIPGCFLRAKAIGLMPMIDQGEKDDKIIAVCADDPEYRHYNDISELPPH
AT4G01480   (   105) PIDVLVIMQEPVLPGCFLRARAIGLMPMIDQGEKDDKIIAVCVDDPEYKHITNINELPPH
AT5G09650   (   176) PVDVVEIGETQRKIGDILKIKPLAALAMIDEGELDWKIVAISLDDPKAHLVNDVEDVEKH
BSP1        (   111) PMDVLVLMQEPVVPGRFLRARAIGLMPMIDQGEKDDKIIAVCADDPEVRHYTDINQLPPH
BSP2        (   108) PMDLLVLMQEPILPGSFLRARAIGLMPMIDQGEKDDKIIAVCADDPEFRHYRELKELPPH
```

Figure 3.25. The partial CLUSTALW alignment (Higgins et al., 1994) of *A. thaliana* sPPases with *B. vulgaris* sPPase isoforms Bsp1 and Bsp2. The conserved cysteine residues are highlighted with red. (*); conserved residues, (+); highly conserved residues.

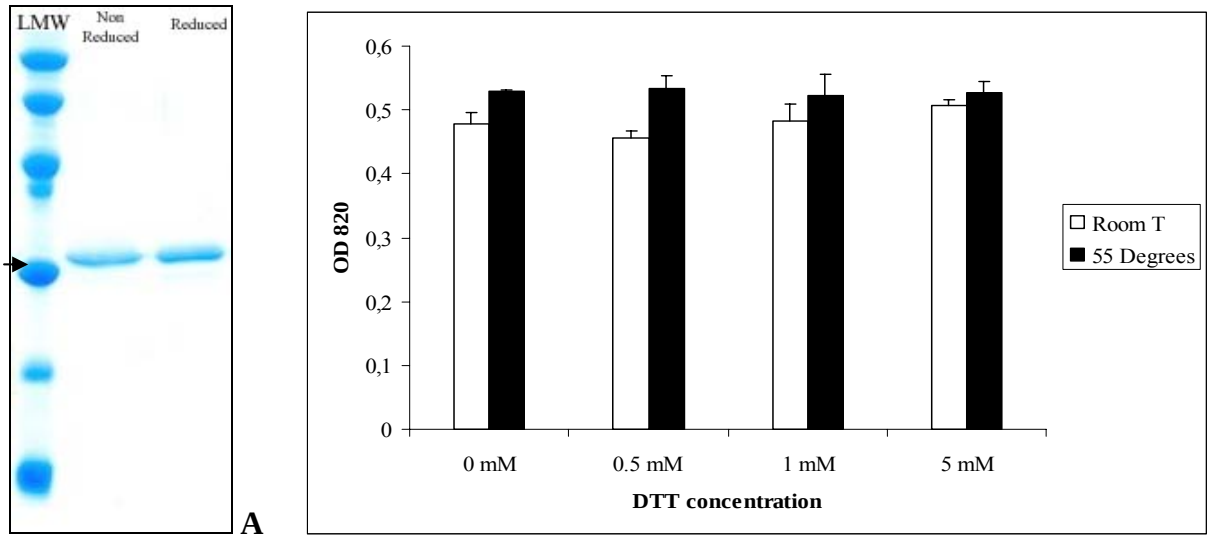

Figure 3.26. Analysis of disulfide bridge formation in recombinant Bsp1 protein. A) 14 % SDS-polyacrylamide gel showing the running size of recombinant Bsp1 protein under reducing and non-reducing conditions. LMW; low molecular weight marker (Amersham). The arrow indicates the location of 30 kDa marker protein. B) The changes in the activity of recombinant Bsp1 protein measured as optical density (OD) at 820 nm with changing concentrations of DTT both at room temperature (Room T) and 55°C. The data is calculated as the average of three independent measurements and error bars are added as ± SD between these measurements.

Although the effect of oxidation to the running size and enzymatic activity of recombinant Bsp1 protein was not studied, the results obtained give hints that although there are two relatively well conserved cysteine residues in soluble pyrophosphatases of *A. thaliana* and *B. vulgaris*, there is no disulfide bridge formation in recombinant Bsp1. This is not enough to conclude if the redox regulation is a post-translational regulation mechanism *in planta* or not, however, it can be said that it is not a likely candidate for post-translational control of sPPase activity.

3.2.1.2 Phosphorylation of Recombinant Bsp1 with Protein Kinase C

The 2D-gel electrophoresis studies of self-incompatibility response of *Papaver rhoeas* revealed a highly phosphorylated 26 kDa protein which was stated as having a high homology to known plant soluble pyrophosphatases (Franklin-Tong and Franklin, 2003).

The *in silico* analysis of *B. vulgaris* sPPase isoform 1 (Bsp1) for the detection of possible phoshorylation sites using the web-based programs Softberry PSITE (for eukaryotic proteins, not plant specific) (http://www.softberry.com) and PROSITE (Hulo et al., 2006) indicates two possible protein kinase C (PKC) phosphorylation sites ([ST]-X-[RK]). The *in vitro* phosphorylation of

recombinant Bsp1 was performed by PKC (Promega) according to manufacturer's instructions and detected by Western blotting (Figure 3.27).

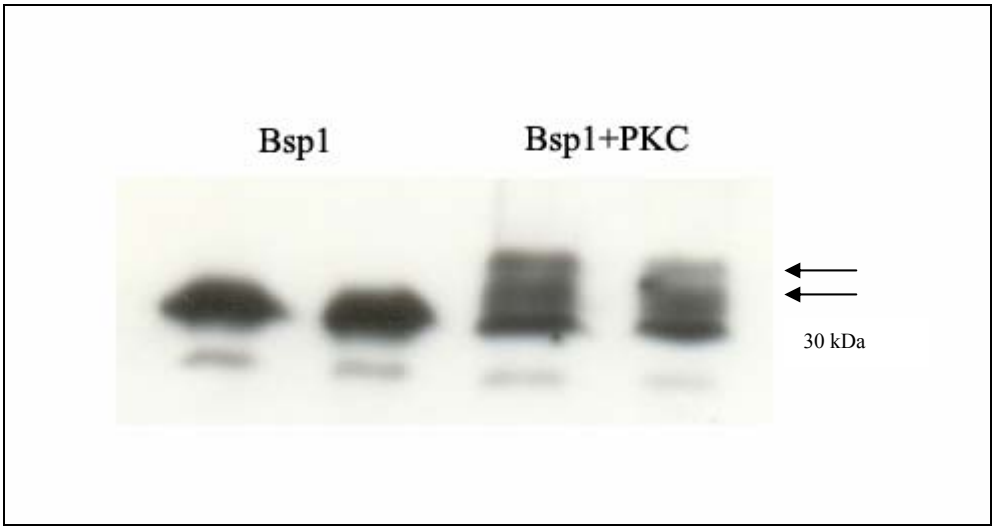

Figure 3.27. The *in vitro* phosphorylation of recombinant Bsp1 by PKC. The phosphorylation was performed according to manufacturer's instructions by using 100 ng of recombinant Bsp1 and 100 ng of PKC (Promega). The location of 30 kDa protein of LMW protein standard (Amersham) on membrane was indicated. The arrows indicate the formation of two further bands in recombinant Bsp1 protein after phosphorylation with PKC.

The Figure 3.27 shows that recombinant Bsp1 signal detected by Western blotting has two further bands after phosphorylation by PKC due to the shift caused by increase in charge after the addition of a single and double phosphate, respectively. No complete phosphorylation of single or double PKC sites of recombinant Bsp1 was achieved through several attempts. The measurements performed before and after phosphorylation of recombinant Bsp1 indicates that the enzymatic activity is not affected by phosphorylation.

Although the recombinant Bsp1 is phosphorylated by PKC *in vitro*, PKC is probably not the enzyme responsible for *in vivo* phosphorylation of soluble pyrophosphatases. The phosphorylation of recombinant Bsp1 has no change in enzyme activity; however, it is possible that the phosphorylation of soluble pyrophosphatases has an effect on protein-protein interactions. The phosphorylation of soluble pyrophosphatases could be a post-translational regulation mechanism for the control of enzyme activity *in planta*.

3.2.2 Effect of Homologous Overexpression of Bsp1 or Bvp1 on Wound Response and Sucrose Loading in *Beta vulgaris*

The transformation of *B. vulgaris* with open reading frames of Bsp1 or Bvp1 in pBinAR (Hofgen and Willmitzer, 1990) constructs was performed by KWS and the selection of transgenic lines overexpressing Bsp1 or Bvp1 in *B. vulgaris* was performed by Markus Schirmer during his PhD study. Based on Northern and Western blotting results of *in vitro* grown seedlings, Bvp1 2-1, 2-5 and 3-2 and Bsp1 4-1, 5-1, 5-4, 5-5 and 5-6 were selected as overexpressing lines. All lines were created by transformation of wild type 6B2840.

In the following studies either *in vitro* grown transgenic *B. vulgaris* plants or seed-grown transgenic sugar beet plants (T1 generation) were used.

3.2.2.1 Post-transcriptional Regulation of Bsp1 and Bvp1 Observed upon Wounding

The harvest of sugar beet taproots requires the decapitation that causes a wound. The wound healing process of plants increases the metabolic activity, which is fed by the sucrose accumulated in the taproot tissue. Therefore, the post harvest sucrose metabolism causes a 100 to 250 g sucrose loss per ton of taproot per day during storage (Leigh et al., 1979).

Accordingly, the transgenic *B. vulgaris* lines overexpressing either Bsp1 or Bvp1 protein were analyzed after wounding. The *in vitro* wounding was performed by slicing the taproot tissue and removing cylinders (R = 0,05 cm) out of the cortex region which is further wounded by cutting into 2 mm thick slices. Afterwards, the wounded tissues were incubated at humidity chambers for different periods.

It is worthy to mention that the expression of Bsp1 and Bvp1 in pBinAR construct is driven by CaMV 35S promoter (Odell et al., 1985) which is known to be induced by wounding. Figure 3.32 shows the wounding response of CaMV 35S promoter driven Nt-VIF (*Nicotiana tobacum* vacuolar invertase construct in pBinAR) transcript in *B. vulgaris* by Northern blotting. The transcript of Nt-VIF is detectable at the unwounded tissue (0 day) and upon wounding it accumulates suggesting the induction of CaMV 35S promoter activity (Figure 3.28).

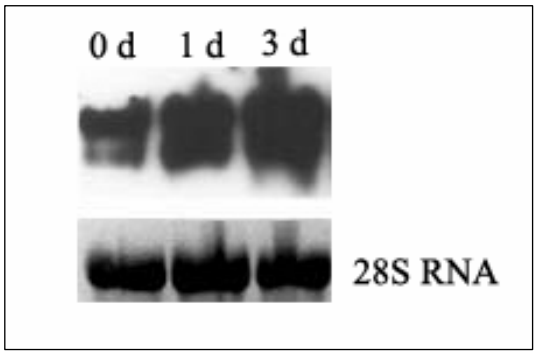

Figure 3.28. The change in the transcript amount of CaMV 35S driven expression of Nt-VIF in sugar beet taproot tissue upon wounding. The unwounded transcription is detectable in '0 day' and it accumulates upon wounding in a manner increasing with time (1 day and 3 days). Twenty micrograms of total RNA was loaded to gel and analyzed by using biotin-dUTP labeled specific Nt-VIF probe. The ethidium-bromide stained 28S RNA band in the agarose gel is given as loading control.

The following figure shows the change in transcript amounts of CaMV 35S promoter driven expression of Bsp1 and Bvp1 in *B. vulgaris* taproot tissue compared to that of wild type upon wounding where Bvp1 overexpressing line 3-2 and Bsp1 overexpressing line 5-6 were used as the representatives of whole groups (Figure 3.29).

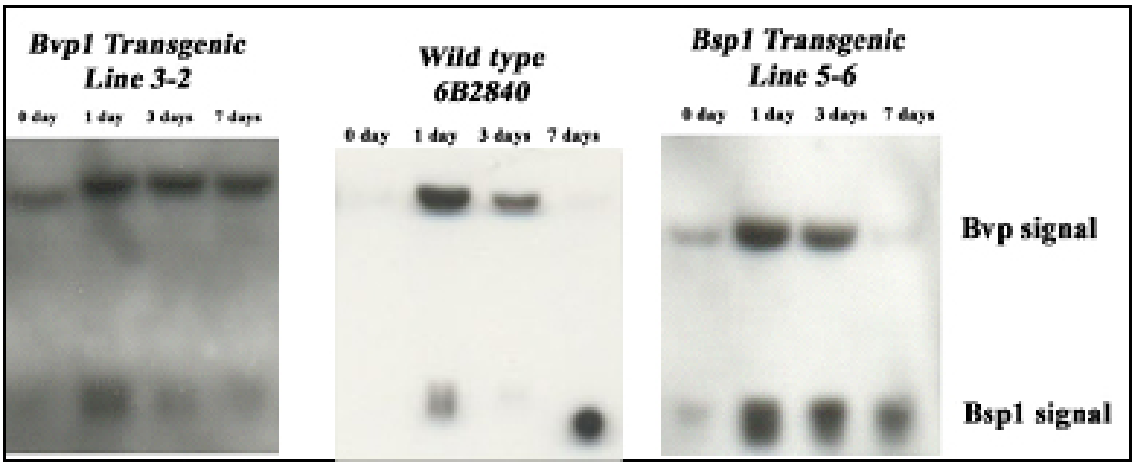

Figure 3.29. Northern blot analysis of changes in transcript amounts of Bvp1 and Bsp1 in wild type, Bvp1 overexpressing line 3-2 and Bsp1 overexpressing line 5-6 observed without wounding (0 day) and 1 day, 3 days and 7 days after wounding. Twenty micrograms of total RNA were loaded to the gel and hybridized with biotin-dUTP labeled Bvp1 3'-UTR and Bsp1 3'-UTR probes.

It is important to mention that several attempts to select transgenic fully-grown sugar beet plants through Northern and Western blotting were unsuccessful using either leaf or unwounded taproot tissues which is in contrast to signals detected in seedling stage (see above). The comparison of '0 day' Bvp1 signals of wild type 6B2840 and Bvp1 overexpressing line 3-2 indicates that the transcript amount in both taproot tissues are relatively similar. The comparison of Bsp1 overexpressing line 5-6 with wild type 6B2840 shows the transcript amount of Bsp1 is the same

in both cases. Therefore, it is not possible to detect the CaMV 35S promoter driven expression of Bvp1 or Bsp1 in unwounded taproot tissues.

There is almost no detectable expression of both endogenous Bvp1 and Bsp1 in unwounded sugar beet taproot tissue. Both Bsp1 and Bvp1 expression induce upon wounding (Figure 3.29, wild type 6B2840). The induction of endogenous Bsp1 is transient; the accumulation is detectable 24 hours after wounding and completely lost 3 days after. On the other hand, the induction of endogenous Bvp1 transcript is relatively stable; the transcription is induced 24 hours after wounding and it returns to unwounded state 7 days thereafter.

The selection of transgenic *B. vulgaris* lines overexpressing Bvp1 or Bsp1 was only possible after wounding (Figure 3.29). The comparison of Bvp1 signal in Bvp1 overexpressing line 3-2 with that of wild type signal indicates the differences occurring due to CaMV 35S driven expression of Bvp1. Both endogenous and CaMV 35S driven expression of Bvp1 are induced upon wounding. The induction in the endogenous Bvp1 signal returns to unwounded state 7 days after wounding as can be seen from Northern blotting result of wild type 6B2840. On the other hand, although the induction in endogenous protein upon wounding is lost after 7 days, the induction due CaMV 35S promoter driven expression is still persistent and enables a clear difference between transgenic and endogenous expressions of Bvp1 only after 7 days upon wounding. It is clear that there is no effect on transcription of Bsp1 in Bvp1 overexpressing lines.

The selection of Bsp1 transgenic lines is also based on the differences in transcript amounts of both endogenous and CaMV 35S promoter driven expression of Bsp1 protein observed upon wounding (Figure 3.29). The endogenous Bsp1 transcription is transiently induced 24 hours upon wounding and completely lost 3 days afterwards. However, in the case of Bsp1 overexpressing line 5-6, the induction occurs both in endogenous and CaMV 35S driven expression upon wounding, which causes a relative stability of the transcript amount which is still detectible 7 days after wounding. The induction behavior of endogenous Bvp1 transcript is the same both in wild type (6B2840) and Bsp1 overexpressing line 5-6 upon wounding.

The comparison of Figure 2.28 with 2.29 (0 day signals) indicates that the CaMV35S driven expressions of Bsp1 and Bvp1 is not detectable in unwounded sugar beet taproots. This market difference can be result of post-transcriptional regulation of Bsp1 and Bvp1 mRNA amount or stability in transgenic sugar beet lines. The wounding induces both endogenous promoter and CaMV 35S promoter activity, thereby disturbs the balance between transcription and regulation which enables the selection of transgenic lines overexpressing Bvp1 or Bsp1 by comparing the transcript behavior observed by Northern blotting as explained above. It is important to mention that although there is an accumulation of transcript amount upon wounding, neither

Bvp1 nor Bsp1 protein is affected by this accumulation as observed by Western blotting using primary antibodies detecting all vacuolar and soluble pyrophosphatases present in the tissue (Figure 3.30 and 31).

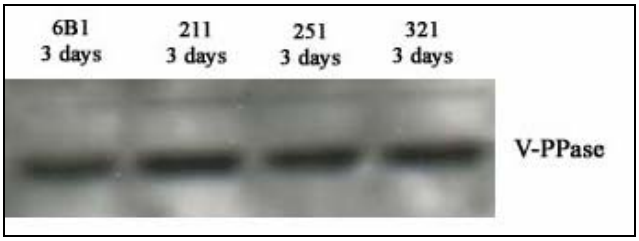

Figure 3.30. Protein amount of vacuolar pyrophosphatases in *B. vulgaris* taproot tissue 3 days after wounding detected with antibodies raised against mung bean pyrophosphatase (Maeshima and Yoshida, 1989). 20 µl out of 300 µl of total protein isolated from fresh weight equivalent of 100 mg taproot tissue was loaded to each well. The primary antibody used in detection cross-reacts with all vacuolar pyrophosphatases (Maeshima and Yoshida, 1989). The size of v-PPase signal is about 70 kDa. 6B1 is the wild type where the others are Bvp1 transgenic lines.

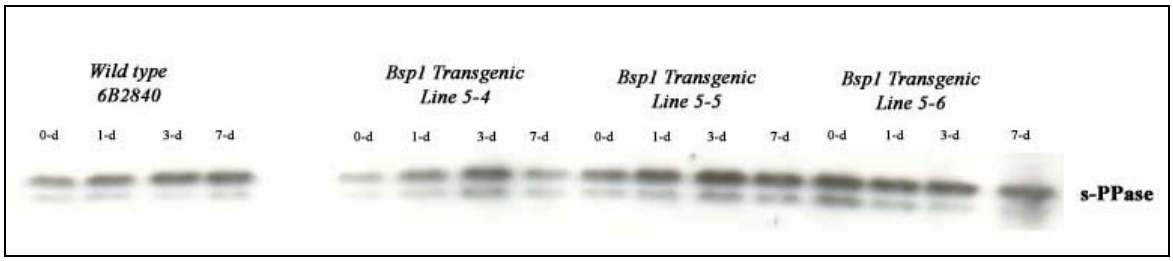

Figure 3.31. The change in soluble pyrophosphatase amount of *B. vulgaris* transgenic and wild type taproot tissues upon wounding. 5 µl out 0f 300 µl of total protein isolated from fresh weight equivalent of 100 mg taproot tissue was loaded to each well. The primary antibody used in detection cross-reacts with all soluble pyrophosphatases except plastidial isoforms. The s-PPase signal is calculated as about 25 kDa.

3.2.2.2 Changes in Sucrose Loading and Hexoses in *B. vulgaris* Taproot Tissue upon Overexpression of Homologous Bvp1 or Bsp1

The overexpression of *E. coli* soluble pyrophosphatase in tobacco, potato and *A. thaliana* plants proved the changes in carbohydrate metabolism of these plants with differences in hexoses and sucrose/starch ratio (Chapter 2.2.2.4). Therefore, it might be possible to achieve a higher sucrose load in sugar beet taproot during growth upon overexpression of endogenous Bsp1 or Bvp1 proteins. The measurements of sucrose concentration in taproot tissues of transgenic *B.*

vulgaris lines with respect to the wild type was performed using 5 months-old seed grown T1 generation plants (Figure 3.32).

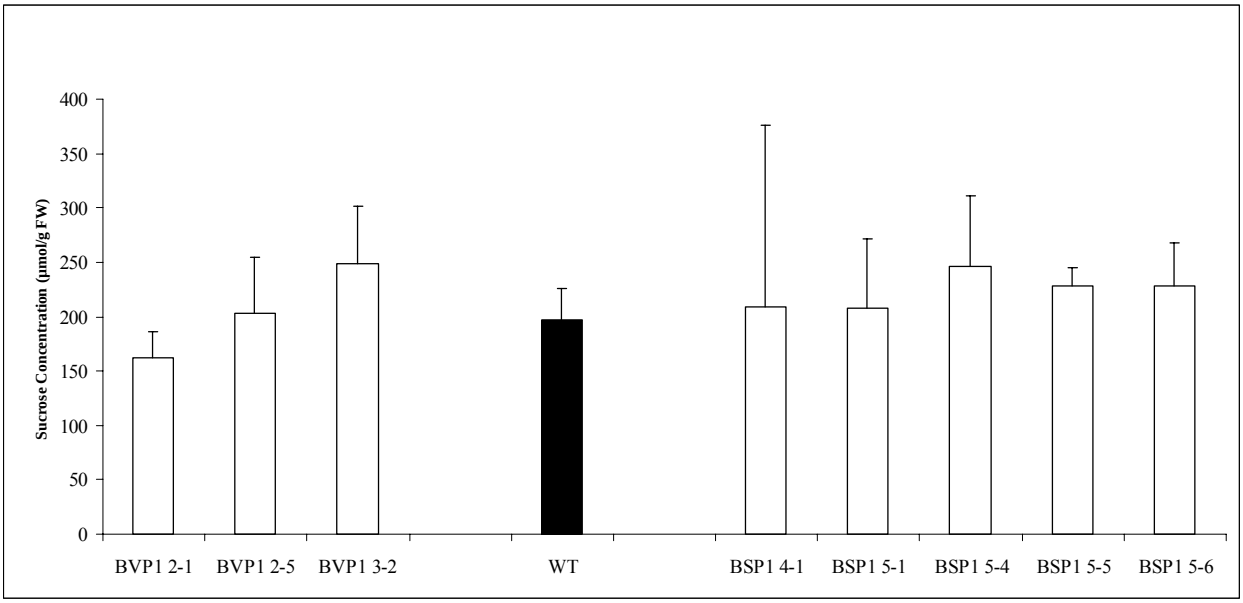

Figure 3.32. Sucrose concentration as µmol/g fresh weight in transgenic lines overexpressing either Bvp1 or Bsp1 compared to their wild type 6B2840. The values are calculated as the average of three independent measurements and error bars indicate ± SD.

Although no clear difference in protein amounts of Bsp1 or Bvp1 in transgenic lines was observed when compared to their wild type in fully developed taproot tissues of *in vitro* grown sugar beets, it seems that the overexpression has an effect on sucrose accumulation. The sucrose concentration in Bvp1 line 3-2 and Bsp1 line 5-4 is approximately 25 % increased by overexpression. The increase in Bsp1 lines 5-5 and 5-6 is about 15 % and no considerable effect on sucrose accumulation was detected in other lines. Although the changes in sucrose amounts of transgenic lines compared to wild type is not statistically significant, the tendency of increase was observable not only Bvp1 but also Bsp1 overexpressing taproot tissues of transgenic *B. vulgaris* lines.

During wound healing after harvesting, sugar beet converts the sucrose stored in taproot tissue into hexoses to be used in metabolic processes like cell wall repair and respiration. This causes an increase in both cytoplasmic glucose and fructose concentrations upon wounding. Therefore, the transgenic *B. vulgaris* lines with CaMV 35S promoter driven expression of endogenous Bsp1 and Bvp1 were analyzed with respect to changes in hexose concentrations upon wounding (Figure 3.33).

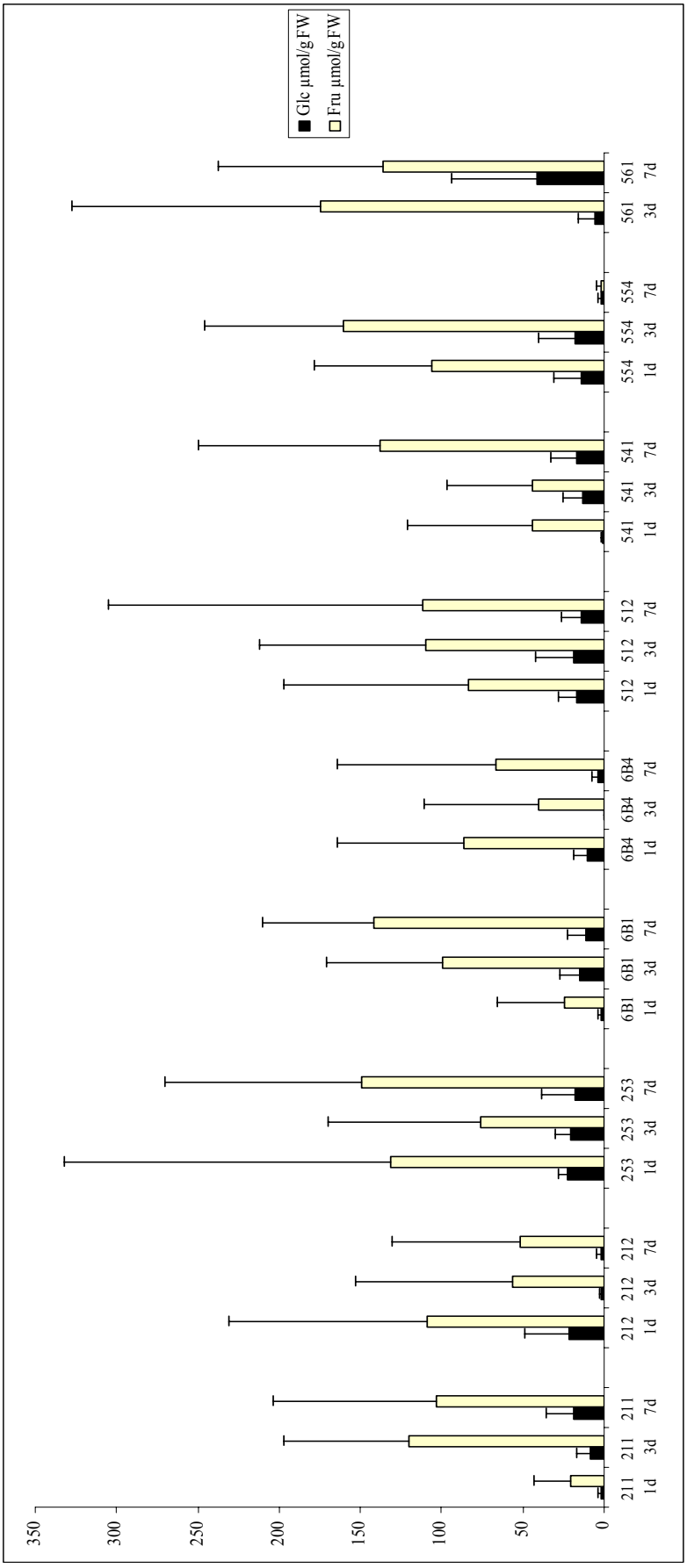

Figure 3.33. Changes in glucose and fructose concentration in sugar beet lines overexpressing either Bsp1 or Bvp1 compared to their wild type. 211 and 212 are two individuals of Bvp1 line 2-1. The 6B4 is a wild type individual. 512 is a representative of Bsp1 line 5-1, whereas 541 is of line 5-4, 554 is of line 5-5 and 561 is of line 5-6. The samples are collected 1 day (1d), 3 days (3d) and 7 days (7d) and isolated carbohydrates were analyzed for hexose content. The values are calculated as the average of three independent measurements and error bars indicate ± SD.

The hexose concentration in unwounded taproot tissue is very low, therefore not measurable with the method that was used for the analysis. However, upon wounding both glucose and fructose concentration increases. Fructose concentration is considerably higher than glucose (Figure 3.33) due to activity of vacuolar invertases induced by wounding which degrades sucrose into fructose and glucose.

Because of the sensitivity of the method used for the extraction and calculation of hexoses in samples, the standard deviation between measurements is considerably high as can be seen from Figure 3.33. This prevents to detect a clear difference between glucose and fructose concentrations of transgenic lines overexpressing Bvp1 or Bsp1 and wild type and to drive any conclusions out of data available. However, it appears that although the overexpression of endogenous Bsp1 or Bvp1 has an affect on sucrose accumulation in the taproot tissue, it does not cause a significant change in the degradation of accumulated sucrose upon wounding.

3.2.3 Effect of Ectopic Expression of Bsp1 and Bvp1 on Salt Resistance of *Arabidopsis thaliana*

The *Arabidopsis thaliana* was stably transformed with pBinAR vector carrying open reading frames of *Beta vulgaris* soluble pyrophosphatase isoform 1 (Bsp1) or *Beta vulgaris* vacuolar pyrophosphatase isoform 1 (Bvp1) and positive lines were selected either by Northern blot (for Bvp1) or by Western blot (Bsp1) analyses (Schirmer, 2004).

The analysis of both Bsp1 and Bvp1 overexpressing *A. thaliana* lines on kanamycin selective ½ MS plates indicated that both lines were defective in root growth when compared to control plant (Figure 3.34).

The inhibition of root growth was observed in all Bvp1 overexpressing lines used for the analysis (V5, V7 and V9, Figure 3.34.A). However, the defectiveness in root growth was not consistent in Bsp1 overexpressing *A. thaliana* lines. Two lines out of six (S11 and S16) had no root formation although they were effectively germinated. The Bsp1 overexpressing lines S3, S6, S7 and S9, on the other hand, were comparatively less rooted than control plant (Figure 3.34.B and C).

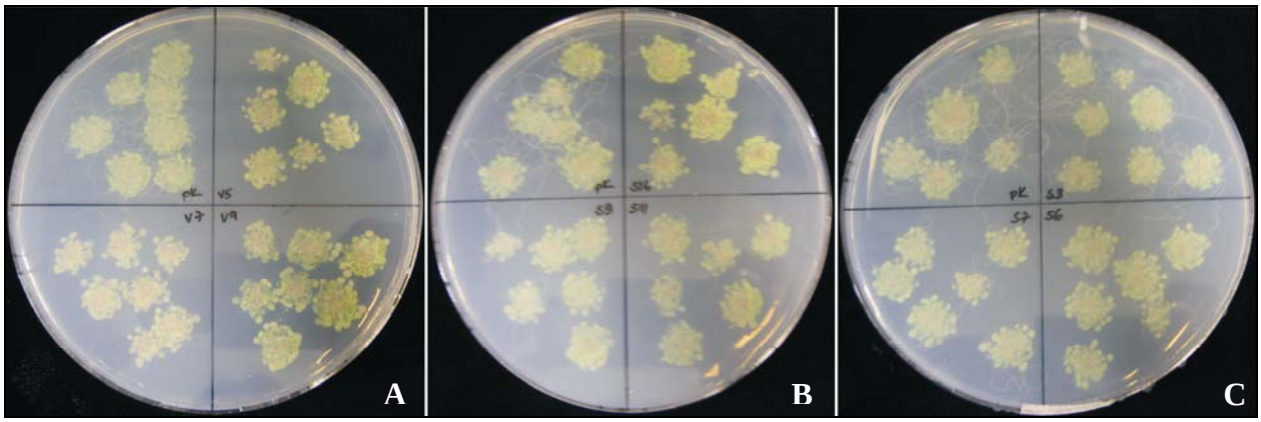

Figure 3.34. Comparison of root formation of *A. thaliana* lines overexpressing either Bvp1 (A) or Bsp1 (B-C) grown for 14 days in kanamycin selective ½ MS plates. As a control plant (pK), a kanamycin resistance line with promotor:GUS fusion was used (top left in each plate). Bvp1 overexpressing (A) V5, V9 and V7 and Bsp1 overexpressing (B) S16, S11 and S9 and (C) S3, S6 and S7 are independent lines obtained from stable transformation of *A. thaliana* with pBinAR construct (written in clockwise starting from top right location).. The seeds used were at least T3 generation of mother plants.

The salt stress resistance of *A. thaliana* seedlings were tested by growing in kanamycin selective ½ MS plates supplemented with either 125 mM or 150 mM NaCl (Figure 3.35). Out of three Bvp1 overexpressing lines, only line V9 survived 150 mM salt treatment (Figure 3.35.A). However, the phenotype was different in Bsp1 overexpressing lines and indicated a clear dependence of salt stress tolerance on root formation. The lines S3, S6, S7 (Figure 3.35.B) and S9 (Figure 3.35.C) that had roots, although comparatively less than control plant, germinated in 150 mM NaCl containing plates. On the other hand, Bsp1 lines S11 and S16 (Figure 3.35.C) having no root formation as in the case of Bvp1 overexpressing plants, had no resistance to salt treatment, and not germinated even in 125 mM NaCl supplemented ½ MS plates.

The results, although preliminary, suggest the involvement of similar mechanisms in phenotypic differences of Bvp1 or Bsp1 overexpression with respect to root formation and salt stress tolerance.

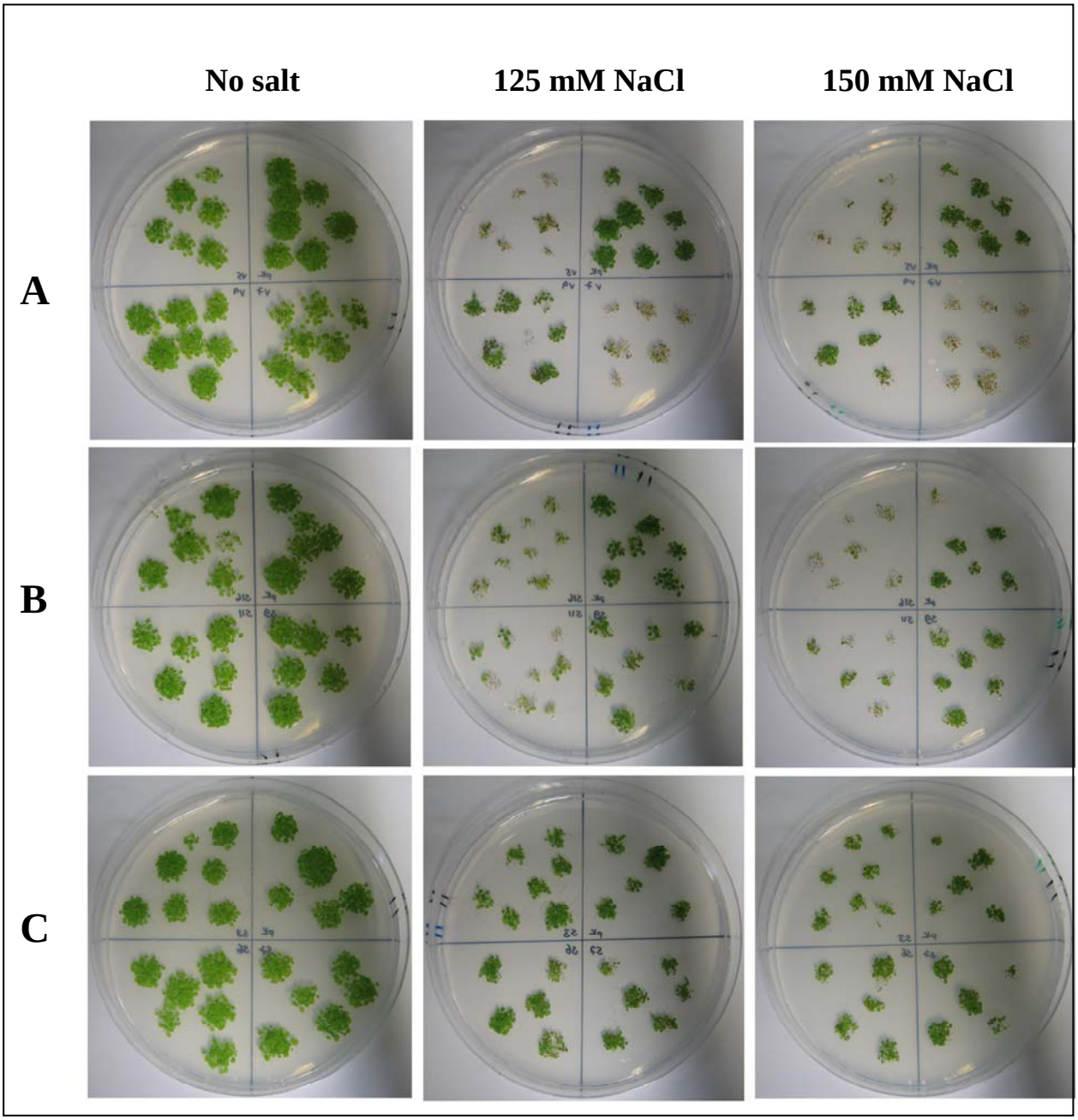

Figure 3.35. Germination of Bvp1 or Bsp1 overexpressing *A. thaliana* seedlings on kanamycin selective ½ MS plates containing different concentrations of sodium chloride. The pictures were taken 14 days after inoculation. A) Bvp1 overexpressing lines V5, V9 and V7; B) Bsp1 overexpressing lines S16, S11, S9 and C) Bsp1 overexpressing lines S3, S6 and S7 (written in counterclockwise starting from top left location). As a control plant (pK), a kanamycin resistance line with promotor:GUS fusion was used (top right in each plate).

4 DISCUSSION

Inorganic pyrophosphate is the by-product of many anabolic reactions, like DNA and RNA synthesis, amino acid acylation, activation of fatty acids and synthesis of carbohydrates (Taiz, 1986; Stitt, 1998). The reversibility of these reactions were demonstrated *in vitro* and therefore, there is a widely accepted hypothesis that they are driven forward by the hydrolysis of pyrophosphate by inorganic pyrophosphatases *in planta* (Taiz, 1986).

Weiner et al. (1987) using fully developed spinach leaves as a model system, showed that plant cytosol contains a significant amount of pyrophosphate (PP_i) (200-300 μM) with a little soluble pyrophosphatase (sPPase) activity, whereas, isolated plastids contain very low PP_i with a high sPPase activity. The importance of high cytoplasmic PP_i pool was proven by the observation that ectopic expression of a bacterial pyrophosphatase in the plant cytosol causes significant alterations in metabolism, growth and development (Jelitto et al., 1992; Sonnewald, 1992; Lee et al., 2006). All of these findings and the occurrence of a H^+-PPase in plant vacuolar membrane (Maeshima, 2000) question the presence and importance of cytoplasmic soluble pyrophosphatases and tonoplast bound proton translocating pyrophosphatase is usually accepted as the sole enzyme responsible for the removal of excess cytoplasmic PP_i (Stitt, 1998).

Currently, little is known about the nature and regulation of plant soluble pyrophosphatases. Therefore, by using *A. thaliana* and *Beta vulgaris* as the model plants, this study aimed to understand localization, regulation, stress responses and importance of plant soluble pyrophosphatases through a functional genomics approach.

4.1 *A. thaliana* sPPases Share a High Homology, Except for the Plastidial Isoform

The sequencing project (The Arabidopsis genome initiative, 2000) revealed the presence of six soluble pyrophosphatases in *A. thaliana* genome (Table 3.1). One of the enzymes contains a signal peptide and was proven to localize in intact plastids by *in vitro* import experiments (Schulze et al., 2004).

The lengths of the five ASP isoforms (ASP1 to ASP4, except ASP5) are very close to each other and usually in the range of approximately 210 amino acids (Table 3.1). The plastidial isoform (ASP5), on the other hand, is slightly larger with a length of 244 amino acids (without signal peptide; Table 3.1). The alignment of known plant sPPases with animal and fungal sPPases showed the presence of two stretches of amino acid sequences that are present in the later but missing in the former (Sivula et al., 1999). Interestingly, plant plastidial sPPases do not contain these deletions (Sivula et al., 1999) and, consequently, are found to be slightly larger. Therefore, they are said to be more 'eukaryotic-like' compared to other isoforms, which are more similar to 'prokaryotic' sPPases (Perez-Castineira et al., 2001). The predicted molecular weights of ASP isoforms (Table 3.1) are approximately the same (about 24 kDa each), except the plastidial isoform, which is a little larger due to additional stretches (26,4 kDa).

The *A. thaliana* sPPase isoforms share a high homology both in nucleic (Figure 3.2) and in amino acid (Figure 3.1) sequences. The coding sequences mainly diverge in 5'- and 3'-UTRs (Chapter 7.2). The multiple alignment of ASP amino acid sequences according to Nikolaev et al. (1997) clearly shows the extra residues in the plastidial isoform (Figure 3.1). Table 3.2 demonstrates the percent homology between *A. thaliana* sPPase isoforms based on the same alignment strategy (Nikolaev et al., 1997). Accordingly, ASP5 shares the least homology with other sPPase isoforms (about 35 %; Table 3.2). Within five paralogous *A. thaliana* sPPases, the homology is significantly high; above 75 % which goes up to 91 % (Table 2.2). Schulze et al. (2004) performed alignment using a different algorithm (MacVector 7.0 program) and reported that plastidial isoform was only 22 % identical to other sPPases isoforms, which shared a homology of at least 69 %.

4.2 *A. thaliana* sPPases Localize in the Cytoplasm, Nucleus and Plastids, but not in Mitochondria

The presence of a tonoplast bound proton translocating pyrophosphatase in the plant kingdom (Maeshima, 1991), and the data showing that plant cytosol has low soluble pyrophosphatase activity (Weiner et al., 1987) led to a hypothesis that plant cell cytosol lacks a soluble pyrophosphatase (du Jardin et al., 1995; Stitt, 1998). There is only one study that claims the presence of a cytosolic pyrophosphatase in plants based on cloning of a sPPase that does not contain any known leader peptide sequence (du Jardin et al., 1995).

The *in silico* predictions for the localization of *A. thaliana* sPPases using several web-based programs (Table 3.3) indicated that ASP1 and ASP5 are localized in mitochondria and chloroplasts, respectively, whereas all other isoforms localize in the cytoplasm. Perez-Castineria et al. (2001b) refers ASP1 to have 'a mitochondrial polypeptide precusor'. It is noteworthy that this 'mitochondrial polypeptide precursor' was detected only by TargetP (Emanuelsson et al., 2000) which is a localization prediction program for eukaryotic organisms and not plant specific. Plant specific programs like PSORT and LOCTree, on the other hand, predict ASP1 to be localized in the cytoplasm (Table 3.3). A further analysis of ASP1 amino acid sequence with MitoProt (Claros and Vincens, 1996) which is a web-based localization prediction program for eukaryotic mitochondrial proteins also fails to detect any mitochondrial precursor protein.

The *in vivo* subcellular localization studies of *A. thaliana* sPPase isoforms using both N-terminal and C-terminal GFP fusions were performed according to Wroblewski et al. (2005). The CLSM analysis of transient overexpression in tobacco epidermal cells using N-terminal GFP fusion constructs revealed aggregate formation in five ASP isoforms (Figure 7.1), except for ASP5 which did not give any fluorescence as expected due to the cleavage of leader peptide and further degradation of GFP. The three dimensional structure of plant soluble pyrophosphatases has not been determined yet, but Sivula et al. (1999) used homologous modeling of the tertiary structure of ASP2B with *E. coli* sPPase. The predicted structure (Figure 7.2) has a N-terminal region close to the enzyme globe (Sivula et al., 1999) and therefore, it is likely that the addition of GFP to the N-terminus may perturb the 3D structure of the enzyme and in this manner can cause aggregation. Both prokaryotic and eukaryotic sPPases were shown to be functional as oligomers (Sivula et al., 1999), therefore, another reason of aggregate formation can be the inhibition of oligomer formation. That is, the addition of GFP protein to the N-terminus may intervene with subunit interfaces, therefore by inhibiting oligomer formation can induce aggregation. Koroleva et al. (2005) in a high-throughput protein localization study of *A. thaliana* proteins by transient transformation of cell suspension cultures showed the same type of aggregate formation in N-terminal GFP fusion construct (in pGWB6 vector) of ASP1 protein.

Figure 3.3 shows the subcellular localization of *A. thaliana* sPPase-GFP chimeras (C-terminal fusion constructs) in transient transformation to tobacco epidermal cells. Accordingly, ASP1, ASP2A, ASP2B, ASP3 and ASP4 are localized in the cytoplasm and/or nucleus, and ASP5 is localized exclusively in the plastids. This is the first proof for *in vivo* localization of ASP5 in plastids, since the localization was mainly predicted based on the signal peptide sequence, and Schulze et al. (2004) used an *in vitro* import approach to show the subcellular localization.

The nuclear localization of ASP isoforms is not conclusive as the nuclear pore complex has an exclusion limit of 60 kDa (Haasen et al., 1999; Koroleva et al., 2005) and the fusion proteins have approximate sizes of about 55 kDa. Hence, the nuclear localization can be the result of free diffusion between the cytoplasm and nucleus. It is worthy to note that the web-based nuclear localization prediction program PredictNLS (Cokol et al., 2000) fails to detect relatively well-known eukaryotic nuclear localization signals (Haasen et al., 1999; Koroleva et al., 2005) in the family of *A. thaliana* sPPases.

It is fairly clear that among the soluble pyrophosphatases revealed from *A. thaliana* genome database (TAIR; http://www.tair.org), none of them was localized in the mitochondria under studied conditions. The presence of mitochondrial sPPases was shown to be essential for the function of this organelle during respiratory growth in animal and fungal cells (Vianello and Macri, 1999; Perez-Castineira et al., 2001). There are a number of studies showing the possible presence of mitochondrial sPPases in plants, all of which were based on crude mitochondrial preparations (Zancani et al., 1995; Vianello et al., 1997; Casolo et al., 2002). Since there are biosynthetic pathways active in plant mitochondria which generate PP_i and thereby necessitate the removal of excess pyrophosphate, either the action of a sPPase or translocation of excess PP_i to the cytoplasm has to be postulated. In conclusion, the absence of sPPases in the mitochondria is quite surprising. The targeting of sPPase to mitochondria may require specific environmental conditions or the mitochondrial localization can be limited to certain tissues (heterotrophic tissues like stem cells; Zancani et al., 1995). A mitochondrial sPPase from plants was shown to be associated with a protein complex at the inner membrane (Vianello et al., 1997); thus, it is also possible that the addition of GFP may interfere with complex formation and thereby can cause mistargeting of the enzyme to the cytoplasm. Although, it is likely that transient overexpression itself can cause an ambiguous localization of the target protein, one cannot rule out the possibility that plants do not have any mitochondrial sPPases and excess PP_i is exported out of this organelle to the cytoplasm by a translocator protein.

4.3 *A. thaliana* sPPases are Differentially Regulated

Several isoforms of an enzyme family localized to the same compartments are known to differ from each other in enzymatic function, regulatory mechanisms of enzyme function, tissue- (or cell-) specificity of gene expression, or growth stage specificity. Thus, the differential regula-

tion of *A. thaliana* sPPases was analyzed using stable transformation of promoter GUS fusion constructs (Chapter 3.1.2.1) and real time PCR (Chapter 3.1.2.2).

4.3.1 Promoter Activities of *A. thaliana* sPPases in Different Tissues Indicate Isoform Specificity and Developmental Stage Dependency

The *A. thaliana* sPPase genes are encoded by different chromosomes but are usually located in heavily gene loaded areas (TAIR; http://www.tair.org). This is especially outstanding in ASP2B, ASP4 and ASP5 genes, whose adjacent promoter regions comprise only about 500 base pairs. The promoter region of ASP1 is approximately 1 kb long, whereas, the promoter regions of ASP2A and ASP3 are more than 3 kb. Therefore, for comparative analysis of promoter activities, the 1 kb region upstream of the start codon of each ASP promoter was fused to β-glucuronidase (GUS) and stably transformed to *A. thaliana*. The histochemical GUS analysis of T2 generation of transgenic plants indicated a distinct staining pattern for ASP isoforms, some of which will be explained in detail in following paragraphs (Figures 3.4 to 3.11).

The promoter activities of *A. thaliana* sPPases were found to be increasing during flower development (Figures 3.4 to 3.9). ASP3 is the only isoform whose promoter drives the expression of GUS in very early stages of flower development (Figure 3.7.C-D). On the contrary, the promoter activity of ASP5 was detected only in fully developed flowers (Figure 3.9.C-D). The histochemical GUS staining of other ASP isoforms indicated that the activity started at a certain stage during flower development where flower buds were still closed, but pollen formation has started (Figures 3.4,5,6, and 8). The AVP1 (*A. thaliana* vacuolar pyrophosphatase) promoter activity was previously shown to be increasing during flower development (Mitsuda et al., 2001). The detection of histochemical staining started at a certain stage during pollen maturation where the tapetum layer surrounding microspores degrades to provide carbohydrates (Mitsuda et al., 2001). The released carbohydrates were reported to be rapidly taken up by the developing pollen grains (Mitsuda et al., 2001). The comparison of GUS staining patterns of AVP1 (Mitsuda et al., 2001) to ASP1 (Figure 3.4.D), ASP2A (Figure 3.5.C-D) and ASP4 (Figure 3.5.C-D) showed a close similarity during flower development. Although, the changes in promoter activities were not analyzed in comparison to precise stages during flower development, based on the similarities to AVP1 promoter activity, it can be hypothesized that the function of ASP1, ASP2A and ASP4 might be important in pollen maturation.

Sucrose is synthesized in the cytosol of mesophyll cells and translocated via companion cells to vascular bundles for transfer to sink organs (Lerchl et al., 1995). To maintain the adequate carbon supply, a small portion of the incoming sucrose is cleaved by sucrose synthase in companion cells (Claussen et al., 1985). Lerchl et al. (1995) proved that cytosolic PP_i is essential for long-distance sucrose transport and excessive removal of PP_i from phloem by ectopic expression of *E. coli* sPPase impairs sucrose hydrolysis via sucrose synthase. In contrast to these data, the activities of all ASP promoters were detected in vascular bundles of source leaves (Figures 3.4 to 3.9). The promoter activities of *E. coli* sPPase transgene (CaMV 35S) and the endogenous sPPase genes are different and, therefore are not comparable. However, this may imply that soluble pyrophosphatase activity can be important for phloem loading or cleavage of sucrose to achieve sufficient sink strength. It is likely that a strictly controlled pyrophosphatase concentration in the cytoplasm is required for regulation of activities of enzymes in carbohydrate metabolism (like sucrose synthase) and/or translocation of sucrose to the phloem.

The staining of funiculus was detected for both ASP1 and ASP4 promoter driven GUS activities (Figures 3.4.F and 3.8.F). There is no direct vascular connection between parental plant and the endosperm and assimilates are transported to the endosperm through funiculi (Bewley et al., 2000; Emes et al., 2003). During early seed development, sucrose is generated by the parental plant, released from the phloem to the apoplast, and hydrolyzed by invertases (Bewley et al., 2000; Emes et al., 2003). The hexose products are then loaded into seed through funiculus and sucrose is resynthesized from the hexose imported into the storage tissues (Bewley et al., 2000; Emes et al., 2003). At later stages of development, however, hydrolysis of sucrose is not an obligatory step for uptake and sucrose itself is imported (Bewley et al., 2000; Emes et al., 2003). The detection of promoter activities of ASP1 and ASP4 in funiculus, therefore, might indicate the direct role of these enzymes in sucrose hydrolysis/resynthesis and/or translocation and imply the possibility of their function in seed filling.

Although the promoter activities of *A. thaliana* sPPases were detected in mesophyll cells in some cases, the dot-shaped staining pattern specific to ASP1 promoter is of particular interest (Figure 3.4.B-C). The GUS staining indicated promoter activity for this isoform specifically in spongy mesophyll cells. Although the reasons for specific activity of ASP1 in some, but not all spongy mesophyll cells, are not known, the lateral differentiation of expression was reported for other specialized cell types like idioblasts, where the calcium oxalate was shown to develop within intravacuolar membrane chambers (Webb, 1999; Franceschi and Nakata, 2005) during the accumulation of crystalline calcium oxalate in response to surplus calcium (Nakata and McConn, 2000; Kostman et al., 2001). The accumulation of polysaccharides in the same vacuoles and in-

volvement of polysaccharides in defining crystal shape was reported in some instances (Webb, 1999; Nakata and McConn, 2000). The function of calcium oxalate accumulation is largely unknown since the mutants lacking the ability to form crystals were shown not to have any phenotype (Nakata and McConn, 2000). This is also true for biosynthetic pathways for calcium oxalate production.

Trichome staining was observed after histochemical GUS assay of stably transformed *A. thaliana* with 1 kb promoter regions of ASP2B and ASP3 (Figure 3.10). Trichomes are specialized unicellular structures derived from epidermal cell layer (Herman and Marks, 1989; Marks, 1997; Gutierrez-Alcala et al., 2000), and normally present on the leaves, stems and sepals of *A. thaliana* (Marks, 1997). They are not able to produce or secrete phytochemicals in *A. thaliana*, but may function as defensive physical structures against herbivores, detoxification of heavy metals and in responses to various other stress conditions (Gutierrez-Alcala et al., 2005). Trichomes are simply expanded single-celled structures, and for that they require several factors like the biosynthesis of new membrane lipids, cell wall components, and proteins and the opposing force of the turgor pressure (Smart et al., 1998). The hydrolysis of sucrose via sucrose synthase is required to produce UDPGlu which is the entering form of carbon for cell wall synthesis (Dennis and Blakeley, 2000), therefore, the promoter activities of ASP2B and ASP3 in trichomes might point out their specific role in the sucrose hydrolysis. The enlargement of trichome cells also requires regulation of turgor pressure by the accumulation of ions to the vacuole. Since the driving force for the secondary transport is provided by both vPPase and V-ATPases, the activity of sPPases might control cytoplasmic level of PP_i and thereby affect the activity of vPPases. Although not very likely, the activity of ASP2B and ASP3 in trichomes might also point out their role in detoxification or stress tolerance metabolisms.

The promoter activities of *A. thaliana* sPPases in root tips (Figure 3.11) indicate the importance of sPPases in root growth. The differences in the staining patterns, on the other hand, reveal the high specificity of sPPase isoforms in function. The ASP1 and ASP5 promoters were found to be active in cell division zone (Figure 3.11.A and F), ASP2B only in the elongating region (Figure 3.11.C) and ASP2A, ASP3 and ASP4 in overall root tip (Figure 3.11.B, D, and E). The failure to detect ASP2B promoter activity in rapidly dividing root cells implies the possibility that the function of this soluble pyrophosphatase isoform might be other than removing accumulated pyrophosphate to drive the anabolism as suggested in literature. It is important to note that only the promoter activity of ASP4 was observed in whole root, whereas the activities of other isoforms were restricted to defined root domains. This may indicate the specificity of ASP4 activity for root function.

The histochemical assay of the stably transformed *A. thaliana* plants expressing 1 kb ASP promoter driven GUS activity demonstrate tissue specificity and developmental stage dependency of each isoform. However, the transcriptional regulation in eukaryotic organisms is known to be dependent not only on cis-elements found in the promoter regions but also in trans-elements that can be localized in different chromosomes. Therefore, using a full promoter region is not enough to prove the active transcription of the gene of interest in that tissue. In addition, gene expression analysis based on promoter driven GUS expression is not sufficient since it does not reflect factors like post-transcriptional regulation or mRNA stability (Tsien, 1998). Finally, GUS enzyme is relatively stable after expression, thereby; even if the transcription was ceased, histochemical staining can still be detected. Therefore, the results obtained from promoter analysis of ASP isoforms, although giving hints about the expression of sPPases in tissues examined, are not comprehensive.

4.3.2 Real Time PCR Analysis Confirms Gene Expression of *A. thaliana* sPPases in Several Plant Tissues

The mean normalized results of real time PCR analysis of each *A. thaliana* sPPase isoform-specific gene expression are given in Figures 3.12 and 3.13. The normalization was performed by using beta actin expression as the reference. Beta actin is a cytoskeletal structural protein, thus, it should have a relatively constant expression level in all tissues (Vandesompele et al., 2002). Although housekeeping gene expression can vary between different tissues or in response to certain environmental conditions, the normalization method used (Muller et al., 2002) is based on the calculation of expression of the target gene relative to the reference gene, thereby minimizes the error range.

As expected, the expression of the plastidial isoform (ASP5) is considerably higher than other ASP isoforms (Figure 3.12). The ASP5 transcript is not only detected in green tissues, but also in heterotrophic tissues like roots and stem, although in relatively low levels. Redrawing of the same graph after removing the ASP5 data (Figure 3.13) clearly shows that other ASP isoforms (which were proven to localize in the cytoplasm and/or nucleus; Figure 3.3), have very low expression levels compared to actin gene, however, they are actively expressed in tissues studied. ASP4 and ASP1 have higher expression rates in whole plant and the transcripts of ASP2A and ASP2B are almost non-detectable in the tissues analyzed. Since the promoter activities of ASP2A and ASP2B were observed by histochemical GUS staining of several plant tissues, the

extremely low levels of their RNA might indicate the presence of a post-transcriptional regulation or a low mRNA stability of these sPPase isoforms.

The results obtained from real time PCR analysis are a further confirmation for the tissue-specific expression of *A. thaliana* sPPases. The data also show that the cytoplasmic soluble pyrophosphatases are actively transcribed not only in tissues of high metabolic activity (like sink leaves), but also in later developmental stages (like source leaves). As indicated before (Chapter 4.2), there is only one study that claims the possible presence of cytoplasmic sPPases in plants based on the cloning of a sPPase gene from potato tuber that does not have any known leader peptide sequence (du Jardin et al., 1995). Perez-Castineria et al. (2001) claimed that the plant soluble pyrophosphatases missing a leader peptide should be expressed in non-photosynthetic tissues like roots based on their similarity to previously cloned sPPase gene from potato tuber (du Jardin et al., 1995). The results obtained by both promoter driven GUS expression and by real time PCR analysis are clearly in opposition to this hypothesis by proving the expression of several ASP isoforms in both photosynthetically active and heterotrophic tissues. In summary, the plant sPPases localized in the cytoplasm and/or nucleus might have a role in the regulation of plant metabolism during all developmental stages.

4.4 Sugar-mediated and Starvation Induced Responses of *A. thaliana* sPPases

The analysis of promoter regions of *A. thaliana* sPPases indicates the presence of several cis-acting regulatory motifs including etiolation, starvation and sugar responsive elements (Table 3.4).

Development in darkness is referred as skotomorphogenesis and characterized by etiolated appearance of seedlings with a fast-growing hypocotyl, presence of an apical hook and small, closed cotyledons or primary leaves (Alabadi et al., 2004). When seeds are germinated in the dark, undifferentiated proplastids enlarge and develop into etioplasts (Mochizuki et al., 1996), and the expression of light-regulated genes is repressed or kept at low, basal levels (Alabadi et al., 2004). Since the light is the source of energy for carbon fixation, plants undergo a starvation response in the absence of light unless supplied with exogenous carbon (Kim and von Arnim, 2006).

The responses of plant genes to changing carbohydrate status is an ancient system of cellular adjustment to critical nutrient availability (Koch, 1996). Being photoautotrophic organisms, plants have a complex carbon metabolism and, thereby, they must coordinate the production of carbon from photosynthesis with its utilization, mobilization and allocation in various tissues at different developmental stages (Jang and Sheen, 1997). Sugar-regulated genes provide a means not only for integrating cellular responses to transport sugars but also for coordinating changes in resource utilization and allocation among different plant parts (Koch, 1996). In addition to playing a central role in metabolism, soluble sugars like glucose and sucrose have shown to be important signaling molecules regulating many developmental and physiological processes in plants (Jang and Sheen, 1997; Rook et al., 1998; Gibson, 2000). Carbohydrate depletion enhances expression of genes for photosynthesis, reserve mobilization and export processes, whereas abundant carbon resources favor genes for storage and utilization and repress the expression of genes for sugar production (Koch, 1996; Jang and Sheen, 1997). In photosynthetic leaves, those genes associated with starch breakdown were shown to be upregulated by carbohydrate depletion and repressed by glucose (Koch, 1996; Jang and Sheen, 1997).

The non-metabolizable sugar analogs are commonly used to prove the role of sugars as signaling molecules (Gibson, 2000). 3-*O*-methylglucose (3-O-methyl-D-glucopyranose) is a glucose analog, which accumulates as 3-*O*-methylglucose-6-phosphate in the cytosol after phosphorylation by hexokinases (Cortes et al., 2003). 3-*O*-methylglucose was reported to be used as glucose analog without triggering any apparent sugar signal (Cortes et al., 2003). In addition, it is not perceived as respiratory substrate, therefore, causes sugar starvation. (Cortes et al., 2003).

4.4.1 ASP2B is Induced by Sugar Starvation

The basal expression level of ASP2B in leaf is quite low when quantified relative to the expression of actin gene (Figure 3.13). The expression of ASP2B in *A. thaliana* cell culture, on the other hand, is influenced by the carbon source in the medium; no expression was detected in cells grown in glucose containing medium but transcript was detectable in cells fed by sucrose (Figure 3.15).

The gene expression of ASP2B was found to be notably induced both in seedlings germinated in dark without exogenous sugar supply (Figure 3.14) and in cell cultures grown in 3-*O*-methylglucose as carbon supply (Figure 3.16). As explained above, when grown in dark, plants experience starvation due to repression of photosynthesis (Kim and von Arnim, 2006). This is

also true for feeding with 3-*O*-methylglucose, since it is a non-metabolizable glucose analog (Cortes et al., 2003). The induction of gene expression of ASP2B was observed 24 hours after transfer of glucose grown *A. thaliana* cell culture to a fresh medium containing 3-*O*-methylglucose as carbon supply (Figure 3.16). Cortes et al. (2003) reported the complete consumption of endogenous sucrose and fructose in sugar starved root tips as 15 hours, therefore, this relatively late response can be due to usage of endogenous sucrose and hexoses until they are totally used up.

The gene expression of ASP2B, therefore, is induced by sugar starvation, indicating the role of this soluble pyrophosphatase isoform in starvation response. The sugar-mediated regulation of ASP2B gene expression is surprisingly similar to the regulation of enzymes responsible from starch breakdown; they were shown to be significantly upregulated by carbohydrate depletion and completely repressed by glucose (Koch, 1996; Jang and Sheen, 1997). Alas, these preliminary results are not sufficient to deduce a specific role for ASP2B in response to starvation.

4.4.2 Light and Photosynthesis Regulation of the Plastidial Isoform

The expression of plastidial isoform (ASP5) was found to be completely repressed both in seedlings germinated in dark (Figure 3.14) and *A. thaliana* cell cultures (Chapter 3.1.3.3), implying the requirement of light for the transcription of ASP5 gene. It is important to note that the failure to detect ASP5 expression in *A. thaliana* cell cultures can also be explained with the low starch turnover in cell cultures.

The *in planta* responses of gene expression of ASP5 is given in Figure 3.17. The data obtained are not correlating with data reported by Schulze et al. (2004) where by Northern blotting analysis, the authors showed the induction of ASP5 transcript by both glucose and sucrose when used only in 300 mM concentrations. The results collected by real time PCR analysis (Figure 3.17), on the contrary, show an induction by 100 mM glucose, as in the case of photosynthesis related genes, which can be deduced from the *in vivo* function of plastidial soluble pyrophosphatases. The variation between results presented in this study and Schulze et al. (2004) can be related with using leaves of different developmental stages (Schulze et al. (2004) did not specify the age of the plant used for collecting leaf discs). It is noteworthy that 300 mM sucrose and 300 mM glucose are non-physiological concentrations of sugars; therefore, the responses obtained by Schulze et al. (2004) can be unrelated with carbohydrate-mediated regulation. In *A. thaliana*

seeds, Gibson (2000) proved that high concentrations of exogenous sugar (300 mM glucose and 300 mM sucrose) severely interfere with seedling development.

In summary, the expression of plastidial *A. thaliana* sPPase isoform is regulated by light at the level of transcription and induced only by glucose as in the case of photosynthesis related genes.

4.4.3 Sugar-mediated Changes in Gene Expressions of *A. thaliana* sPPase Isoforms; Only ASP3 Expression is Regulated by Sucrose

The mean normalized expression values of sPPase isoforms ASP1, ASP3 and ASP4 in *A. thaliana* cell cultures grown either in glucose or sucrose containing medium (Figure 3.15) prove that exogenous carbon supply can significantly influence the expression of ASP3. While the changes in the expression levels of ASP1 and ASP4 are relatively the same in cells grown in glucose or sucrose, the transcript amount of ASP3 in cells grown in glucose is twice the amount of the cells fed by sucrose (Figure 3.15).

This finding is further supported by the *in planta* sugar-mediated responses of gene expressions of ASP1, ASP3 and ASP4 (Figure 3.17). As explained above, the sugar-related signaling can be identified by using sugar analogs that do not prompt any sugar response. In the case of ASP1 and ASP4, removal of leaf discs from parental plant and incubating them in 3-*O*-methylglucose, glucose or sucrose (100 or 300 mM) caused a complete repression of expression in all cases, which show that there is no sugar-mediated regulation of these ASP isoforms. On the other hand, while 3-*O*-methylglucose and glucose (both 100 and 300 mM) do not influence the expression of ASP3, the transcript amount is significantly induced by 100 mM sucrose but not with 300 mM sucrose (non-physiological concentration). That is, the transcription of ASP3 is carbohydrate-regulated and influenced by sucrose, but not by glucose, which might indicate a function of this isoform during sucrose hydrolysis. This finding is further supported by detecting the promoter activity of ASP3 in trichomes, which require the hydrolysis of sucrose via sucrose synthase in order to produce UDPGlu for cell wall enlargement (Chapter 4.3.1). The hydrolysis of sucrose consumes PP_i rather than generates it, therefore, the function of sPPase in this process is hard to explain. It is possible that the cytoplasmic PP_i/P_i concentration might regulate the enzymes functional in sucrose biosynthesis as previously proven for enzymes of starch synthesis (Tiessen et al., 2002 ; Geigenberger, 2003).

The expression of ASP2A was found to be completely repressed in etiolated seedlings and it was not detected in *A. thaliana* cell cultures grown either in glucose or in sucrose (Figure 2.14 and Chapter 2.1.3.3). This might indicate the requirement of light for transcription of ASP2A. The *in planta* response of ASP2A, on the other hand, is quite complex. It is clearly induced by 100 mM sucrose, however, the non-physiological concentrations of both 3-*O*-methylglucose and sucrose (300 mM) cause an unexpected induction in expression. Therefore, it is not possible to presume the regulation of ASP2A in response to carbohydrates with the data collected here.

4.5 Changes in the Expressions of *A. thaliana* sPPases upon Different Environmental Conditions Confirm Their Importance in Stress Responses

Plants are immobile, and therefore they are sessile to changes in environmental conditions like water-logging, drought, high or low temperatures, excessive soil salinity, inadequate mineral nutrients in soil, and too much or too little light (Bray et al., 2000). Stresses trigger a wide range of plant responses, from altered gene expression and cellular metabolism to changes in growth rates depending on the duration and severity of the stress (Bray et al., 2000).

The response is initiated when a plant recognize a stress at the cellular level (Bray et al., 2000). Stress recognition activates signal transduction pathways that transmit information within individual cells and throughout the plant. Ultimately, changes in gene expression, which occur at the cellular level, are integrated into a response by the whole plant that may modify growth and development and even influence reproductive capabilities (Bray et al., 2000). The duration and severity of the stress dictate the scale and the timing of the response (Bray et al., 2000).

There is no data available in literature concerning the stress regulation of plant soluble pyrophosphatases. In the following chapters, the data collected from a preliminary stress experiments in the course of this study will be discussed. It is noteworthy that stress treatments were applied only at one concentration and usually at one time point; therefore, the results are not comprehensive. A detailed analysis to understand the regulation of plant soluble pyrophosphatases under environmental stress conditions is necessary to assign the functions and importance of these enzymes in plant stress tolerance.

4.5.1 ABA, Salt and Cold Specifically Induce Some ASP Isoforms, while Repressing the Others

Table 3.5 summarizes the responses of ASP isoforms to 100 μM ABA (16 hours), 200 mM salt (16 hours) and cold (2 hours at 4°C and 16 hours recovery) treatments. The results indicate both isoform- and tissue-specific responses of *A. thaliana* sPPases implying their importance in plant stress responses.

Abscisic acid (ABA) regulates many aspects of plant growth and development like embryo maturation, seed dormancy, germination, cell division and elongation. A critical function of ABA is to optimize growth during environmental stresses such as drought, salinity, cold and pathogen attack by maintaining osmotic homeostasis (Kang et al., 2002). At the whole plant level, low ABA promotes root growth but inhibits shoot growth, leading to an increased root/shoot ratio. In contrast, high ABA inhibits growth of both roots and shoots, but promotes formation of arrested lateral roots (Kang et al., 2002). At cellular level, ABA can promote tolerance of some abiotic stresses including drought, salinity and cold by changing the expressions of a set of genes (Kang et al., 2002). Extensive studies of stress- and ABA-induced gene expression reveal two waves of response; en early transient response (peaking at about 3 hours) and a late sustained response (from approximately 10 hours onward) (Kang et al., 2002). The early responses include signal transduction cascades and several transcription factors (Kang et al., 2002). The late genes are presumed to contribute to the adaptive aspects of induced tolerance. Many of these encode proteins that accumulate during the acquisition of desiccation tolerance in seeds (late embryogenesis abundant proteins), while others encode proteases, presumed chaperonins, enzymes of sugar or other compatible solute metabolism, ion- and water-channel proteins, and enzymes that detoxify active oxygen species (Kang et al., 2002). ABA regulated genes were shown to be induced by exogenous ABA (Xiong et al., 2001) enabling mimicking of stress response by experimentation.

The vacuolar proton-pumping pyrophosphatases were shown to be regulated by hormones (Ozolina et al., 2001; Yang et al., 2003) and the vacuolar pyrophosphatase from red beet was clearly upregulated upon exogenous ABA treatment (100 μM) (Ozolina et al., 2001). Therefore, it is interesting to observe if hormones also have a role in the regulation of expression of *A. thaliana* soluble pyrophosphatases. Indeed, the exogenous ABA treatment (100 μM, 16 hours) indicate an isoform- and tissue-specific regulation of ASP isoforms.

The responses of ASP isoforms to ABA treatment are quite complex (Table 3.5). For example, ASP1, ASP4 and ASP5 expressions were induced in leaf tissue, but not changed in root, on the other hand, the expressions of both ASP2A and ASP2B were induced in root, while repressed in leaf. These results can be explained by tissue-specific ABA signaling pathways as reported before (Kang et al., 2002). The increased activities of ASP2A and ASP2B in root tissue might indicate their role in root growth arrest. It is noteworthy to mention that ASP2B was shown to be induced under sugar starvation conditions (Chapter 4.4.1), which might indicate the role of this enzyme in cell death. Among ASP isoforms, only ASP3 was upregulated by ABA treatment both in root and leaf tissues. As this isoform was presumed to have a role in sucrose hydrolysis based on promoter activity and sugar response experiments (Chapter 4.4.3), the function of ASP3 might be related with the regulation of enzymes of sugar biosynthesis by changing the cytosolic pyrophosphate concentration. It is important to consider that the induction of transcription after ABA treatment can be an indirect effect due to changes in metabolism rather than a direct regulation. This is quite possible for ASP isoforms, since *in silico* analysis failed to detect any ABA-related element (ABRE) in their promoter regions.

Plants need essential mineral nutrients to grow and develop. However, excessive soluble salts are harmful to most plants. Upon exposure to high salinity, plants may exhibit a reduced growth rate, accelerated development and senescence (Zhu, 2002; Ma et al., 2006). Salinity imposes two stresses on plant tissues: a water deficiency result from the relatively high solute concentrations; and ion-specific stresses resulting from altered K^+/Na^+ ratios and Na^+ and Cl^- ion concentrations that are unfavorable to plants (Blumwald et al., 2000). Thus, the net result of plant responses to elevated sodium concentrations is the maintenance of low cytosolic Na^+ and a high cytosolic K^+/Na^+ ratio (Blumwald et al., 2000). The extrusion of sodium ion from the cytosol to the vacuole is an active process and requires the electrochemical potential (Blumwald et al., 2000; Mimura et al., 2003). Therefore, the proton-pumping vacuolar pyrophosphatases are likely candidates for salt stress response.

The response of vacuolar proton pumping pyrophosphatase to salinity, on the other hand, is rather complex (reports of stimulus, no effect and stimulation of H^+-transport but not hydrolysis activity) and seems to be species-dependent (Otoch et al., 2001; Wang et al., 2001; Maathuis et al., 2003). The ectopic expression of AVP1 (*A. thaliana* vacuolar pyrophosphatase) was reported to increase salt and drought tolerance in Arabidopsis (Gaxiola et al., 2001). The effect was explained with induced vacuolar solute accumulation as a results of increasing the activity of vacuolar proton pump (Gaxiola et al., 2001). Although the vacuolar pyrophosphatase is reported

to be a likely candidate to engineer salt tolerant crop species (Park et al., 2005), why and how salt stress responses are different from each other is not clear yet.

During high salinity, plants accumulate compatible solutes in the cytoplasm to lower osmotic potential as an adaptive strategy. Various compatible osmolytes such as proline, glycine betaine and polyols were shown to greatly reduce stress damage to plant cells (Zhu, 2002). The ionic and osmotic stresses imposed by high salinity on plant may create secondary stresses too. These derived stresses include the accumulation of toxic or unwanted compounds, perturbance in cellular metabolism and nutritional disorder (Zhu, 2002).

The salt stress responses of *A. thaliana* soluble pyrophosphatases are summarized in Table 2.5. The comparison of salt responses with changes obtained by ABA treatment reveals that there is no clear similarity except the root response of ASP3, which was induced in both cases. Since 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 a crosstalk between these two pathways (Zhu, 2000). On the other hand, since no clear similarities were observed in the changes of the expressions of ASP isoforms, the induction or repression of ASP expression may be the consequence of stress injury rather than a direct regulation. Most of the ASP isoforms were repressed upon salt treatment, among them ASP1 and ASP4 were repressed both in leaf and root tissues, which may imply that their function is not required or hazardous to plant metabolism under salt stress conditions. The upregulation of ASP2B expression in leaf tissue might be a cause of nutritional disorder since this enzyme was shown to be induced by starvation. Genes that are regulated by photosynthetic activities may be affected but they are not regulated by salt stress per se (Zhu, 2002), therefore, the induction of ASP5 transcription upon salt stress can not be explained as a stress response but rather show the changes in cellular metabolism upon treatment.

Low temperature was reported as one of the most important environmental constraint for plant growth (Ishinati et al., 1997). Low-temperature inducible genes have been shown to be regulated by both ABA dependent and ABA independent pathways (Yamaguchi-Shinozaki and Shinozaki, 1994). The vacuolar proton-translocating pyrophosphatase of rice seedlings was reported to be induced by chilling (Carystinos et al., 1995). Chilling creates an energy stress due to mitochondrial dysfunction and therefore, PP_i-dependent pathways are favorable under this ATP limiting conditions (Carystinos et al., 1995). Surprisingly, some of the ASP isoforms were found to be induced after cold treatment (2 hours at 4°C, 16 hours recovery). This may, although indirectly, imply that the function of soluble pyrophosphatases are critical for plant function or the *in vivo* function of plant sPPases is not restricted to pyrophosphate cleavage.

The overall responses of ASP isoforms to ABA, salt and cold treatments, although showing some similarities, do not correlate with each other as expected due to the effect of endogenous ABA accumulation to stress responses in the case of salt and cold treatments. This can be explained in three ways; (i) there are ABA-dependent and ABA-independent signaling pathways in response to salinity and cold treatment, thus, the differences can be related with signaling through an ABA-independent pathway, (ii) the changes in the expression of ASP isoforms upon these stresses might be related with secondary effects on cellular metabolism rather than a direct regulation, and (iii) the data compared were collected as response to one concentration and one time point, therefore, are not comprehensive.

The preliminary results clearly indicate that the effects of stress treatments on the expression of ASP isoforms are both tissue- and isoform-specific. Therefore, as mentioned before, the roles of these enzymes in regulation of plant metabolism is likely to be more important previously thought and each isoform might have specific function *in vivo*.

4.5.2 Responses of ASP Isoforms to Heat Stress are Time Dependent

In nature, plants are subject to shifts of temperatures, both during changes in season and more rapidly over the course of individual days (Larkindale and Knight, 2002). Therefore, they have evolved strategies for preventing damage caused by rapid changes in temperature and for repairing it (Larkindale and Knight, 2002). The heat shock response is characterized by a rapid reprogramming of gene expression, leading to a transient accumulation of heat shock proteins that is correlated with enhanced thermotolerance (Lee et al., 1995; Busch et al., 2005). The induction of genes in different functional classes including chaperons other than heat shock proteins, proteins of the degradation pathways, enzymes of carbohydrate metabolism, membrane transporters, transcription factors, and signaling components were also reported (Busch et al., 2005). One of the genes in the plant carbohydrate metabolism that is induced upon increased temperatures is UGPase whose activity is required for the synthesis of galactinol upon heat stress response (Busch et al., 2005).

The changes in the expression of *A. thaliana* sPPase isoforms upon 2 hours heat treatment followed by 16 hours of recovery are summarized in the Table 4.1.

Table 4.1. Percent changes in the expression of ASP isoforms after 2 hours heat stress (37°C) and 16 hours of recovery.

	ASP1	ASP2A	ASP2B	ASP3	ASP4	ASP5
2 hrs Leaf	29	-69	-91	-40	-81	29
2 hrs Root	83	45	813	-90	751	-45
Recovery Leaf	103	214	-90	443	423	475
Recovery Root	-13	-4	-56	257	-1	154

The initial response of most of the ASP isoforms to heat stress in leaf tissue is either no significant change or even strong repression. In roots, the heat treatment for 2 hours at 37°C resulted in a significant induction in the expressions of ASP2B and ASP4 only. Interestingly, the expression of ASP2B, which is presumed to be related with sugar starvation response, is repressed during recovery. During the recovery, the expression of ASP4 in roots returned to initial level, whereas a delayed upregulation was observed in leaves. These two examples indicate that the heat stress response of *A. thaliana* sPPases is not only isoform-specific but also time dependent.

The expression of ASP3 is initially repressed by heat treatment, whereas, it is significantly induced during recovery. As mentioned before, this enzyme seems to have specific role in the regulation of sucrose synthesis via sucrose synthase dependent pathway and is induced upon several other stress treatments (Chapters 4.4.3 and 4.5.1). Since one of the enzymes that is reported to be induced by heat shock is UGPase (Busch et al., 2005), the role of ASP3 might be the regulation of UGPase activity by changing the cytoplasmic PP_i concentration.

It is noteworthy to mention that the effects of heat stress treatment on the expression of ASP isoforms might be a direct course of heat treatment or by secondary effects due to changes in cellular metabolism. At least, the induction of ASP3 expression during recovery but not directly after stress indicates that it is regulated by the changes in the metabolism rather than heat stress itself.

4.5.3 Phosphate Starvation Represses the Expression of *A. thaliana* sPPases

Phosphate is an essential but one of the most limiting nutrients for plant growth because of its low mobility in the soil (Palma et al., 2000; Raghothama, 2000; Sanchez-Calderon et al., 2005). Plant roots absorb phosphate as orthophosphate (P_i, inorganic phosphate) and distribute to

whole plant via phosphate transporters. Inorganic phosphate plays a central role in the plant metabolism both as a substrate for phosphorylation and ATP generation through respiration, as well as a key component of the export of carbon from chloroplasts during photosynthesis (Misson et al., 2005). P_i and nucleoside phosphates participate as substrates or products in numerous enzymatic reactions and also act as allosteric modulators (Misson et al., 2005). Phosphorylation plays an important role in the control of several signal transduction pathways (Misson et al., 2005). It is thus not surprising that P_i deficiency has a profound effect on various aspects of plant metabolism, including photosynthesis, carbon fixation, glycolysis and respiration (Misson et al., 2005).

Because of large decline in cytoplasmic P_i levels that follows severe phosphate starvation, large reductions in intracellular levels of ATP (as much as 80 %) and related nucleoside phosphates occur (Palma et al., 2000). This may hinder carbon flux through enzymes of classical 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, non-phosphorylating NADP-glyceraldehyde-3-phosphate dehydrogenase, phosphoenolpyruvate carboxylase and phosphoenolpyruvate phosphatase) have been reported to be induced upon phosphate starvation (Plaxton, 1996; Palma et al., 2000). The plant vacuolar proton-pumping pyrophosphatase is also known to be upregulated upon phosphate starvation (Palma et al., 2000; Wasaki et al., 2003). There are also several studies indicating the upregulation of soluble pyrophosphatases in plants upon phosphate deprivation (Wasaki et al., 2003; Misson et al., 2005). Thus, PP_i-dependent processes may be a crucial aspect of the metabolic adaptations of plants to environmental extremes causing reduced ATP pools (Palma et al., 2000). Interestingly, plant cytosolic PP_i levels are remarkably insensitive to abiotic stresses such as anoxia or phosphate starvation which elicit significant reductions in cellular ATP pools (Palma et al., 2000).

There is no induction in the expression of ASP isoforms upon phosphate starvation for 5 days (Table 4.2). The expressions of most *A. thaliana* sPPases are found to be repressed upon treatment. This finding can be explained by the induction of PP_i-dependent pathways upon phosphate deficiency and indirectly implies the *in vivo* function of *A. thaliana* sPPase isoforms as degradation of pyrophosphate to two orthophosphates. Although, the induction of sPPase function might cause increase in cytosolic P_i concentration which is decreased upon phosphate starvation, the cytoplasmic PP_i concentration might be vital for the fate of cellular metabolism to facilitate the function of 'bypass' enzymes.

Table 4.2. Percent changes in the expressions of *A. thaliana* sPPase isoforms after 5 days of phosphate starvation.

	ASP1	ASP2A	ASP2B	ASP3	ASP4	ASP5
Leaf	7	-84	-1	-26	-58	4
Root	-85	25	-75	-39	-94	-92

As mentioned above, some of the ASP isoforms were reported to be induced upon phosphate deprivation (Misson et al., 2005). However, phosphate starvation for 5 days caused a strong downregulation of several ASP isoforms (Table 4.2). The difference in the results can be the influence of developmental stage of plants, different growth conditions or treatments. Nevertheless, the hypothesis of sPPase represion upon phosphate starvation in order to maintain the cytoplasmic PP_i pool for PP_i-dependent enzymatic activity appears more plausible as compared to the assumption that sPPase activity is required to increase cytosolic P_i concentration during phosphate stress.

4.5.4 Differential Gene Expression of Soluble and Vacuolar 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 results in localized cell death, loss of water and solutes from exposed surfaces, provide a point of entry for pathogens and can disrupt the vascular system (Cheong et al., 2002; Quilliam et al., 2006).

Many signaling pathways are activated either directly by the wounding process or subsequent to it (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).

4.5.4.1 Indirect Evidence on the Transient Induction of Bsp1 and Bvp1 upon Wounding of *B. vulgaris* Taproot Tissue

The wounding response in 7 days time frame was analyzed using fully developed sugar beet taproot tissues by Northern blotting (Figure 3.29). The results indicated the induction of endogenous Bsp1 (*B. vulgaris* soluble pyrophosphatase isoform 1) and Bvp1 (*B. vulgaris* vacuolar pyrophosphatase isoform 1) expression upon extreme wounding. The increase in the transcript amount of Bvp1 was detected 24 hours after wounding and returned to the initial level 7 days afterwards. On the other hand, the induction in the transcription of Bsp1 was transient, which was increased 24 hours after wounding and completely lost 3 days afterwards.

The Northern blot analysis, therefore, gives indirect evidence on the transient induction of not only vacuolar but also soluble pyrophosphatases upon wounding.

4.5.4.2 Expression Analysis Confirms Induced Expression for Most ASP Isoforms upon Wounding

Table 4.3 summarizes the effects of mechanical wounding of source leaves on the expression of ASP isoforms. The results imply isoform specific and time-dependent changes in the expressions of *A. thaliana* sPPases upon wounding. The time frame of induction of ASP isoforms may reflect whether the enzyme is regulated by wounding or upregulation 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 the stress, but rather the expression is altered due to adaptive changes in the metabolism.

Table 4.3. Percent changes in the expressions of *A. thaliana* sPPase isoforms upon mechanical wounding.

	ASP1	**ASP2A**	**ASP2B**	**ASP3**	**ASP4**	**ASP5**
1 hour	-82	274	15	558	119	178
5 hours	-75	613	558	17	43	152

The transient increase in the expression of ASP3 might be related with the synthesis of cell wall components (like UDPGlu) for wound healing via regulating the UGPase activity as suggested by several other studies (Chapter 4.4.3 and 4.5.1). The induction in the expression of ASP2B was observed only 5 hours after wounding indicating the possibility that the function of this enzyme is related with changes in the metabolism, rather than the direct effect of stress signaling, as explained above. The expression of this isoform was shown to be induced upon starvation (Chapter 4.4.1) and a possible function in cell death was presumed, therefore the induction of ASP2B upon wounding indirectly confirms these hypotheses. It is noteworthy to mention that the promoter activities of ASP2B and ASP3 were also detected in trichomes, which may signify their expression in pathogen related responses and their induction upon wounding might be related with defense mechanism against pathogen invasion through an open wound.

The expression of ASP1 was found to be repressed upon wounding and *in planta* sugar response experiments, which includes excessive wounding of leaf tissue for the removal of discs (Figure 3.17). Therefore, the downregulation of ASP1 expression during *in planta* sugar experiments can be explained by a wounding response, however, since no induction was observed in response to different feeding sugars, it is clearly not regulated with carbohydrates.

These preliminary stress treatments (Chapter 4.5) indicated an isoform-specific regulation of *A. thaliana* sPPases, which might imply the specific functions of each isoform *in vivo*. It is also important to consider the possibility of different substrate specificities of each ASP isoform. That is, the isoform specific expressional changes in response to stress treatments can also be explained by the different substrate specificities of ASP isoforms, since the *in vivo* substrate(s) of plant soluble pyrophosphatases has not been determined yet.

4.6 Both Soluble and Vacuolar Pyrophosphatases are Post-transcriptionally Regulated

First indications for a post-transcriptional regulation of mRNA level of soluble and vacuolar pyrophosphatases were obtained from an exprerimental approach using *B. vulgaris* soluble and vacuolar pyrophosphatases. The selection of transgenic lines expressing CaMV 35S promoter driven Bsp1 or Bvp1 was performed using leaf tissues of *in vitro* grown seedlings by Northern blot analysis (Schirmer, 2004).

CaMV 35S promoter drives an ectopic expression of the transgene, even in dormant tissues like sugar beet taproot (Figure 3.28). On the other hand, the selected transgenic lines did not give any apparent signal, neither in leaf (data not shown) nor in taproot tissues (Figure 3.29; comparison of '0 day' signals) of fully developed sugar beet plants as indicated by Northern blot analysis using 3'-UTR probes. This rather surprising result implies the possibility of post-transcriptional regulation of mRNAs of both Bsp1 and Bvp1 or shows that the transgenes are not functional at all. However, the detection of CaMV 35S promoter driven expressions of Bsp1 and Bvp1 in seedlings of transgenic lines (Schirmer, 2004) rules out the second possibility considering the plants analyzed were obtained from the same seedlings used for the initial selection.

The second indirect evidence for the presence of a post-transcriptional regulation of mRNA levels of Bsp1 and Bvp1 comes from taproot wounding experiments. The comparison of Northern blotting signals of wild type and transgenic lines (either CaMV 35S promoter driven Bsp1 or Bvp1 expression) shows some basic differences in the behavior of transcripts during the course of wounding. The promoter activities of both endogenous Bsp1 and Bvp1 are induced upon wounding (Chapter 4.5.4.1). The transcriptional induction of endogenous Bvp1 is reversed 7 days after wounding and that of endogenous Bsp1 is also transient and lost after 3 days. These results indicate the transcriptional regulation and a high turnover or short half-life of mRNAs of endogenous Bsp1 and Bvp1. However, the Bvp1 signal in transgenic lines expressing CaMV 35S promoter driven Bvp1 is more permanent compared to wild type signal and still visible 7 days after wounding. This is also true for Bsp1 signal in the Bsp1 overexpressing sugar beet plants where the transcript is induced upon wounding and still visible 7 days afterwards, although appears to be diminished in comparison to 3 day signal. These observations can be explained by the accumulation of mRNA due to the induction of CaMV 35S promoter activity upon wounding, since the upregulation in the transcriptions of endogenous Bsp1 and Bvp1 were shown to be transient. That is, upon wounding not only the promoter activities of endogenous Bsp1 and Bvp1 genes but also the CaMV 35S promoter driven transcription were induced. These inductions cause an accumulation in the mRNAs transcribed by both promoters and therefore result in a difference between the wild type and transgenic plants. It is important to mention that the mRNA of Bsp1 in Bvp1 overexpressing transgenic lines and that of Bvp1 in Bsp1 overexpressing lines behaves the same as the wild type upon wounding. These basic differences in the behavior of Northern signals of Bvp1 and Bsp1 enable the detection of transgenic lines and indirectly prove the presence of a post-transcriptional control of mRNA levels of both Bvp1 and Bsp1.

The further proof of possible post-transcriptional regulation of Bvp1 and Bsp1 was obtained by Western blot analyses (Figures 3.30 and 3.31). It is noteworthy to mention that the an-

tibodies used for the detection of Bsp1 and Bvp1 proteins are not specific and therefore detects all vacuolar and soluble pyrophosphatases present in the tissue. The molecular weight of Bvp1 protein is predicted as about 80 kDa (Kim et al., 1994), however it runs at about 70 kDa in SDS-polyacrylamide gel electrophoresis. This was reported for several other vacuolar pyrophosphatase proteins before, known to be a common behavior of hydrophobic membrane proteins, and explained as a result of the exposure of charged amino acid residues and/or irregularities in the shape of the SDS-protein complex (Sarafian et al., 1992; Kim et al., 1994; Nakanishi and Maeshima, 1998; Lopez-Marques et al., 2004). The soluble pyrophosphatase antibody used for Western blot analysis results in two signals of about 25 kDa in size. The detection of two bands of similar sizes was reported earlier (Rojas-Beltran et al., 1999). It is not clear if the second band corresponds to a separate gene product or results from partial degradation. The comparison of Western blot signal intensities of vacuolar and soluble pyrophosphatases in sugar beet taproot tissue upon wounding reveals that although the wounding induces the transcription of both endogenous and CaMV 35S promoter driven expression of Bvp1 and Bsp1 (Figures 3.28 and 3.29), there is no apparent change in the protein amount (Figures 3.30 and 3.31). The lack of correlation between transcript and proteins amounts of vacuolar pyrophosphatases were reported earlier (Otoch et al., 2001; Lopez-Marques et al., 2004). Accordingly, these findings further support the hypothesis that soluble and vacuolar pyrophosphatase mRNA levels are regulated not only transcriptionally but also post-transcriptionally.

The lack of post-transcriptional regulation at seedling stage might indicate that regulation mechanism is developmentally regulated and starts at a certain developmental stage. Since the analyses performed in the course of this study are not comprehensive, it was not possible to determine when and why post-transcriptional regulation of mRNA levels of soluble and vacuolar pyrophosphatases start and if the mechanism is similar in both types of inorganic pyrophosphatases.

It is important to mention that *B. vulgaris* has two known membrane bound proton-translocating pyrophosphatases (Kim et al., 1994) and four soluble pyrophosphatases one of which is possibly located in the plastids (Schirmer, 2004). Since the data obtained were mainly based on the analysis of one isoform from each type of inorganic pyrophosphatases, it is hard to generalize the post-transcriptional regulation concept to all isoforms. If true for all, the post-transcriptional regulation of soluble and vacuolar pyrophosphatases can indicate the importance of the regulation of these enzymes for the fate of plant metabolism during development.

4.7 Protein Kinase C Phosphorylates Bsp1 *in vitro*; Hints on Post-translational Regulation of Plant Soluble Pyrophosphatases

Self-incompatibility is a mechanism that regulates the acceptance and rejection of pollen that lands on the stigma in order to prevent fertilization by unwanted pollen (Rudd et al., 1996; Rudd and Franklin-Tong, 2003). The response is characterized by rapid elevation of cytoplasmic Ca^{2+} concentration and subsequent changes in phosphorylation pattern of pollen proteins whose apparent outcome is the inhibition of pollen tube growth (Rudd and Franklin-Tong, 1999). During the self-incompatibility response studies of *Papaver rhoeas*, a 26 kDa protein was reported to be heavily phosphorylated (Rudd et al., 1996), which was later characterized as a soluble inorganic pyrophosphatase (Rudd and Franklin-Tong, 2003). By SDS-polyacrylamide electrophoresis, it was shown to be localized both in the cytoplasm and the microsomal fractions of pollen extracts (Rudd et al., 1996). The phosphorylation was reported to be Ca^{2+} dependent and probably performed by a calcium-dependent protein kinase (Rudd and Franklin-Tong, 2003). The phosphorylation of an inorganic pyrophosphatase during self-incompatibility response was hypothesized to cause a decrease in its pyrophosphatase activity, therefore by increasing the cytoplasmic PP_i level results in a depletion of biopolymers, such as long chain carbohydrates and proteins that contribute to pollen tube membranes and cell walls, and a consequent inhibition of tip growth (Franklin-Tong and Franklin, 2003; Rudd and Franklin-Tong, 2003).

The amino acid sequence of soluble pyrophosphatase from *P. rhoeas* has not been published yet, however, the analysis of Bsp1 (*B. vulgaris* cytoplasmic soluble pyrophosphatase isoform 1; Schirmer, 2004) amino acid sequence for protein kinase recognition sequences revealed two protein kinase C (PKC) phosphorylation sites; one relatively well conserved among plant soluble pyrophosphatases, whereas the other is specific to Bsp1 (data not shown). Protein kinase C is a Ca^{2+} dependent-protein kinase, i.e., requires Ca^{2+} for phosphorylation activity, on the other hand, inorganic pyrophosphatases are known to be inhibited by Ca^{2+}. Therefore, for activity measurements after phosphorylation, the enzyme was treated with EGTA which was previously shown to reverse the Ca^{2+} inhibition of soluble pyrophosphatases (Maeshima, 1991). Figure 3.27 proves the *in vitro* phosphorylation of recombinant Bsp1 protein by PKC. The measurements performed before and after, on the other hand, revealed no changes in the enzymatic activity upon phosphorylation. Thus, the effect of phosphorylation, in relevant *in vivo*, cannot be enzymatic inhibition, but rather an effect on other regulatory elements like protein-protein interactions. The hypothesis explained above (Franklin-Tong and Franklin, 2003; Rudd and Franklin-

Tong, 2003), therefore, cannot be valid. Unfortunately, it is not possible to assume other possible outcomes of phosphorylation of soluble pyrophosphatases, since there is no information available on possible interacting proteins.

It is noteworthy to mention that the complete phosphorylation of Bsp1 could not been achieved by *in vitro* phosphorylation by PKC, which might indicate that PKC is not responsible from phosphorylation of inorganic pyrophosphatases *in vivo*. Additionally, it is not known if the soluble pyrophosphatase from *P. rhoeas* is exclusive to pollen. If so, phosphorylation might be specific to self-incompatibility response. However, a manganese-dependent inorganic pyrophosphatase (Type II soluble pyrophosphatase) from gram-positive *Streptococcus agalactiae* was shown to be reversibly phosphorylated and was reported to seriously effect the growth, cell segregation and virulence (Rajagopal et al., 2003). This implies that, phosphorylation can be a general post-translational regulation mechanism for soluble pyrophosphatases.

4.8 Neither Bsp1 nor Bvp1 Overexpression Has an Effect on Growth, Development, Sucrose and Hexose Concentrations of Sugar Beet

Engineering crops for better qualities and increased stress tolerance is a major goal of transgenic approaches. The overexpression of endogenous Bvp1 and Bsp1 in *Beta vulgaris* aims to increase sucrose accumulation in the taproot tissue during development and to decrease sucrose loss upon wounding.

In sugar beet, sucrose is stored in the vacuoles and transport is associated with proton efflux (Grof and Campell, 2001). Therefore, overexpression of a vacuolar pyrophosphatase may increase the proton motive force across the vacuolar membrane and thus result in increased accumulation of sucrose in the taproot tissue. The increased activity of soluble pyrophosphatases, on the other hand, may impair the hydrolysis of sucrose upon wounding and therefore might decrease the sucrose loss.

As explained in detail in Chapter 4.6, the CaMV 35S promoter driven overexpression of Bsp1 and Bvp1 in sugar beet are probably subjected to post-transcriptional regulation, therefore no direct change in the protein amount was detected in fully developed sugar beet plants. However, since the transcripts were detectable in the seedling stage, it was considered that the over-

expression might result in an effect on sucrose loading during growth and sucrose loss after wounding.

Figure 3.32 shows the sucrose concentrations of both Bsp1 and Bvp1 overexpressing sugar beet lines in comparison to wild type plants measured as µmoles per gram fresh weight. It implies that there is no statistically significant change in the sucrose loading on taproot tissues of transgenic sugar beet plants.

Wounding causes the hydrolysis of stored sucrose in the taproot tissue and causes an increase in cytosolic glucose and fructose concentrations (Geigenberger et al., 1998). The comparison of hexose concentrations of transgenic lines with wild type shows that, as in the case of sucrose accumulation, there is no significant effect of overexpression.

The results are not surprising considering the presumed post-transcriptional and post-translational regulation mechanisms of both soluble and vacuolar pyrophosphatases revealed in the course of this study (Chapter 4.6 and 4.7). It has frequently been observed that overexpression of genes encoding proteins that are subjected to sophisticated regulation have little or no effect on metabolism and growth (Stitt, 1998).

4.9 Heterologous Overexpression of Bsp1 and Bvp1 in *A. thaliana* Affects Root Growth and Increases Salt Tolerance

The homologous overexpression of AVP1 (*A. thaliana* vacuolar pyrophosphatase) in Arabidopsis was reported to cause altered root and shoot development similar to plants with morphogenetic variations typical of auxin defects (Li et al., 2005). The authors indicated that the overexpressing plants were characterized by extensive root system and increased shoot growth, and reported that the phenotype was caused by changes in uptake and distribution of auxin due to increased vacuolar acidification and decreased apoplastic pH (Li et al., 2005). Based on the results obtained from overexpression of AVP1 in tomato plants which also resulted in extensive root development (Park et al., 2005), it was concluded that AVP1 functions in both shoot and root development (Li et al., 2005; Park et al.,2005).

The selection of *A. thaliana* plants with CaMV 35S promoter driven Bsp1 or Bvp1 expression was performed by Northern and Western blot analyses (Schirmer, 2004). Interestingly, the Bvp1 overexpressing lines revealed reduced root growth (Figure 3.34). Although not emphasized in detail, Gaxiola et al. (2001) indicated several phenotypic differences observed upon ho-

mologous overexpression of AVP1 in their first publication. These phenotypic changes might depend on the number of insertions into the genome. Elevated vacuolar pyrophosphatase activity might cause a very high vacuolar acidification, an intolerable decrease in apoplastic pH or simply the high transcript amount of CaMV 35S driven Bvp1 might cause post-transcriptional silencing of both endogenous (AVP1) and the transgene. A similar phenotype was observed in the loss-of-function mutants of AVP1 (Li et al., 2005), supporting the possibility of post-transcriptional silencing. Another likely explanation of the phenotype might be the extreme decrease in the cytoplasmic PP_i concentration, which was reported to impair plant development (Jelitto et al., 1992; Sonnewald, 1992). It is noteworthy to mention that the promoter analyses of *A. thaliana* sPPases indicated different expression levels of each isoform in root tips (Chapter 4.3.1) suggesting a highly specialized function of soluble pyrophosphatases in root development and/or branching. Therefore, altering the total soluble pyrophosphatase activity is likely to cause changes in root growth.

Although these hypotheses require further proofs, it is noteworthy that the impairment of root growth was also observed in CaMV 35S promoter driven Bsp1 overexpressing *A. thaliana* transgenic lines. The similarity to the Bvp1 overexpressing lines indicates that the cause of the phenotype cannot be related with changes in vacuolar acidification or apoplastic pH, since soluble pyrophosphatases do not have a proton pumping activity. However, the increased activity of soluble pyrophosphatases might cause a decreased vacuolar pyrophosphatase activity due to decrease in cytoplasmic PP_i concentration, thereby may result in the similar phenotype. It is noteworthy to mention that not all CaMV 35S promoter driven Bsp1 expressing *A. thaliana* lines showed the same phenotype. Some of the lines were able to grow a root system, although comparatively less than wild type (Figure 3.34).

Another interesting feature of transgenic *A. thaliana* plants overexpressing either Bvp1 or Bsp1 was the salt tolerance (Figure 3.35). Not only Bvp1 overexpressing lines but also Bsp1 overexpressing lines showed an enhanced salt tolerance. However, there was a direct correlation between root growth and salinity tolerance. The lines that have almost no root formation were susceptible to salt treatment and did not survive even at 125 mM NaCl. On the other hand, the transgenic lines with a root system, although less than wild type, were germinated at 150 mM NaCl containing growth medium.

Although the data obtained are not comprehensive and require further experimentation, it appears that the overexpression of not only vacuolar but also soluble pyrophosphatases can influence salt tolerance of plants. Therefore, it is possible that the enhanced salt tolerance is not a direct effect of increased activity of vacuolar pyrophosphatases but a rather indirect effect, which

was initiated with changes in cytoplasmic PP_i concentration. This may imply the importance of cytoplasmic PP_i concentration and that changes in the PP_i might have a regulatory effect on plant growth and development. Thus, since the activity of cytoplasmic soluble pyrophosphatases has a direct influence on cytoplasmic PP_i concentration, they might act as regulatory enzymes for plant metabolism.

4.10 The Activity of Each ASP Isoform Appears to be Required for Normal Growth and Development

In order to understand the importance of soluble pyrophosphatase activity for plant growth and development, two approaches were used; analysis of SALK insertion mutants (Chapter 7.5) and an RNAi-mediated knockdown of ASP1 and ASP2A together (Chapter 7.6). The results are preliminary and not comprehensive; however, they will be discussed in the concept of *in vivo* functional specificity of plant soluble pyrophosphatases.

The growth of offspring seeds in soil revealed the presence of similar phenotypic differences in each ASP insertion mutant (except ASP5); some of the plants were characterized by extremely diminished growth rate, whereas some of them were growing comparable to wild type and the rest were showing an intermediate phenotype (Figure 7.3). Initially, the plants showing diminished growth rate were considered as homozygous, however, a detailed analysis revealed that there was no correlation between insertion type and growth rate. Although reasons unknown, it appears that the functional knockout of each ASP isoform has an effect on plant growth and development. This may imply that each soluble pyrophosphatase isoform has a specific role in the plant metabolism and the change in the activity of one of the enzymes cannot be comprehended with another one, although all of them are localized in the cytoplasm.

Another indirect proof for functional specificity of ASP isoforms was obtained through RNAi-mediated knockdown of ASP1 and ASP2A (Chapter 7.6). Although several independent transformations were performed, only two viable lines were obtained with knockdown of both ASP1 and ASP2A (Figure 7.4). The Western blot analysis revealed opposing effects on the total soluble pyrophosphatase protein amount in the transgenic lines; R1 had effectively less sPPase protein, on the other hand, R2 had more protein compared to wild type (Figure 7.5). Since the BASTA™ selection of offspring of R1 in soil was failed, the seeds were germinated on non-selective plates either horizontally or vertically. The comparison with wild type revealed that

transgenic line R1 with knockdown of both ASP1 and ASP2A has an impaired root and shoot growth. Unfortunately, no further analysis of plants could be performed since they were failed to grow more than seedling stage.

As indicated before, the results are very preliminary and not comprehensive; however, they indicate the importance of soluble pyrophosphatase activity during plant growth and germination. The accumulation of PP_i in cytoplasm can be toxic and the absence of sPPase activity can be detrimental to plants. On the other hand, the phenotypic differences obtained through SALK insertion mutants and RNAi-mediated knockdown of ASP1 and ASP2A suggest the specificity of the function of each ASP isoform *in vivo* and the loss of the activity of one of the isoforms impairs with plant growth and development, while loss of the activity of two isoforms together is highly detrimental to plant growth.

4.11 Conclusions and Future Perspectives

Inorganic pyrophosphate is the by-product of numerous metabolic pathways for the synthesis of polymers like DNA, RNA, proteins and polysaccharides (Chen et al., 1990). Consequently, the rate of PP_i production in a growing cell is particularly high and has to be counter balanced by efficient catabolic pathways to provide a thermodynamic pull for biosynthetic reactions (Chen et al., 1990; Salminen et al., 1995). This is performed by ubiquitous inorganic pyrophosphatase enzymes (Chen et al., 1990; Salminen et al., 1995; Islam et al., 2004). There are two general types of inorganic pyrophosphatases; soluble pyrophosphatases that are shown to be ubiquitous in bacteria, yeasts and animal; and membrane bound inorganic pyrophosphatases that were reported to be functional in photosynthetic organisms like plants, protests and certain prokaryotes (Jelitto et al., 1992; du Jardin et al., 1995; Perez-Castineria et al., 2001b).

Although plant vacuolar pyrophosphatases were relatively well studied, there is almost no data available on plant soluble pyrophosphatases in the literature. The finding that plant cytosol contains a high PP_i concentration and low sPPase activity and the presence of a proton-pumping vacuolar membrane bound inorganic pyrophosphatase led to a hypothesis that plant cytosol lacks sPPase activity and vacuolar membrane bound proton-pumping pyrophosphatase is the sole enzyme for the removal of excess cytosolic PP_i (Weiner et al., 1987; Stitt, 1998; Farre et al., 2000).

Even though relatively well accepted, this hypothesis has many drawbacks. Weiner et al. (1987) measured the cytoplasmic PP_i concentration of fully developed spinach leaves as 200-300 μM, whereas the data of Farre et al. (2001) proved that the cytosol of potato tuber cells contained

only 23 μM PP_i. That is, there is a certain lack of information on the *in vivo* cytoplasmic PP_i levels depending on the plant species, tissue and developmental stage.

The proof of the importance of high cytoplasmic PP_i concentration comes from the ectopic expression of a bacterial (*E. coli*) sPPase in the plant cytosol (Jelitto et al., 1992; Sonnewald, 1992). These studies merely prove the lack of sPPase activity in the plant cytosol by implying that if there would be a sPPase in plant cytosol, the cytoplasmic PP_i concentration would be lower and this would impair the plant growth and development (Jelitto et al., 1992; Sonnewald, 1992). However, there are certain differences in the enzymatic properties of sPPases in prokaryotic and eukaryotic organisms and the data on *E. coli* sPPase indicated the possible presence of a post-translational mechanism regulating the enzymatic activity to keep the cytoplasmic PP_i concentration at a certain level (Chen et al., 1990). That is, the higher decrease in the cytoplasmic PP_i concentration by ectopic expression of *E. coli* sPPase might be due to the lack of the regulatory mechanism of a bacterial enzyme in plants, and the impairment of the growth with decreased cytoplasmic PP_i concentration does not necessarily prove the lack of sPPase activity in plant cytosol.

On the contrary, the sequencing project revealed the presence of six sPPase isoforms in *A. thaliana* (The Arabidopsis genome genome initiative, 2000) only one of which was predicted to be localized in the plastids (Schulze et al., 2004). Through a functional genomics approach to these isoforms, this study aimed to understand the subcellular localization, regulation and environmental stress responses of plant soluble pyrophosphatases.

The subcellular localization studies of ASP isoforms indicated that five isoforms are localized in the cytoplasm and/or nucleus and the other one in the plastids. Since the importance of mitochondrial sPPases was shown to be essential for the function of this organelle for animal and fungal cells, the first interesting finding was the lack of mitochondrial localization for *A. thaliana* sPPases. Although, the subcellular localization of ASP isoforms should be confirmed with stable transformation, it may imply that plant mitochondria lack a sPPase activity and accumulated PP_i is translocated to the cytosol for removal by cytoplasmic sPPases.

The promoter activity analyses and real time PCR data indicated that *A. thaliana* soluble pyrophosphatase isoforms are transcribed not only in young and growing but also in fully developed tissues implying the importance of the function of plant soluble pyrophosphatases throughout development.

The preliminary data indicated that the plant soluble pyrophosphatases are regulated at several levels: transcriptionally (induction and repression in response to several environmental stresses), post-transcriptionally (degradation of mRNA in overexpressing transgenic plants) and

post-translationally (possibly by phosphorylation). These might indicate the fine regulation of cytoplasmic PP_i concentration by changing the sPPase activity; similar to the observations in animal systems (Hessle et al., 2002; Towler, 2005; Zhang et al., 2005). The effect of changes in cytoplasmic PP_i concentration on PP_i-dependent biosynthetic reactions is not known, however, the data on induction of transcription of ASP3 in relation to possible increasing UGPase activity indicates the potential regulatory role of sPPases.

The environmental stress responses of ASPases indicated the changes in the expression are strictly dependent on the isoform and the stress treatment. The *in vivo* functions of sPPases have not been proven yet, therefore the possibility of another substrate cannot be ruled out; however, the isoform dependent responses may imply the specificity of functions of plant soluble pyrophosphatases. Even though the induction in expression does not necessarily correlates with increased protein amount or enzymatic activity, the finding that ASP2B is only induced in starvation related responses strongly supports this assumption.

The importance of inorganic pyrophosphatases is relatively well studied in other organisms and received a lot of attention as a potential drug targets. For example, the presence of a vacuolar membrane bound pyrophosphatase specific to malaria parasites was considered as a potential target for the development of new antimalarial and/or broad-spectrum anti-protozoan agents (Drozdowicz et al., 1999; McIntosh et al., 2001). Although, the membrane bound inorganic pyrophosphatases were thought to be absent in animal systems, there is a relatively new study showing the importance of the activity of a vacuolar pyrophosphatase in the acidification of yolk granules of *Rhadnius prolixus*, indicating the possible presence of a H^+-PPase also in animals (Motta et al., 2004).

There are a number of studies demonstrating that the function of soluble pyrophosphatase is essential for the growth of *E. coli* and *Saccharomyces cerevisiae* (bakery yeast) (Chen et al., 1990; Salminen et al., 1995; Kankare et al., 1996; Gomez-Garcia et al., 2004). This effect was rather expected since the accumulating PP_i can impair with the cell growth (Chen et al., 1990; Salminen et al., 1995; Kankare et al., 1996; Gomez-Garcia et al., 2004). Interestingly, Chen et al. (1990) reported that *E. coli* cells maintain a constant PP_i concentration whatever the level of soluble pyrophosphatase activity, suggesting that the enzyme can be subjected to controls linking its activity to the surrounding PP_i concentration.

The function of soluble pyrophosphatase was demonstrated to be essential for the development and molting process of *Ascaris suum* (a swine roundworm) (Islam et al., 2002; Islam et al., 2005). The requirement of a sPPase in osmoregulation and virulence in mice was proven for *Trypanosoma brucei* (Lemercier et al., 2004). In addition, an inorganic soluble pyrophosphatase

responsible for the degradation of both pyrophosphate and polyphosphate in *Leishmania major* was shown to play an essential role for its virulence (Gomez-Garcia et al., 2004; Espiau et al., 2006). Thus, sPPase was also considered as a potent immunogenic protein, which can induce a distinct mechanism for host protection and may become a potential target for the development of a vaccine and/or chemotherapeutic agent (Islam et al., 2005; Espiau et al., 2006).

The importance of PP_i and sPPase activity in animal bone formation was proven by several studies (Hessle et al., 2002; Towler, 2005; Zhang et al., 2005). The extracellular PP_i concentrations was shown to be finely tuned by soluble pyrophosphatase activity to maintain steady-state levels of PP_i adequate for controlled mineralization (Hessle et al., 2002; Towler, 2005; Zhang et al., 2005). PP_i in fact was reported to be an active signaling molecule important in the paracrine regulation of apatitic calcium deposition (Towler, 2005). As such, PP_i emerges as a novel vascular paracrine factor, of great potential significance to the calcium vasculopathys of diabetes, dyslipidemia and end-stage renal disease (Towler, 2005).

In conclusion, what is known from the other organisms and the data collected by this preliminary study strongly imply that plants possess cytoplasmic soluble pyrophosphatase activity, which can be essential throughout plant development, and the changes in cytoplasmic PP_i concentration carried out by sPPase activity may act as a signaling molecule for the regulation of several PP_i-dependent biosynthetic reactions.

5 MATERIALS AND METHODS

5.1 Materials

5.1.1 *Arabidopsis thaliana*

Arabidopsis thaliana ecotype Columbia was used in all studies. The plants were grown either under greenhouse conditions or in climate chambers [14 hours light (24°C), 8 hours dark (18°C) with light intensity of 125 µmol $m^{-2}s^{-1}$ and relative humidity of about 50 %].

5.1.1.1 *Arabidopsis thaliana* Plate Culture

The growth plates were prepared from ½ MS medium with or without 20 g/l sucrose (pH 5,8) and solidified with 1,5 % plant agar. *A. thaliana* seeds incubated at -20°C overnight were surface sterilized in 3,75 % NaOCl, 0,01 % Triton X-100 (3 x 5 min) and washed in sterile water (3 x 5 min) before suspending in 0,1 % plant agar solution. After inoculation, the plates are incubated at 4°C overnight and transferred to climate chambers.

5.1.1.2 *Arabidopsis thaliana* Cell Culture

Vitamin solution: 5 g inositol, 25 mg nicotinic acid, 25 mg pyrodixin-HCl, 25 mg thiamin-HCl in 5 ml.
Cell suspension medium: 4,3 g/l MS (basal salt mixture), 20 g/l sucrose or glucose, 1 mg/l 2,4 D (from 1 g/l stock solution in ethanol), 1 ml/l vitamin solution, pH 5,7.

The heterotrophic *A. thaliana* cell cultures were grown in a 100 ml cell suspension medium at dark (25°C) with 90 rpm shaking. The cell cultures were renewed in every 7 days by inoculating approximately 5 gram of cells into fresh cell suspension medium.

5.1.1.3 *Arabidopsis thaliana* in Hydroponics

Hydroponics solution: 25 µM H_3BO_3, 0,06 µM $CuSO_4$, 0,14 µM $MnSO_4$, 0,03 µM Na_2MoO_4, 0,001 µM $CoCl_2$, 0,1 µM $ZnSO_4$, 20 µM Fe-EDTA, 2 mM KNO_3, 1 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4 and 1 mM $MgSO_4$.

The growth of *A. thaliana* in hydroponics was performed according to Noren et al. (2004). The *A. thaliana* seeds were surface sterilized and inoculated onto 1 ml pipette tips filled with growth medium (hydroponics solution solidified with 1 % plant agar). The pipette boxes (mini-greenhouses) were sealed with Microtape. They were transferred to climate chambers 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 about 6 l of hydroponics solution. The plants were grown under these conditions without flowering up to six weeks with renewing solution in every 7 days.

5.1.2 *Beta vulgaris* L.

The diploid inbred (Partie-Nr. VV-I/ZR 10738, KWS Ag) *Beta vulgaris* L. plants were grown in 20 cm high pots under greenhouse conditions.

5.1.3 *Escherichia coli*

For cloning procedures *E. coli* strain XL-1 Blue (Genotype; *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F' *proAB* lac^q *ZΔM15* Tn*10*(Tet^r)]; Stratagene) was used.

For the expression of recombinant proteins, *E. coli* strain Origami (Genotype; *Δara-leu7697 ΔlacX74 ΔphoAPvu* II *phoR araD139 aphC gale galK rpsL* F'[lac^+(lac^q)pro] *gor522*::*Tn10 trxB*::kan pRARE; Novagen) was used. The strain carries an additional plasmid (pRARE, chloramphenicol resistance), coding for six seldom tRNAs to support the expression of eukaryotic genes. Origami strain has loss-of-function mutations in the glutathione reductase and thioredoxin reductase genes, therefore creates a reducing cytosol enabling disulfide bridge formation in recombinant proteins.

5.1.3.1 Bacterial Medium and Antibiotics

LB medium: 1 % tryptone, 0,5 % yeast extract, 0,5 % NaCl, pH 7 (NaOH). When required, the media was solidified with 1 % agar.

The selection of bacteria was performed by adding the appropriate antibiotics in following concentrations; Ampicillin 100 µg/ml, Kanamycin 50 µg/ml, Chloramphenicol 34 µg/ml, Spectinomycin 100 µg/ml.

5.1.3.2 Preparation of Electrocompotent *E. coli*

One liter of LB containing appropriate antibiotics was inoculated with 20 ml of overnight *E. coli* culture and incubated at 37°C with 180-200 rpm shaking until OD_{600} reached 0.7. The culture was then cooled down to 4°C and cells were collected by centrifugation at 2500 *g* for 15 min at 4°C. The pellet was washed twice with 500 ml cold sterile water. The cells were suspended in 20 ml 10 % glycerol and centrifuged at 2500 *g* for 15 min at 4°C. The bacteria were then suspended in 2 ml 10 % glycerol, frozen in liquid nitrogen as 50 µl aliquots and stored at -80°C.

5.1.3.3 Preparation of Glycerol Stocks

After overnight growth in LB medium supplemented with antibiotics, 800 µl of cell suspension was mixed with 200 µl of sterile glycerol and directly frozen in liquid nitrogen. The glycerol stocks were stored at -80°C.

5.1.4 *Agrobacterium tumefaciens*

For stable transformation of *A. thaliana* and transient transformation of *Nicotiana tobacum*, the binary vectors were transformed to *Agrobacterium tumefaciens* strain C58C1 containing Ti-plasmid pGV2260.

5.1.4.1 Medium and Antibiotics

YEB medium: 1 g/l yeast extract, 5 g/l beef extract, 5 g/l peptone, 5 g/l sucrose, 0,493 g/l $MgSO_4$ x $7H_2O$, pH 7,5. When required, the media was solidified with 1 % agar.

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 with 200 rpm shaking (for liquid cultures) for two days.

5.1.4.2 Preparation of Electrocompotent *A. tumefaciens*

A. tumefaciens was incubated in 3 ml YEB medium supplemented with appropriate antibiotics and grown overnight at 28°C with 200 rpm shaking. They were then used to inoculate a 200 ml YEB medium and incubated under the same conditions until OD_{560} reached 0,95. The cells were pelleted at 2000 *g* for 5 min at 4°C and washed with 10 % glycerol, 1 mM Hepes (pH 7) twice. Afterwards the cells were suspended in 2 ml of the same solution, frozen in liquid nitrogen as 50 µl aliquots and stored at -80°C.

5.1.4.3 Preparation of Glycerol Stocks

After overnight growth in YEB medium supplemented with antibiotics, 800 µl of cell suspension was mixed with 200 µl of sterile glycerol and directly frozen in liquid nitrogen. The glycerol stocks were stored at -80°C.

5.2 Methods

5.2.1 Nucleic Acid Methods

5.2.1.1 Separation of DNA by Electrophoresis

5.2.1.1.1 Agarose Gels for DNA

50 x TAE: 242 g/l Tris-base, 57,1 ml/l glacial acetic acid, 100 ml/l 0,5 M EDTA, pH 8.
5 x Loading buffer: 50 % glycerol, 5 x TAE, 1 % Orange-G (w/v).

For the separation of high molecular weight DNA, 1 % agarose gels in 1 x TAE was used. The samples were prepared by mixing with a suitable volume of 5 x loading buffer. As molecular weight marker SmartLadder (Eurogentec) was used. After completing the run, the gels were stained in water containing 0,1 µg/ml ethidium bromide for 20 min at room temperature with slight shaking.

5.2.1.1.2 Agarose Gels for RNA

TBE: 0,98 M Tris base, 0,89 M boric acid, 25 mM EDTA, pH 8,4.
RNA sample buffer: 50 % glycerol, 5 x MOPS, 1 % bromophenolblue.

For the confirmation of quality after purification, 1 µg of RNA sample was mixed with appropriate volume of loading buffer and run with 1 % agarose gels prepared with TBE. The gels were stained with water containing 0,1 µg/ml ethidium bromide and RNA samples were visualized under UV light.

5.2.1.1.3 Polyacrylamide Gels

Native electrophoresis buffer: 3,6 g/l Tris, 14,4 g/l glycine, pH 8,6.
5 x Loading buffer: 50 % glycerol, 5 x TAE, 1 % Orange-G (w/v).

For the separation of small molecular weight DNA fragments 11,25 % polyacrylamide gels were used. Gels were prepared by mixing 3 ml distilled water, 2 ml native gel buffer (1,5 M Tris, pH 8,8), 3 ml acrylamide [29,2 % (w/v) acrylamide, 0,8 % N,N'-methylene bisacrylamide (37,5:1)], 45 µl 10 % ammonium peroxodisulfate and 15 µl TEMED. The samples were prepared by mixing a suitable volume of 5 x loading buffer. As molecular weight marker SmartLadder (Eurogentec) was used. The gels were stained in water containing 0,1 µg/ml ethidium bromide for 10 min at room temperature with slight shaking.

5.2.1.2 Long Method for Genomic DNA Isolation from Plants

Extraction buffer: 200 mM Tris-HCl, pH 9, 400 mM LiCl, 25 mM EDTA, 1 % SDS.
PCI: Phenol:chloroform:isoamyl alcohol (25:24:1; v/v/v).
TNE: 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.
TlowE buffer: 10 mM Tris, 0,1 mM EDTA, pH 8.

100 mg of grinded material was suspended in 500 µl extraction buffer. 550 µl PCI was added and vortexed for 20 s. After centrifugation at 15000 *g* for 5 min at 4°C, the supernatant was transferred to a new tube, mixed with 550 µl PCI and centrifuged again. The supernatant was taken to a new tube and genomic DNA was precipitated by the addition of the same volume of isopropanol and incubating for 30 min at room temperature. DNA was collected by centrifugation at 15000 *g* for 10 min at 4°C. The pellet was air dried and resuspended in 500 µl TNE. 20 µg of RNaseA (from 10 mg/ml stock) was added and total RNA were digested at 37°C for 10 min. RNaseA was removed by adding 550 µl PCI and centrifugation at 15000 *g* for 10 min at 4°C. The supernatant (about 475 µl) was transferred to a new tube and precipitated with 750 µl isopropanol for 30 min at room temperature. Genomic DNA was collected by centrifugation at 15000 *g* for 10 min at 4°C. The pellet was washed once with 70 % ethanol and air-dried. The genomic DNA was dissolved in 50 µl TlowE buffer and stored at 4°C.

5.2.1.3 Short Method for Isolation of Plant Genomic DNA

Extraction buffer: 200 mM Tris-HCl, pH 9, 400 mM LiCl, 25 mM EDTA, 1 % SDS.
TlowE: 10 mM Tris, 0,1 mM EDTA, pH 8.

100 mg of grinded material was mixed with 500 µl of extraction buffer. After centrifugation at 15000 *g* for 5 min, 350 µl of supernatant was transferred to a new tube and mixed with the same volume of isopropanol. Genomic DNA was precipitated at 15000 *g* for 15 min and washed with 70 % ethanol. After air-drying, the pellet was dissolved in 50 µl TlowE buffer and stored at 4°C.

5.2.1.4 Isolation of Total RNA for Northern Blotting

DEPC treatment: 0,1 % (v/v) DEPC was added to distilled water and stirred overnight. DEPC was deactivated by autoclaving twice. All of the solutions used for RNA isolation were prepared using DEPC-treated distilled water.
Extraction buffer: 8 M guanidine-HCl, 20 mM MES, 20 mM EDTA, pH 7. Before use 8 µl/ml β-mercaptoethanol was added to the extraction buffer.
PCI: Phenol:chloroform:isoamyl alcohol (25:24:1; v/v/v).

For Northern blotting, total RNA were isolated using a modified protocol of Logemann et al. (1987). Plant material (500 mg for leaf samples, up to 2 g for root samples) was grinded. To each 500 mg of material 1 ml of extraction buffer was added and vortexed. 1 ml PCI was mixed with the thawed sample and vortexed for 20 s. After centrifugation at 15000 *g* for 10 min at room temperature, the supernatant was transferred to a new tube and precipitated with the addition of 0,2 x vol of 1 M acetic acid and 0,7 x vol of cold ethanol overnight at -20°C. Total RNA was

collected with 30 min centrifugation at 15000 *g* at 4°C and the pellet was washed with 250 µl per 500 mg material cold 3 M sodium acetate (pH 5,2). The RNA pellet was collected by centrifugation at 15000 *g* for 10 min at 4°C and washed with 70 % ethanol. After drying the sample at 37°C, total RNA was dissolved in 50 µl formamide. In order to enhance dissolving, the sample was incubated at 65°C for 10 to 30 min and overnight at 4°C. The insoluble material was removed by centrifugation (10 min at 15000 *g* at 4°C) before use. RNA samples were stored at -80°C.

5.2.1.5 Isolation of Total RNA for cDNA Synthesis

For cDNA synthesis, total RNA was isolated using RNeasy Plant Kit (Qiagen) according to manufacturer's instructions.

5.2.1.6 Plasmid DNA Purification for DNA Sequencing

Plasmid purification for sequencing was performed using QIAprep Kit (QIAGEN) according to manufacturer's instructions. For high copy plasmids 1 to 2 ml and for low copy plasmids 50 to 100 ml of overnight bacterial culture was used.

5.2.1.7 Plasmid DNA Purification for Restriction Digestion and PCR

GTE buffer: 50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.
Lysis buffer: 200 mM NaOH, 1 % SDS.

For restriction digestion and PCR, plasmids were isolated with an alkaline lysis method. For this purpose, 1 to 5 ml of overnight bacterial culture was used. Bacteria was precipitated by centrifugation at 15000 *g* for 1 min at 4°C and resuspended in 300 µl GTE buffer. 300 µl lysis buffer was added to suspension and incubated at room temperature for 5 min. After addition of 300 µl 3 M potassium acetate (pH 4,8), the suspension was mixed by inverting several times and genomic DNA was collected by centrifugation at 15000 *g* for 15 min. The supernatant containing plasmids was transferred to a new tube and mixed with the same volume of isopropanol. Plasmid DNA was collected by centrifugation at 15000 *g* for 15 min at 4°C. The pellet was washed with 500 µl 70 % ethanol and after 5 min of centrifugation, dried at 37°C. Plasmid DNA was dissolved in distilled water and stored at -20°C.

5.2.1.8 Determination of Nucleic Acid Concentration through Spectrophotometry

Concentration of isolated nucleic acid samples was determined photometrically at 260 nm using appropriate dilutions. ε is 20 µl x mg^{-1} x cm^{-1} for double stranded DNA and 25 µl x mg^{-1} x cm^{-1} for RNA. The OD at 230 nm and 280 nm was used to determine the purity of the sample preparation. The ratio between 1,8 to 2,0 was accepted as pure sample preparation.

5.2.1.9 cDNA Synthesis

Reverse transcription of 2 µg of total RNA was performed according to the manufacturer's instructions of Omniscript Reverse Transcriptase kit (QIAGEN). In order to prevent degradation of RNA, RNaseOUT (Invitrogen) was added to the reaction mix. The cDNA was stored at -20°C.

5.2.1.10 Polymerase Chain Reaction Techniques

5.2.1.10.1 Oligonucleotides

All oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). The lyophilized oligonucleotides were dissolved in TE buffer to final concentration of 100 pmoles/µl and stored at -20°C.

For promoter amplification	**5' to 3'**
GPro_asp1_for	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGGATATTGATTGATGAGA
GPro_asp1_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCATCTGTCAAAATCAGAACG
GPro_asp2a_for	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTTGGTACATCACTGTCGT
GPro_asp2a_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCATATCGGATGTTCACTATAA
GPro_asp2b_newfo	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCAACATTTCTTCTGGTA
GPro_asp2b_newre	GGGGACCACTTTGTACAAGAAAGCTGGGTCATCTATTGTCACATCAATCG
GPro_asp3_for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGTCTATGTCTCTTTTTCC
GPro_asp3_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCATTGCTGCAAAACAATGC
GPro_asp4_for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACTCCATATGATAGCGAAA
GPro_asp4_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCATCTAATAAAACAGAGCAAAAC
GPro_asp5_for	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGCTTCACACCTCAAGAAAT
GPro_asp5_rev	GGGGACCACTTTGTACAAGAAAAGCTGGGTCATTATTGTGGAAAGATTGA

For construction of RNAi1/2A	**5' to 3'**
ASP1_3UTR_for	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCTTCTCCTCAGAAGATTTC
ASP1_3UTR_rev	GAAGGGATCCACTAGTGAACTCTTCAAACAAATTAAACC
ASP2A_3UTR_for	GTCGACTAGTGGATCCGAAGAAACCAGTCCTTTTCC
ASP2A_3UTR_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGATTGTACTGGAAATTTTTCATC

For cenventional RT of ASP1 and ASP2A	**5' to 3'**
RTRi1-L	TGG CAT GAT CTT GAG ATT GGA C
RTRi1-R	CAC ACA ATG TGC GAG GAA CAA
RTRi2A-L	GGA TGT CCT GGT ACT GAT GCA GG
RTRi2A-R	GGT GAG GGG GAA GCT CTT TG

For real time PCR	**5' to 3'**
Asp1-RT-L	ACA ATC GGC TGT TTC GTT TC
Asp1-RT-R	TTC CTT TAG TGA TCT CAA CAA CCA C
Asp2A-RT-L	GAT TCT CTG CTT CGG TTT CG
Asp2A-RT-R	CAG TAG GAG CTT CTG GAC CAA TC
Asp2B-RT-L	CAA ATG CTC TGT TTT CTT CTG C
Asp2B-RT-R	CCT TTG TGA TCT CAA CCA CCA C
Asp3-RT-L	TGA GAT CTG TGC TTG CGT TT
Asp3-RT-R	TGG GGC TTC AGG TCC TAT C
Asp4-RT-L	CTC CAC ACT TTC CGC AAG AT
Asp4-RT-R	ACT GGA GCT CCA GGT CCG
Asp5-RT-L	GAG ACA AAC CAG CAA ACA AAG AC
Asp5-RT-R	AAA CAA AAT CCA AAT CCC AAT G
phactin_L	GGT AAC ATT GTG CTC AGT GGT GG
phactin_R	CTC GGC CTT GGA GAT CCA CAT C

For subcellular localization	**5' to 3'**
First step Gateway PCR	
ASP1-5UTR	ACA ATC GGC TGT TTC GTT TC
ASP1-3UTR	CAG AAG GAC CAA ATG ATA AGG A
ASP2A-5UTR	GAT TCT CTG CTT CGG TTT CG
ASP2A-3UTR	TAG AAG GCG GCT TCA GTG AT
ASP2B-5UTR	TCT TTC CAA ATG CTC TGT TTT C
ASP2B-3UTR	GGA TGC TGC GAA GAG AAT CA
ASP3-5UTR	TGA GAT CTG TGC TTG CGT TT
ASP3-3UTR	TCA TTC CAT TGC TTG AGG TG
ASP4-5UTR	CTC CAC ACT TTC CGC AAG AT
ASP4-3UTR	ACC CAA AAA TTG GCA GGA AT
ASP5-5UTR	CCA GTC GAG GGT AAA ACG AA
ASP5-3UTR	CTC ATA AGT CGA CAT TGC AAA A
Second step Gateway PCR	
ASP1-Prof	AAA AAA GCA GGC TTC ATG AGT GAA GAA ACT AAA GAT AAC C
ASP1-Pror	AGA AAG CTG GGT CAC GCC TCA GGG TGT GGA G
ASP2A-Prof	AAA AAA GCA GGC TTC ATG GCT GAA ATC AAG GAT GAA G
ASP2A-Pror	AGA AAG CTG GGT CGC GTT GCA GGC CAG CTT TG
ASP2B-Prof	AAA AAA GCA GGC TTC ATG AGT GAA GAA GCA TAT GAA G
ASP2B-Pror	AGA AAG CTG GGT CTC TCC TCA ACG TGT GGA GAA TAT AC
ASP3-Prof	AAA AAA GCA GGC TTC ATG GCG CCA CCG ATT GAG
ASP3-Pror	AGA AAG CTG GGT CAC GTC TTA GGT TCT CCA CGA CG
ASP4-Prof	AAA AAA GCA GGC TTC ATG AAT GGA GAA GAA GTG AAA AC
ASP4-Pror	AGA AAG CTG GGT CTC TCC TCA GGG TGT GAA GAA TG
ASP5-Prof	AAA AAA GCA GGC TTC ATG GCG GCT ACT AGA GTG
ASP5-Pror	AGA AAG CTG GGT CGT AAA GTG AAA GGT CTC CAG

For SALK insertion lines	**5' to 3'**
1Salk_LP	TTT GTT TCA AAC TTG AAG GCG
1Salk_RP	AAT CTC AAG ATC ATG CCA TGG
Asalk_LP	ATG ACG TTG TGG AGG ATT TTG
Asalk_RP	AAT ACT GTG CAG GAG CCT GTG
Salk_LBb1	GCG TGG ACC GCT TGC TGC AAC T

For biotinylated probe preparation	**5' to 3'**
BVP1(S)-L	ATG AAT GAT CGG CGC AAA ATC
BVP1(S)-R	TAG ATC CAA TCT GCA AAA TGA G
Bsp3'L	GGC ACT TTC AAT TAT TGT CAT TC
Bsp3'R	AGC CTC CTA AAC CAA ACA TGA

5.2.1.10.2 Standard PCR for Amplification from Plasmids or cDNA

For amplification through plasmids or cDNA samples, a 50 µl of PCR was prepared. The reaction was set up using 1 µl of template, 1 µl of dNTPs (10 mM each), 1 µl of each primer (100 pmole/µl), 5 µl 10 x PCR buffer, 3 µl of $MgCl_2$ (50 mM) and 0,1 µl of Taq polymerase and appropriate volume of distilled water.

For the amplification RoboCycler Gradient 40 (Stratagene) was used with the following PCR program: 95°C x 5 min, 35 x {94°C x 1 min, T_m x 45 s, 72°C x 1-2 min}, 72°C x 10 min. The annealing temperature (T_m) and elongation time were adjusted according to primer pairs used.

5.2.1.10.3 Amplification from Genomic DNA

For amplification through genomic DNA, a 50 µl of PCR was prepared. The reaction was set up using 1 µl of template (1:10 dilution), 1 µl of dNTPs (10 mM each), 1 µl of each primer (100 pmole/µl), 5 µl 10 x PCR buffer, 3 µl of $MgCl_2$ (50 mM), 0,25 µl of Taq polymerase and appropriate volume of distilled water.

For the amplification RoboCycler Gradient 40 (Stratagene) was used with the following PCR program: 95°C x 5 min, 35 x {95°C x 2 min, T_m x 1,5 min, 72°C x 1-2 min}, 72°C x 10 min. The annealing temperature and elongation time were adjusted according to primer pairs used.

5.2.1.10.4 Real Time PCR

The real time polymerase chain reactions were prepared in 25 µl volume containing 1 µl cDNA sample, 0,25 µl of each primer (50 µM), 1,25 µl $MgCl_2$ (50 mM), 2,5 µl 10 x PCR buffer, 0,5 µl dNTPs (10 mM each), 1 µl FITC (1:40 dilution from 1 mM stock in 1 x PCR buffer, Bio-Rad), 0,25 µl SYBR Green (1:40 dilution from 1:10 dilution in DMSO, Invitrogen), 0,1 µl Platinum Taq DNA polymerase (5 U/µl, Invitrogen) and appropriate volume of distilled water.

For quantitative expression analysis real time PCR was performed using iCycler (Biorad). The efficiency of amplification was optimized separately for each primer combination. The standard curve to determine amplification efficiency was prepared using 1:5, 1:10, 1:20 and 1:40 dilutions of cDNA sample.

The PCR cycle used was 95°C x 3 min, 45 x {95°C x 30 s, T_m x 30 s, 72°C x 22 s}. The annealing temperature was adjusted according to primer pairs used.

5.2.1.10.5 Amplification for Biotinylated Probes

Biotinylated probes for Northern blotting were generated through standard PCR from isolated plasmids with changing the common dNTPs with a dNTP mixture containing 0,25 mM Biotin-16-dUTP (Roche), 0,75 mM dTTP and 1 mM dATP, dGTP and dCTP. The biotinylation was confirmed by agarose gel electrophoresis by comparison to a non-biotinylated probe according to the size difference.

5.2.1.10.6 2-Step PCR for Generation of Gateway Compatible Overhangs

For the generation of Gateway compatible overhangs in amplicon, a two step PCR method was followed. In the first round of PCR, template specific primers containing a short stretch of Gateway compatible ends were used. The PCR was performed as a standard PCR but only for 10 cycles.

In the second step, 10 µl of first step PCR was used as a template with primers containing appropriate Gateway overhangs and specific region towards the first primer set. The PCR was performed as a standard PCR for 20 cycles at 52°C annealing temperature.

5.2.1.10.7 Direct Purification of PCR Amplicons

Purification of amplicons from PCR mixture was performed by PCR Purification Kit (QIAEN) according to manufacturer's instructions.

5.2.1.10.8 Gel Extraction of PCR Fragments

After agarose gel electrophoresis of PCR samples, the desired fragment was cut from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN).

5.2.1.11 Cloning Methods

5.2.1.11.1 Vectors

The following vectors were used for different purposes; pGEM-T (Promega) or TA Cloning Kit (Invitrogen) for subcloning of PCR fragments; pQE30 (Qiagen) for recombinant protein expression in *E. coli*; pDONR201 (Invitrogen) as Entry vector of Gateway system; pK7FWG2 (Destination vector of Gateway system, Invitrogen) as N-terminal GFP-protein construct for transient expression in tobacco; pK7WGF2 (Destination vector of Gateway system, Invitrogen) as C-terminal GFP-protein construct for transient expression in tobacco; pBGWFS7 (Destination vector of Gateway system, Invitrogen) for promoter driven GUS expression and pB7GWIWG2(I) (Destination vector of Gateway system, Invitrogen) for initiation of RNAi effect.

5.2.1.11.2 Restriction Digestion

All restriction enzymes were purchased from New England Biolabs (NEM) and used with supplied buffers. 5 to 20 µg of vectors were used for preparative digestion. For each µg of DNA, 3 units of restriction enzyme were added. The digestion was carried out in 10 µl volume for 2 hours at recommended temperature.

5.2.1.11.3 DNA Ligation

DNA fragment ligations were performed with T4-DNA Ligase (NEB) overnight at 4°C or in a PCR cycler with 99 rounds of alternating temperatures at 10°C and 30°C, 30 sec each. The ligase was heat denatured by incubating at 65°C for 20 min.

5.2.1.11.4 Gateway Recombination

To 100 ng of pDONR201 vector, 1 to 3 µl purified PCR product with Gateway compatible overhangs, 1 µl of 5 x BP clonase reaction buffer (Invitrogen) and 1 µl of BP clonase mix (Invitrogen) was added. The reaction was carried out at 25°C for 1 h, then stopped by adding 0,5 µl Proteinase K (2 µg/µl) and incubating at 37°C for 10 min.

The recombination to destination vectors was carried out by adding 1 µl of BP reaction mix, 1 µl 5 x LR clonase reaction buffer (Invitrogen) and 1 µl of LR clonase mix (Invitrogen) to 100 ng of destination vector and incubation at 25°C for 1 h. The reaction was terminated by adding 0,5 µl Proteinase K (2 µg/µl) and incubating at 37°C for 10 min.

The reactions were either directly electroporated to host cells or stored at -80°C.

5.2.1.11.5 Electroporation to Host Cells

SOC medium: 20 g/l tryptone, 0,5 g/l yeast extract, 0,5 g/l NaCl, 0,186 g/l KCl, 2,03 g/l $MgCl_2$, 3,96 g/l glucose monohydrate, pH 7.

The vectors were transformed to host cells (either *E. coli* or *A. tumefaciens*) by electroporation with GenePulserII (Bio-Rad) with following settings; 200 Ω, 1,8 kV, 25 µF. The cells were collected in 1 ml SOC medium and incubated at 37°C for 1 h (*E. coli*) or 28°C for 2 h (*A. tumefaciens*). Variable volumes were inoculated to selective plates and incubated at 37°C overnight in case of *E. coli* and 28°C for 2 days for *A. tumefaciens*.

5.2.1.12 Northern Blotting

RNA sample buffer: 50 % glycerol, 5 x MOPS, 1 % bromophenolblue.

RNA sample mix: 3,3 µl formaldehyde (37 %), 1 µl 20 x MOPS, 2 µl RNA sample buffer, 1 µl ethidium bromide (1 mg/ml).

Denaturing gel: 1,4 % agarose, 1 x MOPS, 5,5 % (v/v) formaldehyde (37%).

Electrophoresis buffer: 1 x MOPS, 1 % formaldehyde (37 %).

20 x MOPS: 0,4 M MOPS, 0,1 M sodium acetate, 20 mM EDTA, pH 7 (adjusted with 5 M NaOH).

10 x SSC: 1,5 M NaCl, 0,3 M sodium citrate, pH 7.

10 x PBS: 80 g/l NaCl, 2 g/l KCl, 14,4 g/l Na_2HPO_4, 2,4 g/l KH_2PO_4, pH 7,2.

Prehybridization solution: 30 % formamide, 1 % SDS, 1 M NaCl, 6 % polyethyleneglycole 6000, 250 µg/ml salmon sperm DNA.

Low stringency wash: 2 x SSC, 0,5 % SDS.

High stringency wash: 0,2 x SSC, 0,5 % SDS.

Blocking buffer: 1 x PBS, 0,5 % SDS, 0,2 % I-Block (Tropix).

Conjugate buffer: 1:20,000 Streptavidin-HRP conjugate (1 mg/ml) in blocking buffer.

Wash buffer: 1 x PBS, 0,5 % SDS.

The volume of 20 µg of RNA sample was adjusted to 12,7 µl with formamide and combined with 7,3 µl RNA sample mix. The samples were denatured at 65°C for 10 min and kept on ice until usage. They were loaded to denaturing agarose gel and run at 70 V.

When the run was completed, the gel photographed under UV light and washed with 10 x SSC buffer twice for 10 min each. The RNA was transferred to a nylon membrane (Duralon-UV, Stratagene) overnight by capillary blotting. The membrane was completely dried the following morning and total RNA was crosslinked to the membrane by UV Stratalinker (Stratagene).

The membrane was quickly washed in low stringency buffer and subsequently incubated in prehybridization solution at 42°C for 60 min. The hybridization solution was prepared by denaturing the biotinylated probes in 1 ml prehybridization solution at 95°C for 5 min and adding to the prehybridization solution. Hybridization was allowed to complete at 42°C for 16 h.

The membrane was then washed twice in low stringency buffer for 15 min each at room temperature. High stringency wash was performed at 55°C for 1 h and the membrane was transferred into blocking buffer. The blocking was performed twice for 5 min and then for 30 min at room temperature. The conjugation was done at room temperature for 40 min. The membrane was cleaned with wash buffer twice, 5 min each. Finally, the membrane was incubated with the chemiluminescent substrate (North2South Chemiluminescent Substrate for HRP, Pierce) for 10 min in a plastic bag. After exposure overnight at 4°C, the film was developed with a Optimax TR developing machine.

5.2.3 Protein Methods

5.2.3.1 Soluble Protein Isolation

Extraction buffer: 100 mM Hepes/KOH (pH 7,1), 250 mM sorbitol, 10 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF (freshly added from 0.2 M stock), 2 mM DTT (freshly added from 1 M stock).

200 mg tissue powder was mixed with 750 µl cold extraction buffer and vortexed for 20 seconds. After centrifugation at 15000 *g* for 20 min at 4°C, the supernatant was taken to a fresh tube and stored at -20°C.

5.2.3.2 Total Protein Isolation

Extraction buffer: 56 mM Na_2CO_3, 56 mM DTT, 2 % SDS, 12 % sucrose, 2 mM EDTA.

100 mg grinded tissue was mixed with 500 µl extraction buffer and incubated at 70°C for 15 min. After centrifugation at 15000 *g* for 10 min at 4 °C, the supernatant was taken to a fresh tube and either directly reduced or stored at -20°C.

5.2.3.3 Protein Concentration Determination with Bradford Method

The protein concentration was determined with Bradford method. The protein assay solution (Bio-Rad) was diluted 1:5 with distilled water. 1 to 10 µl of protein extract was added to 1 ml of diluted assay solution and incubated for 10 min at room temperature. The protein concentration was calculated with a standard curve prepared using known concentrations of Bovine Serum Albumin by measuring the absorbance at OD_{595}.

5.2.3.4 SDS-Polyacrylamide Gel Electrophoresis

4 x Stacking buffer: 0,5 M Tris-Cl, pH 6,8, 0,4% SDS.
4 x Resolving buffer: 1,5 M Tris-Cl, pH 8,8, 0,4% SDS.
Stacking gel (4,5 %): 3 ml distilled water, 1,5 ml stacking buffer, 0,75 ml acrylamide [29,2 % (w/v) acrylamide, 0,8 % N,N'-methylene bisacrylamide (37,5:1)], 60 µl 10 % ammonium peroxodisulfate and 8 µl TEMED.
Resolving gel (12 %): 2,45 ml distilled water, 1,75 ml resolving buffer, 2,8 ml acrylamide [29,2 % (w/v) acrylamide, 0,8 % N,N'-methylene bisacrylamide (37,5:1)], 30 µl 10 % ammonium peroxodisulfate and 7 µl TEMED.
1 x Electrophoresis buffer: 10 mM glycine, 2,5 mM Tris-base, 0,1 % SDS.

The protein isolations were run by SDS-polyacrylamide gel electrophoresis, where proteins were collected in a single line through running in stacking gel and further running through separating gel resulted in discrimination of different sizes. The samples were denatured at 95°C for 5 min after the addition of 4 x SDS-sample buffer (Roti-Load1, Roth) for reduction, or mixed with non-reducing sample buffer (Roti-Load2, Roth) for detection of disulfide bridges. The gels were run at 100 V until the bands pass to resolving gel and completed by running at 200 V. As protein marker LMW Calibration kit (Amersham) was used.

5.2.3.5 Coomasie Staining

Coomasie solution: 0,2 % Coomasie Brilliant Blue G-250 in 45 % (v/v) ethanol and 10 % (v/v) acetic acid.
Colloidal Coomasie solution: 0,2 % Coomasie Brilliant Blue G-250 in 20 % ethanol, 1,6 % phosphoric acid and 5 % ammonium sulfate.

For visualization, SDS-polyacrylamide gels were stained with Coomasie solution and then destained in 45 % ethanol, 10 % acetic acid solution. They were stored in 45 % methanol, 2,5 % (v/v) glycerol at 4°C.

For more sensitive detection, SDS-polyacrylamide gels were stained using colloidal Coomasie staining method. For that, gels were incubated in colloidal staining solution overnight and destained with water. They were stored in 0,1 % acetic acid.

5.2.3.6 Silver Staining

Fixing solution: 50 % ethanol, 10 % acetic acid, 0,05 % formaldehyde (37 %).
Staining solution: 0,2 % $AgNO_3$, 0,075 % formaldehyde (37 %).
Developing solution: 6 % Na_2CO_3, 0,0004 % $Na_2S_2O_3$ x $5H_2O$, 0,05 % formaldehyde (37 %).

For silver staining, the gels were fixed in fixing solution for 1 h at room temperature and washed twice with 50 % ethanol, 25 min each. The gel was then sensitized for 1 to 2 min in 0,02 % sodium thiosulfate ($Na_2S_2O_3$ x $5H_2O$), then twice rinsed with water. The gel was incubated in staining solution for 20 min at room temperature and rinsed three times with water. The incubation in developing solution was performed until the bands are visible and then the development was stopped by 12 % acetic acid.

5.2.3.7 Western Blotting

Transfer buffer: 48 mM Tris-base, 39 mM glycine, 20 % methanol, 0,0375 % SDS (w/v).
Blocking buffer: 5 % skim milk powder in 1 x TBST, 0,02 % NaN_3.
10 x TBST: 20 mM Tris-HCl, pH 7,5, 150 mM NaCl, 0,05 % Tween 20.
Primary antibody for sPPase detection: 1:10000 dilution of crude final bleeding in 1 % skim milk powder in 1 x TBST, 0,02 % NaN_3.
Primary antibody for vPPase detection: 1:1000 dilution of purified antibody in 1 % skim milk powder in 1 x TBST, 0,02 % NaN_3.
Secondary antibody: 1:20000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce) in 1 % skim milk powder in 1 x TBST, 0,02 % NaN_3.
Amino black solution: 0,1 % amino black, 45 % ethanol, 10 % acetic acid.

After the proteins were separated by SDS-polyacrylamide gel electrophoresis, the resolving gel was incubated in transfer buffer for 20 min at room temperature. During incubation, the membrane (Immobilon-P, Millipore) was prepared by first rinsing with methanol, then washing with water and incubating in transfer buffer for 5 min at room temperature. The transfer of proteins from gel to membrane was done by "semidry-blotting" method with TransBlot SD apparatus (BioRad) at 15 V, 3,5 mA/cm^2 for 45 min.

After blotting, the membrane was incubated in blocking buffer for 1 h at room temperature. The membrane was then transferred to primary antibody solution and kept overnight at 4°C. The following morning, the membrane was washed four times in 1 x TBST and then incubated in secondary antibody solution for 1 h at room temperature.

The membrane was then incubated with substrate solution (Super Signal DUra, Pierce) for 5 min in a nylon bag. After exposure (3 min for sPPase antibody or 1 h for vPPase antibody), the film was developed with an Optimax TR developing machine.

5.2.3.8 Overexpression and Ni-NTA Purification of Bsp1 in *E. coli*

Lysis buffer: 50 mM MOPS, 300 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole, pH 8,5 µl/ml lysozyme (100 mg/ml stock).
Wash buffer: 50 mM MOPS, 300 mM NaCl, 5 mM $MgCl_2$, 10 mM imidazole, pH 8, and different concentrations of imidazole (20 mM, 50 mM, 75 mM, 100 mM and 250 mM).

The *E. coli* Origami strain carrying pQE30 vector with full coding Bsp1 sequence insert was used for recombinant Bsp1 purification. A 5 ml LB supplemented with 10 µg/ml Ampicillin and 34 µg/ml Chloramphenicol was inoculated from glycerol stock and was grown overnight at 37°C at 190 rpm. A 200 ml LB supplemented with the same antibiotics was inoculated with overnight culture the following morning and when OD_{600} reached 0,6 induced with 1 mM IPTG and left for overnight growth under the same conditions.
The following morning, the cells were collected by centrifugation for 10 min at 4000 *g* at 4°C. The pellet was dissolved in 500 µl lysis buffer and after briefly vortexing, incubated on ice for 30 min. The insoluble material was removed by centrifugation at 15000 *g* for 10 min at 4°C and supernatant containing soluble proteins was mixed with appropriate volume (usually 250 µl) of Ni-NTA agarose matrix (Qiagen). The mixture was loaded to IMAC-column and incubated at 4°C for 1 h with slight shaking. The flow through was collected and the column was first washed with 2 ml wash buffer containing 25 mM imidazole. Following washes were performed by increasing volumes of imidazole (1 ml for 50 mM and 75 mM imidazole containing wash buffers, repeated twice; and 500 µl for 100 mM and 250 mM imidazole containing wash buffers, repeated four times). The elutions were stored at 4°C.

5.2.3.9 Magnesium-dependent Soluble Pyrophosphatase Activity Assay

Reaction buffer: 50 mM Tris pH 7, 1 mM $Na_2H_2P_2O_7$, 2,5 mM $MgCl_2$.
Stop buffer: 3,4 mM ammonium molybdate in 0,5 M sulfuric acid, 0,5 M SDS, 0,6 M ascorbic acid (6:2:1; v/v/v).

Enzyme activity was assayed at room temperature for 15 min. To 200 µl reaction buffer, 5-10 µl of elution (see 4.2.3.8) was added and mixed. After 15 min, the reaction was terminated with 750 µl stop buffer and OD_{820} was measured. The data were calculated as average of triplicates.

5.2.3.10 *In vitro* Phosphorylation of Recombinant Bsp1

Phosphorylation buffer: 20 mM Hepes, pH 7,4, 0,6 µg/µl phosphaditylserine, 10 mM $MgCl_2$, 1,7 mM $CaCl_2$, 0,15 mM ATP, 1 mM DTT, 5 µl Protein kinase C (Promega, out of 60 µl).

For *in vitro* phosphorylation of recombinant Bsp1 protein, 100 ng of protein was mixed with 60 µl of phosphorylation buffer and incubated at 30°C for 1 h. In order to recover Bsp1 activity (which is inhibited by Ca^{2+}), 3,5 µl 100 mM EGTA added to the mixture and after vortexing incubated at room temperature for 5 min. As a negative control, recombinant Bsp1 protein was incubated in phosphorylation buffer without protein kinase C.

5.2.4 Plant Transformation Methods

5.2.4.1 Stable Transformation of *Arabidopsis thaliana* with Floral Dip

5.2.4.1.1 Preparation of Agrobacterium tumefaciens for Floral Dip

YEB medium: 1 g/l yeast extract, 5 g/l beef extract, 5 g/l peptone, 5 g/l sucrose, 0,493 g/l $MgSO_4$ x $7H_2O$, pH 7,5.

The *A. tumefaciens* cells containing the vector were grown in 50 ml YEB medium supplemented with 100 µg/ml Rifampicin, 50 µg/ml Carbenicillin and 100 µg/ml Spectinomycin at 28°C with 200 rpm shaking for 24 hours. The following morning, the overnight culture was used to inoculate 800 ml YEB culture supplemented with the same antibiotics and *A. tumefaciens* were grown for another 24 hours.

5.2.4.1.2 Floral Dip Transformation

DIP medium: ½ MS, 5 % sucrose, pH 5,8, 10 µl/l BAP, 0,05 % Vac-In Stuff (Silwet L-77, Lehle Seeds).

The *A. tumefaciens* cells were collected by centrifugation at 4000 *g* for 10 min and resuspended in DIP-medium to have OD_{600} 0,9. The inflorescence of *A. thaliana* plants (approximately 7 plants per construct) were dipped in this solution twice and left inside a plastic box overnight (Clough and Bent, 1998). The transformation was repeated three times in one week periods.

5.2.4.1.3 Screening of Transformants

The seeds of floral dipped plants were collected and T1 generation was used for screening of transformants. The selection was based on the resistance on the vector used which was BASTA™ in most cases.

5.2.4.2 Transient Transformation of Tobacco Leaves

YEB medium: 1 g/l yeast extract, 5 g/l beef extract, 5 g/l peptone, 5 g/l sucrose, 0,493 g/l $MgSO_4$ x $7H_2O$, pH 7,5.

The *A. tumefaciens* cells containing the vector were grown in 50 ml YEB medium supplemented with 100 µg/ml Rifampicin, 50 µg/ml Carbenicillin and 100 µg/ml Spectinomycin at 28°C with 200 rpm shaking for 24 hours. The following morning, cells were collected by centrifugation at 4000 *g* for 10 min and resuspended in de-ionized water to have OD_{600} 1. The *A. tumefaciens* suspension was infiltrated into the abaxial side of *Nicotiana tobacum* cv. Petite Havanna SNN leaves with a needleless 10 ml syringe until the tissue was filled with liquid (Wroblewski et. al., 2005). The plants were left in greenhouse conditions for 2 days for the accumulation of construct.

5.2.4.3 Histochemical GUS Staining

GUS staining buffer: 100 mM sodium phosphate buffer, pH 7, 10 mM Na_2(EDTA), 0,5 mM $K_3[Fe(CN)_6]$, 0,5 mM $K_4[Fe(CN)_6]$, 0,08 % X-GlcA (freshly added after dissolving in DMSO).

The tissues were incubated in GUS staining buffer overnight at 37°C with slight shaking. The visualization was performed after decolorizing the tissues in 70 % ethanol.

5.2.4.4 Confocal Laser Scanning Microscopy

For localization studies, leaf discs were analyzed with a Zeiss META LSM 510 confocal laser scanning microscope. Excitation wavelength for GFP was 488 nm and detection bandpass was 505-530 nm. Chlorophyll autoflorescence had excitation wavelength of 488 nm and detection was performed with longpass 650 nm.

5.2.5 Determination of Soluble Sugars

5.2.5.1 Soluble Sugar Extraction from Tissue

Ethanolic extraction buffer: 80 % ethanol, 10 mM Hepes, pH 7,5.

100 mg of grinded tissue was mixed with 500 µl of ethanolic extraction buffer and incubated at 80°C for 40 min. After centrifugation at 15000 *g* for 3 min at room temperature, the supernatant was taken to a fresh tube and stored on ice. The extraction was repeated by adding same volume of ethanolic extraction buffer to the pellet. The supernatants from both extractions were combined and stored -20°C.

5.2.5.2 Determination of Sucrose Concentration

Reaction buffer: 100 mM imidazole, 3 mM $MgCl_2$, pH 6,9.
Master mix per sample: 1 µl 30 mM NADP, 2 µl 30 mM ATP, 0,4 µl glucose-6-phosphate dehydrogenase (700 U/ml, Roche), 0,4 µl hexokinase (1500 U/.ml, Roche) and 155,2 µl reaction buffer.

The measurement was performed in 96-well format using 96-well plate-reader (Fluostar Optima, BMG Labtech) according to Spackman and Cobb (2001) with slight modifications. To each well, 160 µl of master mix and 10 µl ethanolic extract (usually 1:40 dilution) was added. The plate was vigorously mixed after insertion to plate-reader and at 15^{th} min background absorption was measured (abs1, 340 nm). 10 µl of invertase (2 mg/ml in reaction buffer, Sigma) was injected to each well at this step and thoroughly mixed. The reaction was incubated for 60 min and the final absorption at 340 nm was measured (abs2). The absorption was calculated with subtracting abs2 from background absorption (abs1). The standard curve prepared using known concentrations of sucrose (0,1 to 0,8 mg/ml in ethanol) was used for determination of concentration.

5.2.5.3 Hexose (Glucose and Fructose) Measurements

Reaction buffer: 100 mM imidazole, 3 mM MgCl2, pH 6,9.
Master mix: 2 µl 30 mM NADP, 2 µl 30 mM ATP, 0,4 µl glucose-6-phosphate-dehydrogenase (700 U/ml, Roche), 155,6 µl reaction buffer.

The hexose measurements were performed only for wounded taproot tissues (Spackman and Cobb, 2001) using 96-well plate-reader (Fluostar Optima, BMG Labtech). To each well 160 master mix and 2 µl of undiluted ethanolic extract was added. The plate was vigorously mixed after insertion to plate-reader and background absorption at 340 nm was taken immediately (abs1). Afterwards 4 µl of hexokinase (62,5 U/ml, Sigma) diluted in reaction buffer was injected to each well and incubated for 15 min. A second absorption value was taken (abs2) before injecting 4 µl of phosphoglucoisomerase (44 U/ml, Roche) diluted in reaction buffer. The last absorption (abs3) was measured after 30 min incubation. The glucose absorption was calculated by subtracting abs1 from abs2 and fructose by subtracting abs2 from abs3. The standard curve prepared using known concentrations of glucose and fructose (0,1 to 0,8 mg/ml in ethanol) was used for determination of hexose concentrations.

5.2.6 Stress Treatments

5.2.6.1 Wounding of *Beta vulgaris* Taproot

Wounding of taproot tissue of *B. vulgaris* was performed according to Rosenkranz et al. (2001). A fully developed taproot was removed from soil, thoroughly washed and decapitated. 0,05 cm in diameter cylinders were removed from cortex and further cut into 2 mm thick slices

with a set of fixed razor blades. The wounded tissues were incubated in a humid environment at dark at room temperature.

5.2.6.2 Etiolation of *A. thaliana* Seedlings

The *A. thaliana* seeds were surface sterilized and inoculated onto ½ MS plates without sucrose. The plates were kept at 4°C overnight and transferred to growth chamber. All plates were left in light for 24 hours. The etiolation seeds were then covered with aluminum foil, whereas control seeds were left under normal conditions. The tissues were collected 7 days after germination of control seeds.

5.2.6.3 Sugar Response in *A. thaliana* Cell Culture

5 grams of *A. thaliana* cell culture (in exponential growth) was washed twice with sugar-free medium and then inoculated to 100 ml of cell suspension medium containing glucose, sucrose or 3-*O*-methylglucose. Cell cultures were grown at dark (25°C) with 90 rpm shaking. The samples were taken 7 days after inoculation for sucrose or glucose grown cells and 3 hours, 1 day and 3 days for cell cultures inoculated in 3-*O*-methylglucose containing suspension culture.

5.2.6.4 Sugar Response *in planta*

For *in planta* sugar response experiment, *A. thaliana* grown in hydroponics (5 week after transfer to hydroponics solution) were used. 0,05 cm in diameter discs were removed from fully developed rosette leaves and incubated in 50 ml of either 100 or 300 mM sucrose, glucose or 3-*O*-methylglucose dissolved in deionized water overnight at dark at room temperature with slight shaking. The control tissue was incubated in deionized water under the same conditions.

5.2.6.5 Stress Treatments in Hydroponics

Stress treatments of *A. thaliana* grown in hydroponics were performed 5 weeks after transfer to hydroponics solution. For each treatment, the hydroponics solution was renewed.

The ABA and salt treatments were performed by adding 100 µM ABA or 200 mM NaCl to hydroponics solution and incubating at growth chamber conditions for 16 h. The control tissues were collected at the same time points.

The cold treatment was performed by keeping whole plants at 4 °C for 2 hours and afterwards 16 hours at growth chamber conditions. The control and stress samples were collected after recovery.

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

The phosphate starvation was performed by removing the phosphate source from hydroponics solution (by replacing KH_2PO_4 with K_2SO_4 and keeping $[K^+]$ and $[SO^{2-}]$ constant). The control plants were incubated in normal hydroponics solution. After 5 days of treatment in growth chamber, tissues were collected from phosphate deficient and control plants.

The rosette leaves were wounded with a leaf-piercing device opening 100 needle holes in 1 cm^2 area. The control and wounded tissue samples were collected 1 hour and 5 hours after treatment.

5.2.6.6 Salt Stress Treatment of *A. thaliana* Seedlings in Plates

A. thaliana seeds were surface sterilized and inoculated to ½ MS plates containing 125 mM or 150 mM NaCl. The control plates were prepared without salt. After inoculation, all plates were incubated at 4°C overnight and transferred to growth chamber.

The visualization was performed 14 days after inoculation.

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7 APPENDICES

7.1 Alignment of Coding Sequences of *A. thaliana* sPPases

The coding sequence alignment of *A. thaliana* sPPases according to Morgenstern (2004) is given below. The alignment clearly shows the extra stretches in the plastidial isoform (ASP5).

```
ASP1            ATGAGTGAAG AAACTAAAGA taa....... .........C C........A
ASP2A           ATGGCTGA.. AATCAAG... .........G ATGAAGGAAG CGCCAAGGGC
ASP2B           ATGAGTGAAG AAGCATATGA agaaactcaG GAATCAAGTC AATCTCCt..
ASP3            atg....... .......... .......... .........G CGCCACCGAT
ASP4            ATGAATGGAG AAGAAGT... .........G AAAACGAGTC AACCTCAGAA
ASP5            .......... .......... .......... .....GGTTC A........A

ASP1            GAGGCTGC.. ......AGCG AC........ ........CA GCTCCTCGTC
ASP2A           TATGCTTTCC CTCTCAGGAA CC........ ........CT AATGTTACGC
ASP2B           .......... ........CG TC........ ........CG GTTCCAAAAC
ASP3            TGAGGTTTCT ACCAAAAGCT ACgttgagaa acatgtttCA CTTCCTACTC
ASP4            GAAGCTTC.. ......AGAA CC........ ........CT ACTCCACGTT
ASP5            GAAGAAGG.. ......TCCG GC........ ........CG AATCCCTAGA

ASP1            TTAACGAGAG GATTCTCTCA TCCTTGTCAA GAAGATCCGT AGCTGCTCAT
ASP2A           TGAATGAGAG AAACTTTGCA GCCTTCACTC ACAGATCAGC TGCTGCTCAT
ASP2B           TGAACGAGAG GATTCTCTCA ACACTATCCA GGAGATCTGT AGCTGCACAT
ASP3            TTAATGAGAG GATACTTTCG TCCATGAGTC ACAGATCAGT AGCTGCACAC
ASP4            TAAACGAGAG GATTCTCTCA TCTTTGTCTA AGAGATCGGT TGCTGCACAT
ASP5            TTATCGAGTT TTCTTCCTCG ATGGTTCTGG AAAGAag... ....GTTTCT

ASP1            CCATGGCATG ATCTTGAGAT TGGACCTGGA GCTCCACAGA TTTTCAATGT
ASP2A           CCTTGGCATG ACTTGGAGAT TGGTCCAGAA GCTCCTACTG TTTTCAACTG
ASP2B           CCATGGCACG ACCTTGAGAT TGGTCCTGAA GCTCCATTGG TCTTCAATGT
ASP3            CCATGGCATG ATCTCGAGAT AGGACCTGAA GCCCCAATTA TCTTCAATTG
ASP4            CCATGGCATG ATCTTGAAAT CGGACCTGGA GCTCCAGTGA TTTTCAATGT
ASP5            CCATGGCATG ATATacca.. TTGACCTTAG GCgatgga.G TTTTCAACTT

ASP1            GGTTGTTGAG ATCACTAAAG GAAGCAAGGT CAAATACGAG CTTGACAAAA
ASP2A           TGTTGTTGAA ATTAGCAAAG GTGGAAAGGT TAAGTACGAG CTAGACAAGA
ASP2B           GGTGGTTGAG ATCACAAAGG GAAGCAAAGT GAAATATGAA CTCGACAAAA
ASP3            TGTGGTTGAG ATAGGAAAAG GGAGCAAGGT GAAATATGAA CTCGACAAAA
ASP4            GGTTATTGAG ATCTCAAAGG GGAGCAAAGT CAAATATGAA CTTGACAAAA
ASP5            TATAGTTGAA ATACCTAAAG AGTCAAAAGC AAAAATGGAG GTTGCTACTG
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ASP1            AGACAGGACT CA.TCAAGGT TGATCGTATT CTC....... ..........
ASP2A           ACAGTGGCCT TA.TTAAGGT TGATCGCGTT CTC....... ..........
ASP2B           AGACCGGTCT TA.TCAAGGT TGACCGGATC TTG....... ..........
ASP3            CTACGGGTCT CA.TTAAGGT CGACCGTATT CTT....... ..........
ASP4            AAACAGGTCT CA.TCAAGGT TGATAGGATT CTT....... ..........
ASP5            ATGAAGATTT CAcTCCTATT AAGCAGGATA CTAagaaggg gaagctcaga

ASP1            TACTCATCAG TTGTGTACCC TCACAACTAT GGTTTTGTTC CTCGCACATT
ASP2A           TACTCATCCA TTGTGTACCC CCACAACTAC GGTTTCATCC CTCGAACTAT
ASP2B           TACTCCTCCG TTGTTTATCC ACACAATTAC GGATTCATCC CACGGACATT
ASP3            TACTCATCTG TCGTATACCC ACACAACTAT GGGTTCATTC CGCGTACCCT
ASP4            TATTCTTCGG TTGTGTATCC TCACAACTAC GGGTTTGTCC CACGCACACT
ASP5            TATTATCCGT ATAACATTAA CTGGAACTAT GGGTTGCTTC CTCAAACATg

ASP1            .......... .......... .......... .........G TGTGAAGACA
ASP2A           .......... .......... .......... .........C TGTGAAGACA
ASP2B           .......... .......... .......... .........G TGTGAAGACA
ASP3            .......... .......... .......... .........T TGTGAGGACA
ASP4            .......... .......... .......... .........A TGTGAAGACA
ASP5            ggaagatcca tctcatgcaa attctgaagt tgaaggatgT TTTGGGGATA

ASP1            ATGACCCCAT TGATGTCTTA GTCATCATGC AGGAACCTGT GCTTCCGGGT
ASP2A           GTGATCCAAT GGATGTCCTG GTACTGATGC AGGAGCCTGT GCTAACCGGA
ASP2B           ACGATCCTCT TGATGTCCTT GTCCTTATGC AGGAACCAGT GCTTCCCGGA
ASP3            GTGACCCTAT TGATGTTCTT GTCATTATGC AGGAACCGGT GATCCCAGGA
ASP4            ATGACCCTAT AGATGTTTTA GTTATCATGC AGGAGCCTGT GCTTCCGGGT
ASP5            ATGATCCAGT TGATGTTGTT GAGATTggtg AAACACAAAG GAAGATAGGC

ASP1            TGTTTTCTGC GTGCCAGAGC CATTGGATTA ATGC..CTAT GATTGACCAG
ASP2A           TCATTCCTCC GTGCCCGTGC TATTGGTCTA ATGC..CCAT GATTGATCAG
ASP2B           TGTTTCCTCC GTGCTAGAGC CATTGGATTA ATGC..CCAT GATTGATCAG
ASP3            TGCTTTCTTC GGGCCAAAGC TATTGGTCTG ATGC..CAAT GATTGATCAG
ASP4            TGTTTTCTTC GCGCCCGAGC TATTGGTTTA ATGC..CTAT GATTGACCAG
ASP5            GATATTCTAA AGATAAAGCC TTTAG..CTG CTttagCTAT GATTGATGAA

ASP1            GGTGAAAAAG ATGACAAGAT CATTGCAGTG TGTGTTGATG ATCCTGAATA
ASP2A           GGTGAGAAAG ACGACAAGAT CATTGCAGTA TGTGCTGATG ATCCCGAGTT
ASP2B           GGAGAGAAAG ACGACAAAAT CATAGCCGTA TGTGCTGATG ATCCAGAGTA
ASP3            GGTGAGAAAG ACGACAAGAT CATTGCTGTC TGCGCTGACG ATCCAGAGTA
ASP4            GGTGAAAAAG ATGACAAGAT CATTGCAGTT TGTGTTGATG ATCCTGAGTA
ASP5            GGTGAGCTAG ACTGGAAGAT TGTTGCCATT TCTTTGGATG ACCCAAAAGC

ASP1            TAAGCACTAC A......... CTGACATCAA AGAACTTCCT CCTCACCGTC
ASP2A           CCGTCACTAC A......... GAGACATCAA AGAGCTTCCC CCTCACCGTC
ASP2B           CAAACATTTC A......... CAGACATCAA ACAACTCGCT CCTCATCGTC
ASP3            TCGCCATTAC A......... ACGACATCAG TGAGCTTCCG CCTCATCGTA
ASP4            TAAGCACATC A......... CTAACATCAA TGAACTTCCT CCTCATCGTC
ASP5            TCATCTTGTG Aatgatgttg AAGATGTTGA GAAACATTTC CCGGGTACAC
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ASP1            TCTCTGAAAT CCGTCGTTTC TTCGAAGACT ACAAGAAAAA CGAGAACAAG
ASP2A           TAGCTGAAAT CCGTCGCTTC TTTGAGGACT ACAAGAAGAA CGAGAACAAG
ASP2B           TCCAAGAAAT CCGCCGTTTC TTCGAAGACT ATAAGAAGAA CGAGAACAAG
ASP3            TGGCTGAGAT CCGCCGTTTC TTTGAAGACT ATAAGAAAAA CGAGAACAAG
ASP4            TTTCTGAAAT CCGTCGATTC TTTGAAGACT ACAAGAAGAA TGAGAACAAG
ASP5            TAACAGCCAT TAGAGACTGG TTTAGAGACT ACAAGATCCC AGATGGAAAG

ASP1            .......... GAAGTTGCAG TGAATGATTT TCTGCCATCT GAGTC..TGC
ASP2A           .......... AAAGTCGACG TTGAAGCTTT CCTTCCCGCT CAAGC..TGC
ASP2B           .......... AAAGTGGCTG TCAACGATTT CTTGCCATCA GAGAg..TGC
ASP3            .......... GAAGTAGCCG TTAACGACTT CCTTCCGGCA ACTGC..AGC
ASP4            .......... GAAGTTGCAG TGAATGATTT TCTACAACCT GGTCC..TGC
ASP5            cctgctaaca GATTTGGTCT TGGAGACAAA CCAGCAAACA AAGACtaTGC

ASP1            GGTTGAAGCT ATCCAGTACT CAATGGACCT CTATGCTG.. ..........
ASP2A           CATAGACGCT ATCAAGGACT CCATGGATCT TTACGCAG.. ..........
ASP2B           ACATGAAGCT ATTCAGTACT CCATGGATCT ATACGCTG.. ..........
ASP3            CTACGACGCA GTTCAGCATT CCATGGATCT CTATGCAG.. ..........
ASP4            TATTGAAGCC ATTCAGTACT CAATGGATCT TTACGCTG.. ..........
ASP5            TTTGAAGATC ATCCAAGAAA CAAATGAATC ATGGGCTaaa cttgtgaaga

ASP1            ....AATACA TTCTCCACAC CCTGAGGCGT TGA..
ASP2A           ....CTTACA TCAAAGCTGG CCTGCAACGC TAA..
ASP2B           ....AGTATA TTCTCCACAC GTTGAGGAGA TGA..
ASP3            ....ACTACG TCGTGGAGAA CCTAAGACGT TGA..
ASP4            ....AGTACA TTCTTCACAC CCTGAGGAGA TAg..
ASP5            gatcAGTTGA TGCTGGAGAC CTTTCACTTT Actga
```
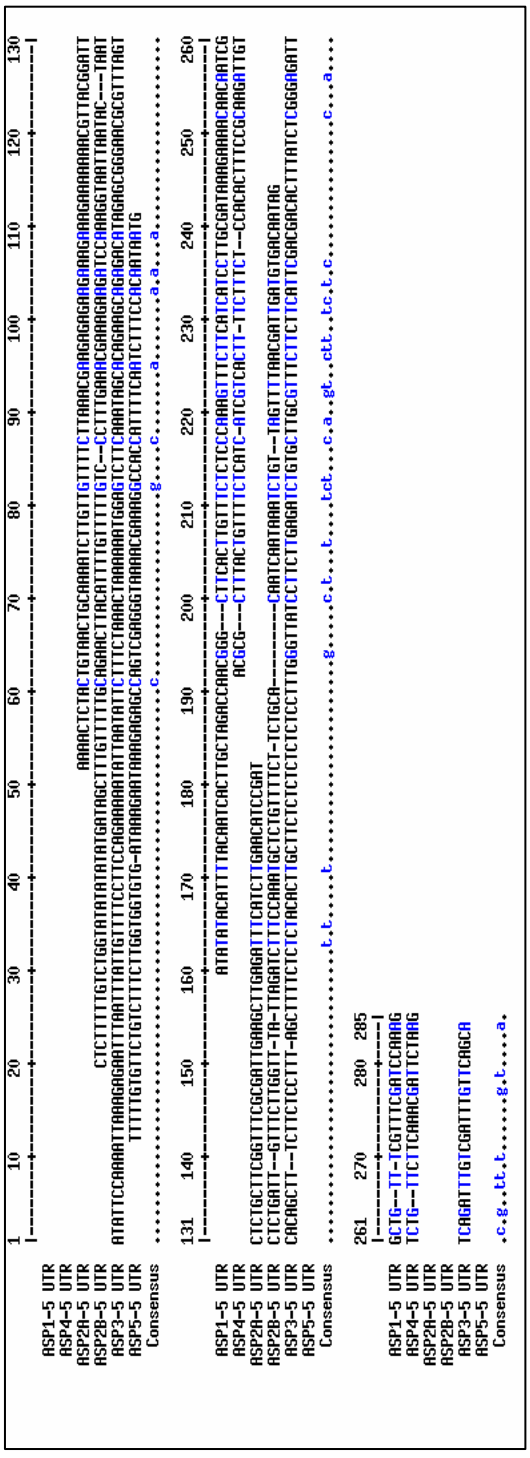
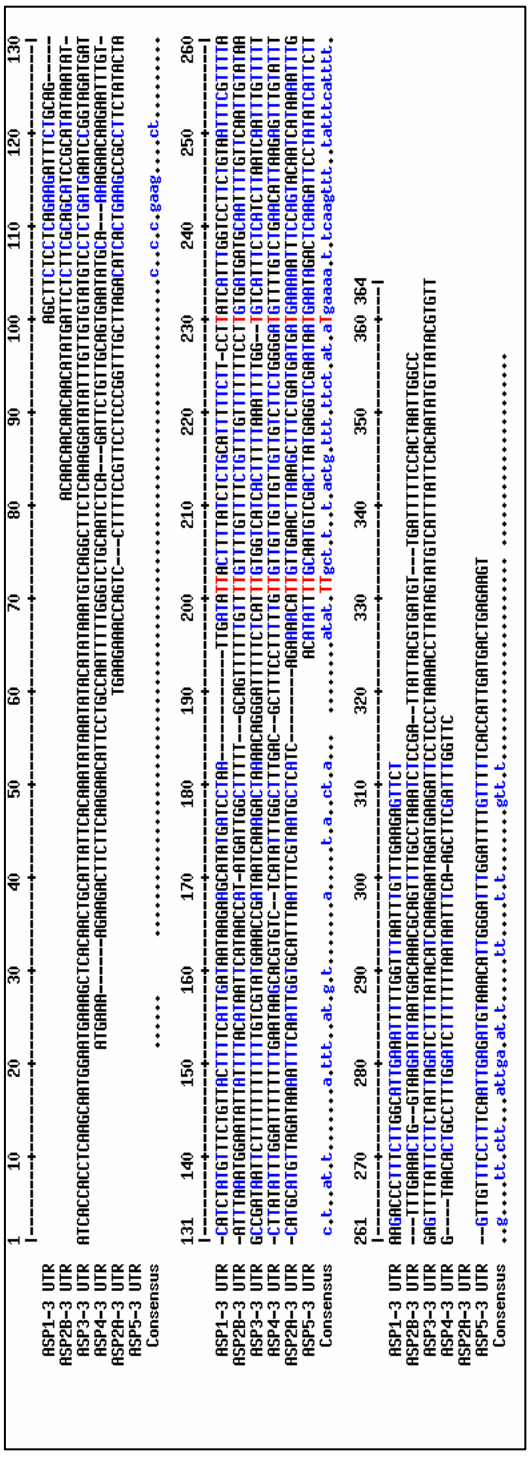

7.2 Alignments of 5'- and 3'-UTR Sequences of *A. thaliana* sPPases

The alignments were performed according to Corpet (1998). The conserved bases were highlighted with red and highly conserved bases with blue.





7.3 Examples of Aggregate Formation in N-terminal GFP-Protein Fusions after Transient Expression in Tobacco

As explained in detail in Chapter 3.1.1.2 and 4.2, the N-terminal GFP constructs of *A. thaliana* sPPases caused aggregate formation after transient overexpression in tobacco leaf epidermal cells. The following picture gives two examples of the images obtained from CLSM analysis.

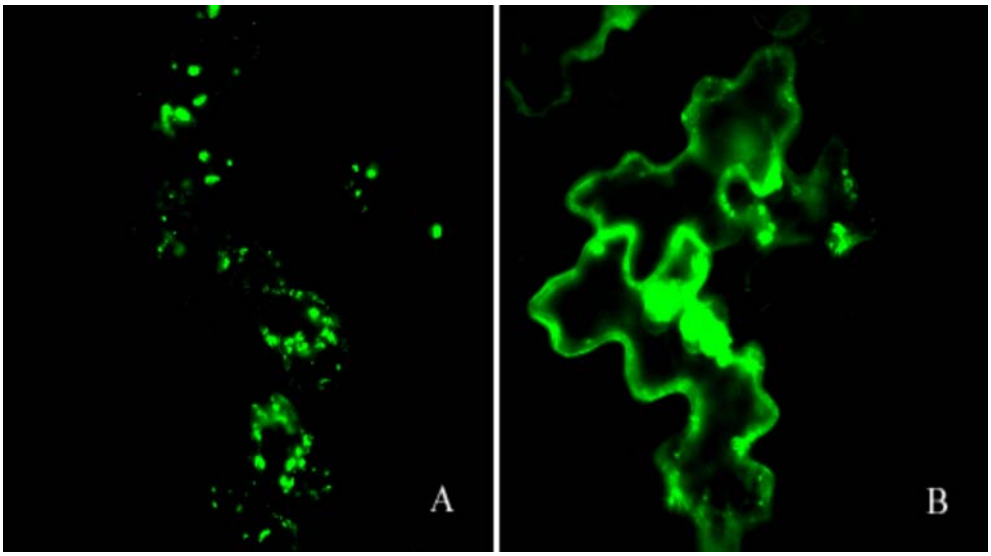


Figure 7.1. Aggregate formation in N-terminal GFP *A. thaliana* sPPase constructs. A) GFP-ASP1, and B) GFP-ASP4 chimera after transient overexpression in tobacco.

7.4 Predicted 3D Structure of ASP2B

The 3D structure of ASP2B was predicted according to homology to *E. coli* sPPase (Sivula et al., 1999).

Figure 7.2. A stereo view of the predicted 3D structure of ASP2B. Some residues at the N-terminus and C-terminus are not shown as they have no analogs in *E. coli* sPPase (Sivula et al., 1999).

7.5 Phenotypic Differences in SALK T-DNA Insertion Mutants of *A. thaliana* sPPase Isoforms

The T-DNA insertion mutants of *A. thaliana* sPPase isoforms except the plastidial isoform (ASP5) are available from the SALK Institute (Sequence indexed library of insertion mutations in the *Arabidopsis* genome) seed collections (Table 7.1) (Alonso et al., 2003).

Table 7.1. SALK T-DNA insertional mutants of *A. thaliana* sPPase isoforms used in detection of possible phenotypic differences.

	SALK Clone	Location of T-DNA Insertion
ASP1	093663.35.35.x	5'-UTR
ASP2A	093839.45.30.x	Exon
ASP2B	072719.56.00.x	5'-UTR
ASP3	124702.55.75.x	Exon
ASP4	014647.55.75.x	Exon

The comparison of the offspring of insertion mutant seeds supplied from SALK Institute with the wild type *A. thaliana* genotype Columbia was given in Figure 7.3. In each insertion mutant line, the offspring plants were of three different sizes when compared to the wild type; relatively small, of middle size and similar to wild type. The initial screening of insertion mutant lines as homozygous, heterozygous or wild type plants showed that the size difference observed was not well correlated with homozygous plants.

The detailed analysis of homozygous insertion mutants with respect to change in the expression levels of other *A. thaliana* sPPase isoforms should be performed to understand the effect of knockout of one ASP isoform to phenotype, however, the initial analysis suggests that not only the removal of cytoplasmic pyrophosphate but also the removal of the activity of one of the cytoplasmic sPPase isoforms significantly alters plant growth and development.

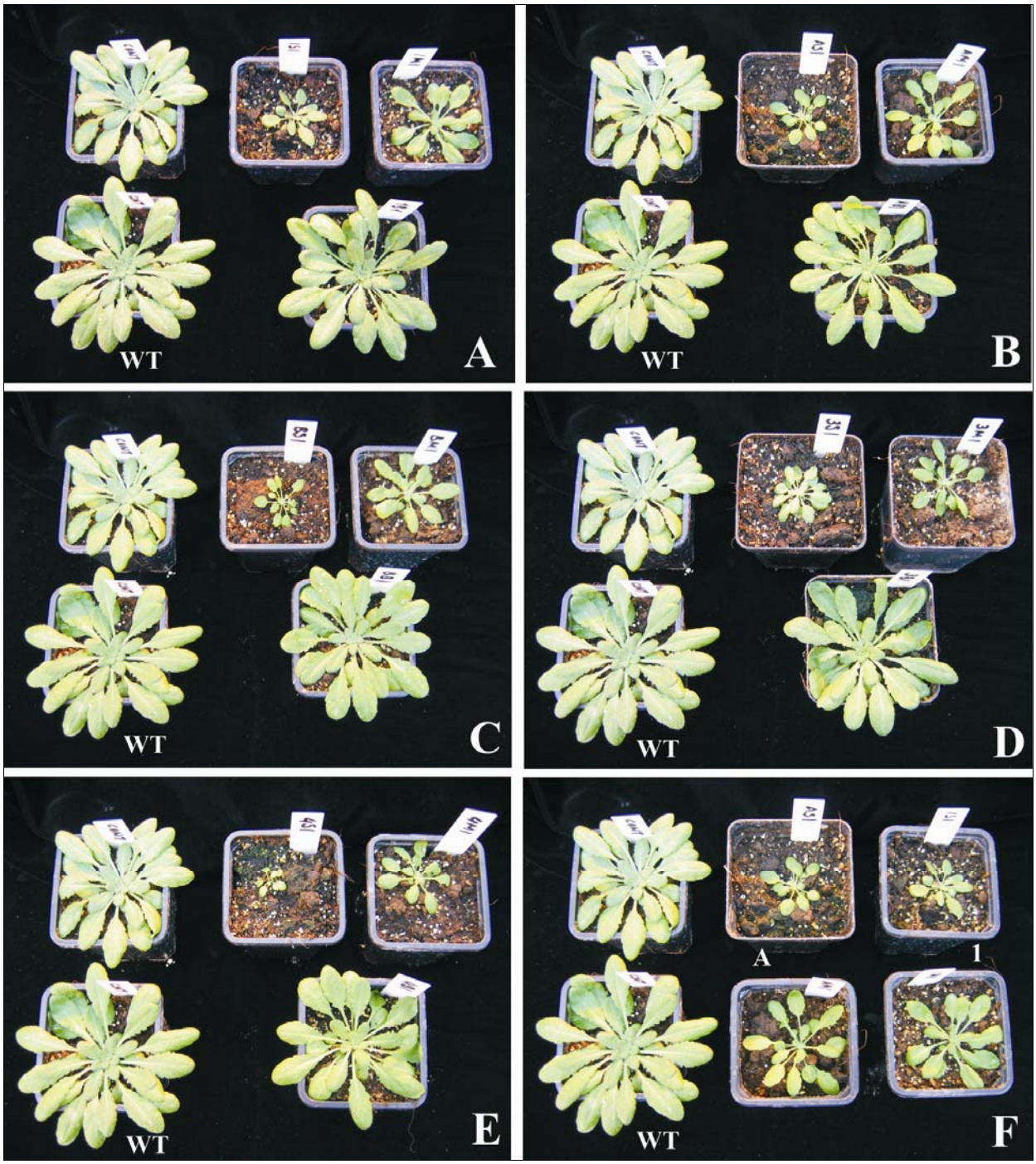


Figure 7.3. Comparison of *A. thaliana* sPPase insertion mutants with wild type plants. A) ASP1, B) ASP2A, C) ASP2B, D) ASP3, E) ASP4, and F) ASP1 (on the right side; 1) and ASP2A (in the middle; A) together. In each picture two wild type plants on the left side were indicated as WT.

7.6 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 7.2.

The mostly encountered *A. thaliana* isoform was ASP5 (plastidial isoform; by 24 ESTs out of 196000; Table 7.2), similar to the real time PCR analysis performed using several plant tissues (Figure 3.12). However, because the activity of the plastidial isoform is possibly essential for plant growth (Weiner et al., 1987; Farre et al., 2001), there was no intention to use it 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 last updated on 2005. Therefore, even though the result of real time PCR analysis indicates a very low expression of ASP2A in different tissues (Figure 3.13), 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. Since the coding sequences of *A. thaliana* sPPase isoforms are very similar to each other (Figure 3.2), in order to be able to specifically knockdown these two isoforms without affecting the expression of others, the 3'-UTRs of ASP1 (184 bp) and ASP2A (176 bp) were amplified from leaf cDNAs with the addition of an artificially created restriction digestion site (BamHI/SpeI). The amplicons were ligated with each other using this restriction site before insertion in Gateway entry vector pDONR201 (Invitrogen). After confirmation of the insert sequence, it was introduced in the Gateway destination vector pB7GWIWG2(I) (Invitrogen) (Karimi et al., 2002) which initiates RNAi effect through a hairpin expression of the insert (Helliwell and Waterhouse, 2003) and carries the *bar* gene enabling the selection of positive plants by spraying BASTA™ solution (0,02 % v/v) to the leaves. The construct was then mobilized in *A. tumefaciens* strain C58C51 containing Ti plasmid pGV2260 and stably transferred to *A. thaliana* by flower dip method (Clough and Bent, 1998).

Table 7.2. Digital Northern (Koo and Ohlrogge, 2002) analysis of *A. thaliana* sPPases using UniGene database (http://www.ncbi.nlm.nih.gov/UniGene) updated on 2005. EST libraries were grouped according to the source tissues where the cDNA library was derived from.

	Library	Mixed	Flower buds	Root untreated	Siliques	Not known	Seedling	Rosette dehydration	Whole plant stress	Rosette tissue	Inflorescence	Silique and flower	Rosette cold stress	Leaf senescence	Leaf untreated	Leaf infected	Germinating seeds	Others	Total
	Total Number of ESTSs in the Library	*36.396*	*5.827*	*23.023*	*13.436*	*8.049*	*4.552*	*7.025*	*184*	*2.723*	*2.478*	*3.404*	*5.102*	*6.164*	*1.714*	*3.719*	*12.424*	*72.205*	*196.001*
	% of the Library in Total	*19*	*3*	*12*	*7*	*4*	*2*	*4*	*0*	*1*	*1*	*2*	*3*	*3*	*1*	*2*	*6*	*37*	
ASP1	**Number of ESTs**	**8**	**1**	**4**	**1**	**2**	**2**	**3**											**21**
	Percentage	***0,022***	***0,1373***	***0,0347***	***0,0595***	***0,0994***	***0,1757***	***0,1139***											***0,01071***
ASP2A	**Number of ESTs**	**7**		**3**	**2**			**1**	**1**	**1**	**1**								**16**
	Percentage	***0,0192***		***0,013***	***0,0149***			***0,0142***	***0,5435***	***0,0367***	***0,0404***								***0,00816***
ASP2B	Number of ESTs											2							2
	Percentage											*0,059*							*0,00102*
ASP3	Number of ESTs				1		5		3	2									11
	Percentage				*0,0074*		*0,1098*		*1,6304*	*0,0734*									*0,00561*
ASP4	Number of ESTs	2		4	1				3	1			1						12
	Percentage	*0,0055*		*0,0174*	*0,0074*				*1,6304*	*0,0367*			*0,02*						*0,00612*
ASP5	Number of ESTs	9		3	3			1	1	1				1	1	3	1		24
	Percentage	*0,0247*		*0,013*	*0,0223*			*0,0142*	*0,5435*	*0,0367*				*0,016*	*0,058*	*0,081*	*0,00805*		*0,01224*

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 7.4).

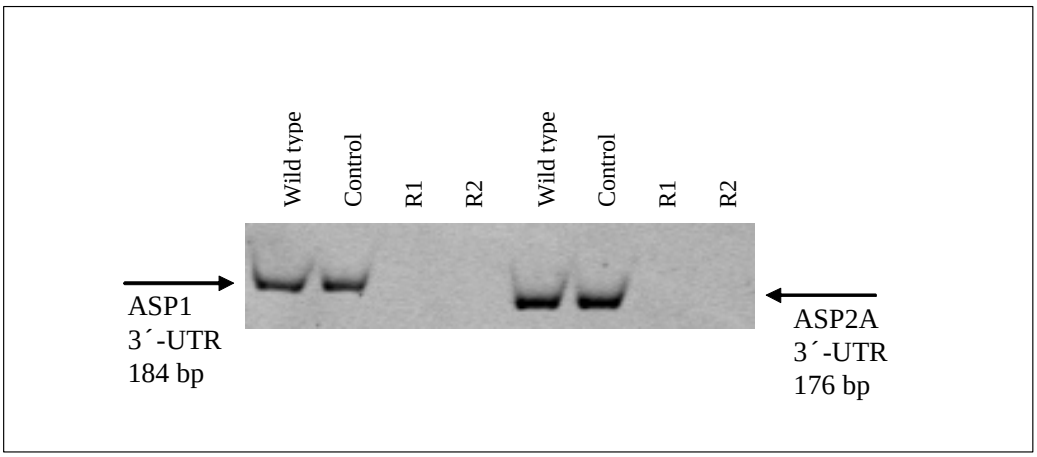


Figure 7.4. Conventional reverse transcriptase PCR of RNAi1/2A lines R1 and R2 proving loss of both ASP1 and ASP2A transcript. 500 ng of total RNA isolated from leaf tissue was used for reverse transcription.

The Western blotting results of soluble protein isolated from fully developed rosette leaves of R1 and R2 plants revealed an opposite effect; R1 having a lower amount of ASP protein and R2 with a higher amount compared to the wild type (Figure 7.5). Note that the primary antibody used for detection in Western blotting was raised against recombinant *Beta vulgaris* soluble pyrophosphatase isoform 1 protein (Bsp1), and is known to cross-react with all soluble pyrophosphatase isoforms of *A. thaliana* except the plastidial isoform (data not shown). Therefore, what is observed after Western blotting represents all ASP protein present in the tissue.

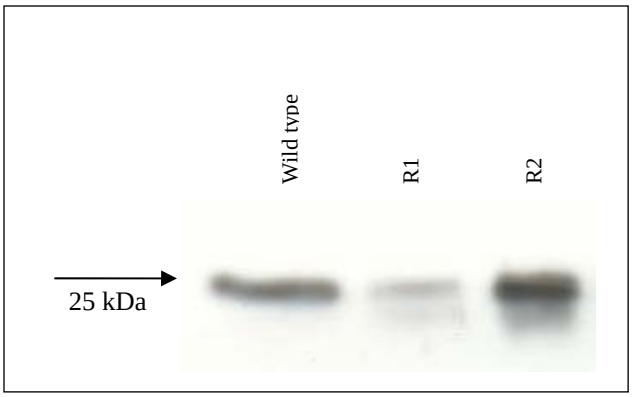


Figure 7.5. Western blot membrane of RNAi1/2A R1 and R2 lines showing the total ASP protein in their leaves. 15 µl out of 300 µl of soluble protein from fresh weight equivalent of 100 mg leaf was loaded to each well. The primary antibody used in detection was raised against recombinant Bsp1 protein and cross-reacts with all cytoplasmic ASP proteins.

In order to understand the effect of decrease of total *A. thaliana* soluble pyrophosphatase protein amount on plant growth and development, the RNAi1/2A line R1 with less ASP protein compared to the wild type (Figure 7.6) was selected as the main focus of further analysis. It is worthy to note that the following results are not comprehensive since only a single transformant line was used for experimentation.

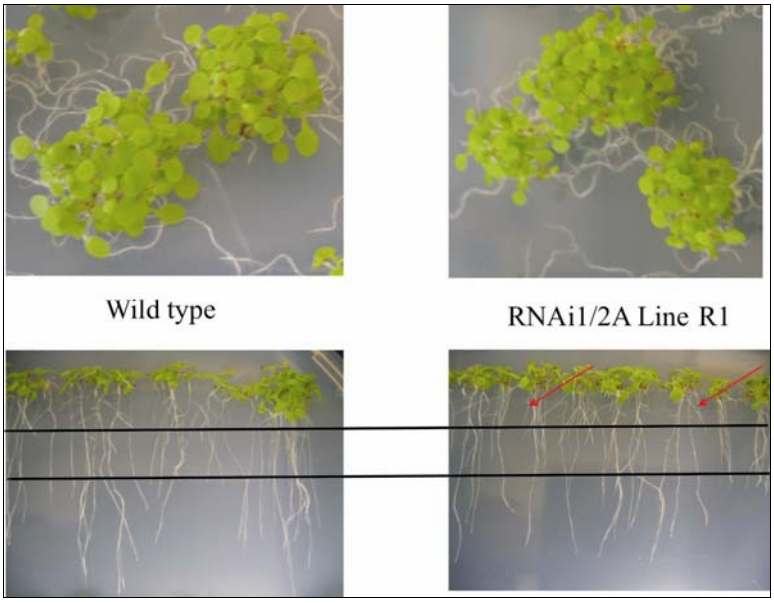


Figure 7.6. Comparison of wild type and R1 seedlings grown in non-selective ½ MS plates. The figure shows the status of seedlings grown either horizontally (top line) or vertically (bottom line) ten days after germination. The lines were drawn to differentiate two distinct root length populations in 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.

Figure 7.6 shows the T1 generation seeds of R1 germinated on non-selective plates either horizontally or vertically. The comparison of horizontally grown R1 seeds with wild type seeds ten days after germination reveals that in transgenic line, there are a number of relatively smaller plants still having no true leaves. The same comparison of vertically grown seeds reveals that in R1 line there are a number of plants having much shorter root lengths. These small plants were collected from plate and shown to be positive with respect to knockdown effect of both ASP1 and ASP2A transcripts proven with reverse transcription PCR (data not shown). The selected representatives of these small plants were transferred to soil at the same time with wild type seedlings. A week after transfer to pots, all transgenic seedlings were dead, whereas wild type seedlings were adopted to soil and grown to rosette leaves.

Based on the phenotypic differences observed in SALK insertion lines (Chapter 7.5) and in RNAi1/2A transgenic plants (having less ASP protein compared to wild type), it was proposed that the activity of plant soluble pyrophosphatases can be highly specialized in their function and their activity can be essential for plant growth and development in contrast to what was proposed in the literature (Stitt, 1998). This hypothesis requires more experimentation, however, since the transgenic lines with impaired root growth is not grown after transfer to soil, the results are not complete.

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