

# **Exploring plant tolerance to biotic and abiotic stresses**

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**Doctoral thesis  
Swedish University of Agricultural Sciences  
Uppsala 2007**

**Acta Universitatis Agriculturae Sueciae**  
2007: 58

ISSN 1652-6880  
ISBN 978-91-576-7357-2  
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Tryck: SLU Service/Repro, Uppsala 2007

*To my parents*

*To Lipa & Naayav*

**Front cover:** GUS expression in a senescing *Arabidopsis* plant

## Abstract

Karim, S. 2007. Exploring plant tolerance to biotic and abiotic stresses.  
Doctor's dissertation.  
ISSN 1652-6880, ISBN 978-91-576-7357-2

Plants are exposed to many stress factors, such as drought, high salinity or pathogens, which reduce the yield of the cultivated plants or affect the quality of the harvested products. *Arabidopsis thaliana* was used as a model plant to study the responses of plants to different sources of stress. With *Agrobacterium* T-DNA mediated promoter tagging, a novel di-/tripeptide transporter gene *AtPTR3* was identified as a wound-induced gene. This gene was found to be induced by mechanical wounding, high salt concentrations, bacterial infection and senescence, and also in response to several plant hormones and signalling compounds, such as salicylic acid, jasmonic acid, ethylene and abscisic acid. *Atptr3* mutants of two *Arabidopsis* ecotypes, C24 and Col-0, were impaired in germination on media containing a high salt concentration, which indicates that *AtPTR3* is involved in seed germination under salt stress. Wounding caused local expression of the *AtPTR3* gene, whereas inoculation with the plant pathogenic bacterium *Erwinia carotovora* subsp. *carotovora* caused both local and systemic expression of the gene. *Atptr3* mutants showed increased susceptibility to infection caused by bacterial phytopathogens, *E. carotovora* and *Pseudomonas syringae* pv. *tomato*, and the *P. syringae* type III secretion system was shown to be involved in suppression of the *AtPTR3* expression in inoculated plants. Moreover, the *Atptr3* mutation was found to reduce the expression of the marker gene for systemic acquired resistance, *PR1* and the mutants accumulated reactive oxygen species (ROS) following the treatment of the plants with ROS generating substances. Overall results and observations suggest that the *AtPTR3* is a novel and versatile stress responsive gene needed for defence reactions against many stresses.

In a second part of the study, the yeast (*Saccharomyces cerevisiae*) trehalose-6-phosphate synthase gene (*ScTPS1*) was utilized to improve the drought tolerance of *Arabidopsis*. This gene codes for the first enzyme in the trehalose biosynthesis pathway of yeast, and expression in plants leads to improved drought tolerance but also growth aberrations. In this study, the ScTps1 protein was expressed in *Arabidopsis* using the constructs containing chloroplast targeting transit peptide sequence that facilitated the import of the ScTps1 into the chloroplast. The drought tolerance and growth phenotypes of *Arabidopsis* transgenics transformed with *ScTPS1* with or without transit peptide, were characterized. The plants with cytosolic localization of the ScTps1 protein showed aberrant root phenotype, but the plants with the chloroplast targeted ScTps1 protein caused no aberration in root morphology. Even though both the transgenic lines showed enhanced drought tolerance, the relative water content of the lines was found to be similar to the wild type control. Moreover, both the transgenic lines showed slightly better water holding capacity or reduced water loss over time compared to wild type plants. The overall results indicated that the growth aberrations caused by cytosolic localization of ScTps1 could be uncoupled from the enhanced drought tolerance in the transgenic plants when the ScTps1 was targeted to chloroplast.

**Keywords:** *Arabidopsis thaliana*, abiotic stress, biotic stress, drought tolerance, peptide transporter, plant pathogen, promoter trapping, salt stress, T-DNA tagging, trehalose, trehalose-6-phosphate, trehalose-6-phosphate synthase.

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## Appendix

This thesis is based on the following articles, which will be referred to by their Roman numerals:

- I.** Karim, S., Lundh, D., Holmström, K-O., Mandal, A. & Pirhonen, M. 2005. Structural and functional characterization of AtPTR3, a stress-induced peptide transporter of *Arabidopsis*. *Journal of Molecular Modeling* 11, 226-236. (DOI: 10.1007/s00894-005-0257-6).
- II.** Karim, S., Holmström, K-O., Mandal, A., Dahl, P., Hohmann, S., Brader, G., Palva, ET. & Pirhonen, M. 2006. AtPTR3, a wound-induced peptide transporter needed for defence against virulent bacterial pathogens in *Arabidopsis*. *Planta* 2 December 2006, 1-15. (DOI: 10.1007/s00425-006-0451-5).
- III.** Karim, S., Aronsson, H., Ericson, H., Pirhonen, M., Leyman, B., Welin, B., Mäntylä, E., Palva, ET., Van Dijck, P. & Holmström, K-O. 2007. Improved drought tolerance without undesired side effects in transgenic plants producing trehalose. *Plant Molecular Biology* 28 February 2007, 1-16. (DOI: 10.1007/s11103-007-9159-6).

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## Abbreviations

ABA	Abscisic acid
ABRE	ABA-responsive element
ACC	1-aminocyclopropane-1-carboxylate
ADH	Alcohol dehydrogenase
AGPase	ADP-glucose pyrophosphorylase
AtHXK	<i>Arabidopsis</i> hexokinase
<i>avr</i>	Avirulence gene
<i>COR</i>	Cold-responsive
DRE	Dehydration responsive element
DREB	DRE binding protein
ET	Ethylene
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HR	Hypersensitive response
Hrc	Hrp conserved
<i>hrcC</i>	Hrc mutant
Hrp	Hypersensitive response and pathogenicity
<i>hrpA</i>	Hrp mutant
HSP	Heat shock proteins
<i>Inh II</i>	Proteinase inhibitor II gene
ISR	Induced systemic resistance
JA	Jasmonic acid
LEA	Late embryogenesis abundant
MAPK	Mitogen-activated protein kinase
OPT	Oligopeptide transporter
PCD	Programmed cell death
PDC	Pyruvate decarboxylase
PR	Pathogenesis related
PRR	Pathogen recognition receptors
PsTP	Trehalose phosphorylase ( <i>Pleurotus sajor-caju</i> )
PTR	Peptide transporter
<i>R</i>	Resistance gene
ROS	Reactive oxygen species
RuBisCO	Ribulose-1,5-bisphosphate carboxylase
SA	Salicylic acid
SAR	Systemic acquired resistance
<i>ScTPS1</i>	<i>Saccharomyces cerevisiae</i> TPS gene
<i>ScTPS2</i>	<i>S. cerevisiae</i> TPP gene
SOS	Salt-Overly-Sensitive
T6P	Trehalose-6-phosphate
T-DNA	Transfer DNA
TPS	Trehalose-6-phosphate synthase
TPP	Trehalose-6-phosphate phosphatase
TSase	Trehalose synthase ( <i>Grifola frondosa</i> )
TTSS	Type III secretion systems

## Introduction

Sustainable crop production is extremely important in the context of declining natural resources and population growth especially in developing countries. According to UN figures, about 60% more food will be needed to feed the world's growing population in the next three decades (BBC News, 2004; Myers & Kent, 2001). A range of abiotic and biotic stresses are the major limiting factors for crop production. Sustaining and improving crop production in spite of abiotic and biotic stresses may be possible with the new knowledge and techniques developed in the field of plant molecular biology. While the leading question in biology is how organisms deal with or respond to their surrounding environment, plant species become the vital model system to study the responses to both physical and biological stress factors.

The various physical stresses that a plant can experience are scarcity of water or drought stress, salinity or salt stress, low temperature or cold stress, heat shock stress by extreme temperature and anaerobic stress by melting snow or a water logged condition due to monsoon rain or flood water and a wide range of biotic stress factors plants try to avoid and adapt them through their defence response are bacteria, fungi, viruses, nematodes, protozoa, insects and grazing animals (Agrios, 2005). Plants exposure to and perception of different biotic and abiotic stresses in their ambient condition are the crucial determinant of growth and production. As an adaptive and defence mechanism plants have developed complex metabolic, hormonal and signalling networks which are responsive in different ways to these stress conditions. Primarily, the signalling compounds salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) as well as reactive oxygen species (ROS) play an important role through their synergistic and antagonistic actions that also lead to complex networks and multi direction crosstalk during stress responses. In the downstream of the biotic and abiotic defence pathways the players are the highly conserved mitogen-activated protein (MAP) kinase (MAPK) cascades and groups of stress responsive transcription factors, which are also the central regulators of diverse molecular and biochemical processes in the cells, such as growth, inhibition and stress responses (Walling, 2000; Knight & Knight, 2001; León *et al.*, 2001; Pieterse *et al.*, 2001; Thomashow, 2001; Zhu, 2001a, b; Kunkel & Brooks, 2002; Mittler, 2002; Blokhina *et al.*, 2003; Dolferus *et al.*, 2003; Wang *et al.*, 2003a; Nürnberger *et al.*, 2004; Shinozaki & Yamaguchi-Shinozaki, 2007). The morphology and distribution of visible abiotic and biotic stress symptoms in plant parts such as the leaves, branches and crown levels, provide a primary diagnostic indication to verify the stress types and plant responses as well as to differentiate the stress symptoms from natural senescence symptoms (Vollenweider & Günthardt-Goerg, 2005).

## Abiotic stress signalling

### *Drought*

A major abiotic stress factor that leads to reduction in crop production is drought or dehydration due to a water deficit. Plants respond to drought and make changes in cellular, metabolic and molecular levels to cope with the stress (Zhu, 2001a; Shinozaki & Yamaguchi-Shinozaki, 2007). The main outcome of drought shock is that it causes metabolic and osmotic imbalance in plants that leads to turgor loss and closure of the stomata, followed by repression of cell growth and inadequate photosynthesis because of limited carbon dioxide uptake due to the closure of stomata as well (Shinozaki & Yamaguchi-Shinozaki, 2007).

A large number of genes are expressed in plants to protect the cells from stress damage and to restore the metabolic disorder caused by desiccation. These genes, can be classified into two functional groups, the first group encodes proteins giving improved stress tolerance. They code for proteins involved in the production of antifreeze proteins, water channels, transporters, detoxifying enzymes, all having a protective function in the cell. This group even contains proteins involved in the production of osmoprotecting molecules, such as amino acid derivatives and sugars and many late embryogenesis abundant (LEA) proteins having uncharacterized function. LEA proteins are the most abundant hydrophilic proteins produced at the onset of embryo maturation and the final desiccation stage of seed development. They were found to be working as molecular chaperones protecting proteins from aggregation due to desiccation (Ingram & Bartels, 1996; Goyal *et al.*, 2005). The second group includes different proteins having a regulatory role, such as transcription factors, protein kinases and phosphatases, enzymes involved in phospholipids metabolism and ABA biosynthesis (Chen & Murata, 2002; Yamaguchi-Shinozaki & Shinozaki, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007).

Often the same genes are expressed in plants when they are exposed to cold, drought or salt, most probably because all these stresses lead to physiological drought and the main function of the encoded proteins is to protect the cells from cellular dehydration under all these stress conditions (Knight & Knight, 2001; Knight *et al.*, 2004). In all these situations, phytohormone ABA is the main plant hormone regulating stress-related gene expression, causing closure of the stomata and the subsequent expression of drought related genes (Zhu, 2002; Chinnusamy *et al.*, 2004; Vinocur & Altman, 2005; Umezawa *et al.*, 2006; Valliyodan & Nguyen, 2006; Xiong *et al.*, 2006; Shinozaki & Yamaguchi-Shinozaki, 2007). Several *rd* (responsive to dehydration) and *erd* (early responsive to dehydration) genes encoding a wide range of proteins are ABA dependent and contain the ABA-responsive element (ABRE) motif. In ABA-deficient (*aba*) or ABA-insensitive (*abi*) *Arabidopsis* mutants some genes are also induced by both dehydration and low temperature, which suggest that also ABA-independent gene expression is possible in response to dehydration and cold stress. Transcription systems regulating ABA-independent gene expression in response to dehydration

and cold stress include cis-acting elements DREs (dehydration responsive element) also referred as CRTs (C-repeat) and their DNA-binding proteins encoding transcription factors DREBs (DRE binding protein) or CBFs (C-repeat binding factor), respectively (Shinozaki & Yamaguchi-Shinozaki, 2000; Valliyodan & Nguyen, 2006).

Many of the drought responsive genes are co-regulated not only by related stresses such as cold and salt, but also by wounding and pathogens (Zhu, 2002; Vinocur & Altman, 2005; Shinozaki & Yamaguchi-Shinozaki, 2007). In the plant, a distinct family of ubiquitous 14-3-3 proteins, which act as regulators for different regulatory proteins through protein-protein interactions, play an important role in cross talk between many stress responses as well as regulating many membrane localized proteins such as ion channels and pumps, for example 14-3-3 regulates the activation of plasma membrane pump  $H^+$ -ATPase (Bunney *et al.*, 2002; Roberts *et al.*, 2002). 14-3-3 proteins regulate the different proteins in the signal transduction pathways in response to different biotic and abiotic stress factors such as wounding and pathogen attack, salt stress and nutrient deficiency, as well as modulating the biochemical pathways by regulating the metabolic and nutritional functions such as carbon and nitrogen assimilation and starch synthesis. 14-3-3 proteins are found to interact with elements of the ABA-induced gene expression machineries, which crucially regulate plant development and seed germination. Moreover, the presence of 14-3-3 proteins also within the chloroplast suggests that they may be involved in trafficking the thylacoid proteins (Fulgosi *et al.*, 2002; Roberts *et al.*, 2002; Xu & Shi, 2006).

## ***Temperature***

One of the most common environmental stresses is unfavourable change in the ambient temperature. From the equatorial regions to the arctic region plants can experience daytime temperatures as high as 60°C to as low as -70°C. Local topography and altitude also have a significant influence on the temperature of an area. Each plant species has an optimum temperature for growth and development, and the distribution of plant can be determined to a major extent by the temperature zone in which it can survive (Iba, 2002).

When the temperature falls below zero, ice forms first in the intercellular spaces of plant tissues because of higher freezing point in intercellular fluid than in the intracellular fluid. This ice formation results in adhesions between the cell walls and membranes and thereby causes severe disruption of cells (Olien & Smith, 1977). At subzero temperatures there is a decrease in the water potential outside the cell because of ice formation. Consequently, the unfrozen fluid or water moves out of the cell to the intercellular spaces and causes desiccation inside the cells, which then results in freezing injuries (Thomashow, 1999). Cold-induced desiccation can cause several physiological effects such as protein denaturation, precipitation of various molecules, membrane damage and lysis of the cells. The additional factor that causes cellular damage is the production of ROS during cold-induced injury (Thomashow, 1998, 1999; Pearce, 1999; Smallwood & Bowles, 2002).

The mechanisms that contribute to freezing or cold tolerance mainly act by preventing the induction of the factors damaging to the plant tissues. The initial factor responsible for triggering enhanced freezing tolerance in plants is exposure to low, but non-freezing temperatures, the phenomenon known as cold acclimation (Mohapatra *et al.*, 1988; Hughes & Dunn, 1996). Cold acclimation is the first line of defence and has been found to be involved in the stabilization of plasma membranes against cold-induced injury. During cold acclimation, the hydrophilic and LEA proteins help to stabilize membranes against cold-induced damage. Low temperature also reduces the water absorption by roots and water transport in shoots and as a result desiccation shock and wilting of the plant (Uemura *et al.*, 1995; Thomashow, 1998, 1999; Pearce, 1999; Smallwood & Bowles, 2002). Therefore, many of the functional proteins such as LEA and antifreeze proteins as well as regulatory proteins which are involved in drought signalling are also involved in cold or salt stress signalling (Pearce, 1999; Thomashow, 1999; Novillo *et al.*, 2004; Goyal *et al.*, 2005; Shinozaki & Yamaguchi-Shinozaki, 2007). Plants are divided into three groups according to their cold responsiveness. The first group is susceptible and damaged by temperatures below 12°C whereas the second group is able to acclimate to temperatures below 12°C but unable to survive freezing, and the last group is freeze-tolerant and able to acclimate to survive temperatures significantly below freezing (Pearce, 1999; Thomashow, 1999; Sharma *et al.*, 2005).

Cold acclimation or the application of ABA can induce the cascade of many cold-responsive (COR) genes (Mohapatra *et al.*, 1988; Hughes & Dunn, 1996; Thomashow, 1999). The COR genes, in *Arabidopsis* and in other plants are also designated as *lti* (low temperature induced), *kin* (cold-inducible), *rab* (responsive to ABA), *rd* or *erd*, encode proteins homologous to LEA proteins, dehydrins and cryoprotective proteins (Hughes & Dunn, 1996; Steponkus *et al.*, 1998; Thomashow, 1999; Thomashow, 2001; Gilmour *et al.*, 2004; Knight *et al.*, 2004; Maruyama *et al.*, 2004). Some of the COR genes encode hydrophilic polypeptides that have little or no homology to previously characterized proteins. These COR polypeptides are homologous to cryoprotective proteins which protect the plasma membrane from cold shock and destabilization and lead to freezing tolerance (Steponkus *et al.*, 1998). One of the COR genes, *kin1* from *Arabidopsis*, was reported to be homologous to the antifreeze protein of fish (Kurkela & Franck, 1990).

In *Arabidopsis*, during cold, drought and salt stress responses, the DREB1A or DREB1B transcription factors bind to the same *cis*-acting DRE elements. Therefore DRE element binding site could be the cross talk point between cold, drought and salt stress signalling in *Arabidopsis*. Along with DRE, the promoter element ABRE plays an integral part in this cross talk (Knight & Knight, 2001; Gilmour *et al.*, 2004; Xiao *et al.*, 2006). DREB1 family of regulatory genes possibly works as a “master-switch” in COR gene induction and cold acclimation during cold stress (Thomashow, 1999, 2001). More genes will most likely be identified in the future, as in *Arabidopsis* transcriptome profiling using Affymetrix GeneChips revealed about 939 cold-regulated genes of which 655 genes were upregulated (Lee *et al.*, 2005).

Plants cope with extreme high temperatures by the induction of a group of genes called heat shock genes. Proteins encoded by heat shock genes enable the plant cell to survive in two ways. One group of heat shock proteins (HSPs) act as molecular chaperons that counteract protein denaturation and aggregation, and other HSPs, including ubiquitin and certain proteases, target non-native proteins for degradation (Gurley, 2000). *Arabidopsis* mutants that have a mutation in heat shock gene *Hsp101* are unable to acquire tolerance to high temperature (Queitsch *et al.*, 2000; Hong & Vierling, 2001). Maize heat shock protein Hsp101 accumulated in higher levels in response to 40°C heat shock but negatively influenced the growth rate of the primary root (Nieto-Sotelo *et al.*, 2002). In *Arabidopsis*, Hsp70 showed 2 to 20 fold induction by 40°C heat shock and conferred tolerance to heat and drought stress (Sung *et al.*, 2001; Sung & Guy, 2003). However, some of the HSPs, such as *Spinacia oleracea* Hsp70 and *Brassica napus* Hsp90, are also involved in cold acclimation of the plant (Anderson *et al.*, 1994; Krishna *et al.*, 1995; Thomashow, 1999). Not all HSPs are stress-inducible, but all known stresses, if sufficiently intense, were able to induce HSP expression (Feder & Hofmann, 1999). Plant perception, signal transduction, transcriptional activation as well as metabolic and physical responses to low or high temperature and tolerance reveal that plants have different independent and non-overlapping as well as parallel and similar responses to such conditions (Sung *et al.*, 2003).

## **Salt**

The uptake of minerals and nutrients from the soil is essential for plant growth; however, excessive presence of soluble salts can cause severe osmotic stress in plants. Saline soils generally constitute large amounts of water soluble salts such as sodium chloride (NaCl), sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>), carbonates of sodium (NaHCO<sub>3</sub> and Na<sub>2</sub>CO<sub>3</sub>), sodium nitrate (NaNO<sub>3</sub>), magnesium chloride (MgCl<sub>2</sub>), magnesium sulphate (MgSO<sub>4</sub>), potassium sulphate (K<sub>2</sub>SO<sub>4</sub>), calcium carbonate (CaCO<sub>3</sub>) and calcium sulfate (CaSO<sub>4</sub>). However, in salt-prone soils NaCl (the main ingredient of table salt) causes the most harmful inhibitory effects on seed germination and plant growth as a result of osmotic and ionic stress (Flowers *et al.*, 1977).

Salt stress in general reduces the water uptake capacity of the plant, which as a consequence reduces growth rate and metabolic activity. The initial growth reduction could be due to hormonal signals generated by the roots encountering salinity (Munns, 2002). As a more long term impact of salinity, the excessive salt toxicity levels lead to senescence and reduce the photosynthetic capacity due to the closure of stomata and limited carbon dioxide uptake, which cannot sustain proper growth (Zhu, 2001a; Munns, 2002).

Up to certain level, the salt or NaCl concentration can enhance plant growth, but a harmful excessive level of salt can cause severe damage to plant growth and development. The main two effects of salinity in the physiological and biochemical phases of the plant growth are the osmotic stress due to changes in

osmotic potential and the ionic imbalance and toxicity in the cell. The presence of a higher salt concentration in the saline soil changes the osmotic potential of the cell, which inhibits the ability of the plant to take up water as well as necessary minerals and ions, such as  $K^+$  and  $Ca^{2+}$ . Secondly, inhibition of growth and photosynthesis by  $Na^+$  and  $Cl^-$  toxicity is the most common effect when plants are exposed to salinity.  $Na^+$  causes the primary damage due to ion toxicity. High  $Na^+$  in soil competes with other nutrients and causes their deficiency in the plant. Metabolic toxicity occurs because  $Na^+$  competes and substitutes the  $K^+$  that is essential for many enzyme activities and protein synthesis (Hasegawa *et al.*, 2000; Tester & Davenport, 2003; Munns *et al.*, 2006). These primary damaging effects are followed by subsequent secondary stresses or inhibitory effects such as the production of ROS during salinity that cause oxidative damage to membrane lipid and proteins and eventually lead to programmed cell death (PCD). The inhibitory effect on cell growth, photosynthesis, membrane destabilization, metabolic toxicity and even acceleration of cell death are also evident during salt stress (Hasegawa *et al.*, 2000; Apse & Blumwald, 2002; Lin *et al.*, 2006). Slower growth rate is an adaptive mechanism for plant to survive in stress situation, which allows the plant to maintain and manipulate energy resources and survive the stress. During stress one of the most important effects is the inhibition of cell division and expansion, and consequently, the slower plant growth leading to significant loss of plant productivity (Zhu, 2001a).

The ability to maintain the right cellular cytosolic sodium concentration is crucial for the growth of the plant in high salt concentration. The most essential way of maintaining the cellular homeostasis is by keeping the cytosolic  $Na^+$  concentration at a low level by minimizing  $Na^+$  influx and maximizing  $Na^+$  efflux.  $Na^+$  detoxification by compartmentalization into vacuoles and cellular osmotic adjustment are also essential for the plants ability to deal with salt stress. Most of the higher plants try to exclude  $Na^+$  by  $Na^+/H^+$  antiport at the plasma membrane.  $Na^+/H^+$  antiporters utilize the proton motive force generated by the plasma membrane  $H^+$ -ATPase pump for  $H^+$  influx into the cell along its electrochemical gradient and  $Na^+$  efflux against its electrochemical gradient.  $H^+$ -ATPase pumps  $H^+$  out of the cell by ATP hydrolysis through an electrochemical  $H^+$  gradient (Blumwald *et al.*, 2000; Tester & Davenport, 2003).

The potassium ion  $K^+$  is the most abundant cation and an important macronutrient in higher plants. It is needed for enzyme activation, protein synthesis and photosynthesis and mediates osmoregulation during cell expansion, stomatal movements and tropisms. In contrast, closely related  $Na^+$  that compete with  $K^+$  has a toxic effect and causes osmotic and ionic stresses and inhibits vital enzyme activities. Therefore, it is very important to maintain the right  $K^+:Na^+$  ratio in the cytosol for the normal function of cells. It has been suggested that  $Na^+$  could use the  $K^+$  transport channel and possibly the mechanisms for  $Na^+$  entry into roots is through  $K^+$  and  $Ca^{2+}$  transporters (Mäser *et al.*, 2002; Xiong & Zhu, 2002).

Like potassium, the calcium ion  $Ca^{2+}$  also plays an important role in signalling cell metabolism responses to abiotic stresses including temperature stress, salt stress, oxidative stress and anoxia. In *Arabidopsis*, cellular calcium mediates the

signalling that leads to an increased expression of abiotic stress responsive genes which may encode proteins having protective functions.  $\text{Ca}^{2+}$  uptake and transport are severely affected during high salinity, and as a result  $\text{Ca}^{2+}$  deficiency can be observed during salt stress (Rengel, 1992; Knight *et al.*, 1998; Knight & Knight, 2001; Zhu, 2002). A salt stress-induced calcium signal is most likely to induce the distinct Salt-Overly-Sensitive (SOS) pathways in plants in response to the ionic stress, particularly  $\text{Na}^+$  stress. In *Arabidopsis*, the *sos* mutants, *sos1*, *sos2* and *sos3* are found to be hypersensitive to salt and reveal a novel calcium-regulated protein kinase signalling complex in response to the ionic stress (Zhu, 2000, 2003; Chinnusamy *et al.*, 2004). Extensive genetic analysis reveals the mechanism within the SOS pathways. To maintain the  $\text{K}^+$  and  $\text{Na}^+$  intracellular homeostasis during salt stress, a calcium-binding protein SOS3 binds and activates a regulatory protein, protein kinase SOS2, which is found to be essential to salt tolerance, in a  $\text{Ca}^{2+}$ -dependent manner. Under salt stress the SOS3–SOS2 kinase complex regulates the expression and activity of ion transporters such as SOS1, a plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter to re-establish the cellular ionic homeostasis. In addition to transporter activity SOS1 is possibly a sensor of  $\text{Na}^+$  (Halfter *et al.*, 2000; Liu *et al.*, 2000; Shi *et al.*, 2000; Zhu 2000, 2001a, b, 2003; Chinnusamy *et al.*, 2004, 2005).

During salt stress a cascade of molecular and biochemical changes occur as functions of different salt tolerant genes (Borsani *et al.*, 2003). Many genes and transcriptional regulatory mechanisms function simultaneously in regulating drought, cold, or high salinity stress signal transduction pathways. The transcription factors could govern the expression of all these stress-inducible genes either cooperatively or independently, and may constitute gene networks in *Arabidopsis* (Zhu, 2001b; Shinozaki & Yamaguchi-Shinozaki, 2007). In fact, like in cold acclimation, whole plants can also get improved salt tolerance when being exposed to high but non-lethal concentrations of salt. Exposure of seedlings of *Sorghum bicolor* to a sublethal NaCl concentration (75-150 mM NaCl) for 20 days induced an ability to grow them at 300 mM NaCl, a lethal concentration for non-treated plants (Amzallag *et al.*, 1990).

Plant species are divided into two groups according to their ability to grow in high salinity. Halophytes are tolerant to high levels of salt, such as 400 mM which can be as high as or even higher than sea water. On the other hand, most of the plant species classified as glycophytes cannot withstand high salinity (Hasegawa *et al.*, 2000; Zhu, 2001a; Chinnusamy *et al.*, 2004; Flowers, 2004). The two main mechanisms for salt tolerance utilized by halophytes are minimizing the entry of salt into the plant, and minimizing the concentration of salt in the cytoplasm and cell wall. Salt tolerant plants protect themselves from salinity by lowering the rate of  $\text{Na}^+$  and  $\text{Cl}^-$  transport to leaves and compartmentalizing these ions to vacuoles other than cytoplasm or cell walls to avoid salt toxicity, while the salt-sensitive glycophytes have poor ability to exclude salt and thus accumulate toxic levels of salt in the cytosol (Munns, 2002).

During salt stress one of the complex molecular responses in the plant is to produce stress proteins and compatible osmolytes that may have protective and scavenging functions against cell damaging ROS molecules produced during the

stress (Zhu, 2001a). Transgenic tobacco expressing bacterial glycine betaine (an osmolyte) pathway genes encoding choline dehydrogenase (CDH) and betaine aldehyde dehydrogenase (BADH) accumulated small amounts of glycine betaine and thus exhibited increased tolerance to salt stress as well as significantly improved photosynthetic capacity (Holmström *et al.*, 2000). Transgenic rice with trehalose biosynthesis genes *otsA* and *otsB* from *Escherichia coli* showed enhanced growth in 100 mM salt treatment compare to nontransformed plants (Garg *et al.*, 2002). In tomato, wounding enhances salt tolerance through wound signalling peptide systemin and JA-dependent pathways, and a Ca<sup>2+</sup> dependent protein kinase LeCDPK1 might be involved in this wounding-salt stress tolerance signalling (Capiati *et al.*, 2006). Finally, a halophytic plant called salt cress (*Thellungiella halophila*) is a small winter annual crucifer and can be used as a model system like *Arabidopsis* to elucidate the molecular and biochemical aspects of salt tolerance in plants (Inan *et al.*, 2004; Volkov *et al.*, 2004).

### ***Hypoxia***

Higher plants require access to free water for easy nutrient uptake but excess water in the root surroundings can be suffocating, injurious or even lethal because it blocks the transfer of free oxygen and other gases between the soil and the atmosphere (Drew, 1997). The limitation or lack of free oxygen is common in waterlogged soil as a result of excess rainfall, flooding, and in ice encasement during winter due to snow melting and refreeze which forms solid ice layer on the soil surface. In all these situations the gas exchange is reduced to extremely low level (Andrews, 1996; Drew, 1997; Vartapetian & Jackson, 1997; Dennis *et al.*, 2000; Blokhina *et al.*, 2003; Jackson & Colmer, 2005).

The partial deprivation of the free oxygen in soil is termed as hypoxia, whereas anoxia is the complete absence of free oxygen and both are detrimental to the growth and development of aerobic organism like higher plants (Crawford & Braendle, 1996; Vartapetian *et al.*, 2003). Plants adapt to hypoxia and anoxia by avoidance of or adaptation to oxygen deficits. Avoidance occurs through morphological adaptation and provision of oxygen *via* leaves. Adaptation to oxygen deficit includes a by lowering of metabolic rates, removal of toxic products and compensating the metabolic changes such as alternation of metabolic pathways, endogenous oxidation, adaptive enzyme synthesis, preservation of membrane integrity and metabolism of anaerobic products (Crawford & Braendle, 1996; Vartapetian *et al.*, 2003). In hypoxic conditions like seasonal flooding, the first plant response is closure of stomata and wilting, similar responses to those found in drought stress. Plants also show reduced photosynthetic and transpiration rates during hypoxia. The closure of stomata is regulated by ABA accumulation during drought, whereas during hypoxia the closure of stomata is rather due to the release of ET despite repressed ABA accumulation (Else *et al.*, 1996; Blanke & Cooke, 2004; Yordanova *et al.*, 2005). One of the characteristic consequences of hypoxia is the generation of ROS in plant tissues due to oxygen deprivation and reoxygenation (Blokhina *et al.*, 2003). In plant, oxygen insufficiency causes altered cellular metabolism and can dramatically reduce productivity. During hypoxia plants adopt to the altered carbohydrate consumption and anaerobic

metabolism. Under low oxygen condition the enzyme alcohol dehydrogenase (ADH) metabolizes the anaerobic fermentation, an alternative way of sugar metabolism leading also to production of ROS (Fukao & Bailey-Serres, 2004).

In *Arabidopsis*, exposure to moderate hypoxia (5% O<sub>2</sub>) works as hypoxic acclimation and helps the plant to survive in extreme hypoxia (0.1% O<sub>2</sub>) but hypoxic acclimation did not improve tolerance to anoxia (0% O<sub>2</sub>) (Ellis *et al.*, 1999). Two enzymes in the glycolytic (alcoholic fermentation) pathway, ADH and pyruvate decarboxylase (PDC) are the members of the anaerobic polypeptides, are strongly induced in hypoxic conditions. Expression of the genes *adh* and *pdh* enhances the ethanol production and result in an increased rate of glycolysis that compensates the metabolic changes through altered carbohydrate consumption during hypoxia. In the anaerobic glycolytic pathway the first enzyme PDC non-oxidatively decarboxylates the pyruvate into acetaldehyde and in the subsequent reaction ethanol is produced from acetaldehyde by the enzyme ADH (Andrews, 1996; Hossain *et al.*, 1996). In *Arabidopsis* *adh* and *pdh* genes are induced in roots and leaves during low oxygen conditions. *Arabidopsis adh1* gene is induced by many stress factors such as hypoxia, cold, drought, wounding and hormone ABA (de Bruxelles *et al.*, 1996; Dolferus *et al.*, 1997, 2003).

ET signalling is involved for the induction of *adh* during the later stages of hypoxia in *Arabidopsis* (Peng *et al.*, 2001). The reduced expression of *adh* has been observed in hypoxic condition in two ET insensitive mutants in ET-signalling pathways *etr1* and *ein2*. During hypoxic conditions, ET precursor 1-aminocyclopropane-1-carboxylate (ACC) is produced due to the increase of ACC synthase in plant roots and ACC is further converted to ET by ACC oxidase in shoots. In hypoxia, ET production is increased because of the partial availability of the molecular free O<sub>2</sub> which converts ACC to ET (Drew, 1997). Recently, Xu *et al.*, (2006) described the identification of a cluster of three genes at the *Sub1* locus in the rice (*Oryza sativa*) chromosome, encoding putative ET response factors. Overexpression of one of these genes, *Sub1A-1*, enhance the expression of *adh* genes in a submergence-intolerant *O. sativa* ssp. *japonica* resulting in the enhanced submerge tolerance.

### ***Reactive oxygen species (ROS)***

In plants, a wide range of abiotic and biotic stress factors can induce oxidative stress. Salt, drought, heat, hypoxia and oxidative stress are accompanied by the formation of ROS molecules such as superoxide anion O<sub>2</sub><sup>-</sup>, hydrogen peroxide H<sub>2</sub>O<sub>2</sub>, and hydroxyl ion OH<sup>-</sup>, which damage membranes and macromolecules (Mittler, 2002; Blokhina *et al.*, 2003).

ROS substances that are activated and accumulated in response to different sources of stress may have dual roles, causing hypersensitive response-programmed cell death (HR-PCD) in plant host cells and defence response to pathogenic invaders. The activation of protein kinase-NADPH oxidase enzyme cascades are involved in the release of ROS substances in plant cells and ROS production is the very early response in the process of cell or tissue necrosis

(Bolwell & Wojtaszek, 1997; Király, 1998; Heath, 2000; Kwak *et al.*, 2003; Patel *et al.*, 2006). The important plant defence signalling molecule SA has a protective role on H<sub>2</sub>O<sub>2</sub> production. SA inhibits the activity of H<sub>2</sub>O<sub>2</sub> scavenging enzymes catalases (CAT) or ascorbate peroxidase, APX (Chen *et al.*, 1993, 1995; Durner & Klessig, 1995, 1996). However, H<sub>2</sub>O<sub>2</sub> might have a role as second messenger of SA to activate the induction of defence related gene expressions that lead to plants systemic defence or resistance response (Chen *et al.*, 1995; Durner & Klessig, 1996; Ryals *et al.*, 1996).

Plants have developed several antioxidation strategies to scavenge these toxic compounds. Enhancement of antioxidant defence in plants can thus increase tolerance to different stress factors. The main way to reduce damage caused by ROS in plant cells is accomplished by several antioxidants (ROS scavengers) that include enzymes such as CAT, superoxide dismutase (SOD), APX and glutathione reductase, as well as non-enzyme molecules such as ascorbate, glutathione, carotenoids, and anthocyanins (Bowler *et al.*, 1992; Gould *et al.*, 2002; Mittler, 2002; Blokhina *et al.*, 2003;). Additional compounds, such as osmolytes, proteins (e.g. peroxiredoxin) and amphiphilic molecules (e.g. tocopherol), also showed functions as ROS scavengers (Noctor & Foyer, 1998; Mittler, 2002; Blokhina *et al.*, 2003).

### **Trehalose production in plants**

Different stress conditions such as drought, salinity, low and high temperature and hypoxia cause a decrease in turgor pressure at the cellular level. During stress, plants usually respond by accumulating organic compounds known as compatible solutes or osmolytes. Osmolytes protect the cell turgor and restore the water status of the cell by maintaining the cellular water potential as well as acting as chaperones to stabilize the membranes or scavengers of ROS. Osmolytes accumulating during stress in different plant species can be polyols and sugars, such as mannitol, trehalose, and amino acids such as proline or quaternary amines such as glycine betaine or dimethylsulfoniopropionate. In most cases the expression of genes leading to the production of these compatible solutes in transgenic plants may improve stress tolerance (Holmström *et al.*, 1996, 2000; Bajaj *et al.*, 1999; Nuccio *et al.*, 1999; Chen & Murata, 2002).

Simple sugars, such as sucrose and trehalose, have been shown to stabilize biomaterials of various composition and origin (Liao, 2002; Pereira *et al.*, 2004). Trehalose is a non-reducing disaccharide of glucose ( $\alpha$ -D-glucopyranosyl-1,1- $\alpha$ -D-glucopyranoside) formed by two pyranose rings in the same configuration. The sugar possesses unique physical qualities like high hydrophilicity, and high chemical and heat stability. Trehalose is ubiquitous in a wide variety of organisms, including bacteria, yeast, fungi, insects, other invertebrates, and lower and higher plants. (Elbein, 1974; Wingler, 2002; Elbein *et al.*, 2003). It has been shown that in a wide variety of organisms, accumulation of trehalose can protect proteins and cellular membranes from inactivation or denaturation caused by a variety of stress conditions such as drought, salinity, heat and cold and oxidation (Elbein *et al.*, 2003). Besides the role of trehalose as a compatible solute, it is also believed to

have an important role as a carbohydrate reserve in many organisms, which serve as a source of energy and carbon. The genes in trehalose biosynthesis pathways in yeast, bacteria and desiccation tolerant plants have been widely used in different plant species to confer stress tolerance (Holmström *et al.*, 1996; Goddijn & van Dun, 1999; Zentella *et al.*, 1999; Yeo *et al.*, 2000; Garg *et al.*, 2002; Blumwald *et al.*, 2004).

### ***Trehalose biosynthesis***

Although most of the plants do not accumulate detectable amount of trehalose, it has been speculated that the trehalose biosynthesis pathway is present in a wide range of plant species (Wingler, 2002). Recent studies show that there could be five different trehalose biosynthesis pathways present in bacteria while only one can be found in eukaryotes (Avonce *et al.*, 2006). Generally, in the metabolic pathway of trehalose biosynthesis, UDP-glucose or glucose-6-phosphate are catabolized to trehalose-6-phosphate (T6P) by the enzyme trehalose-6-phosphate synthase, TPS (yeast, *Saccharomyces cerevisiae*: *ScTPS1/tps1* and *E. coli*: *otsA*) and T6P is subsequently converted to trehalose by the second enzyme, trehalose-6-phosphate phosphatase, TPP (yeast: *ScTPS2/tps2* and *E. coli*: *otsB*). In yeast, two regulatory proteins TPS3 and TSL1 are also necessary for the stabilization and maximal activity of the trehalose synthase complex (Vuorio *et al.*, 1993; Reinders *et al.*, 1997; Bell *et al.*, 1998; Argüelles, 2000). In an alternative pathway in *E. coli*, an enzyme T6P hydrolase can convert T6P to glucose-6-phosphate (G6P) and glucose (Vuorio *et al.*, 1993; Argüelles, 2000). In bacteria, such as *Mycobacterium smegmatis* and *M. tuberculosis* the TPS can utilize either GDP-glucose or UDP-glucose, while in yeast the TPS is mostly specific for UDP-glucose (Klutts *et al.*, 2003). In all these organisms, trehalose can be broken down to glucose by trehalase (yeast: *NTH1* and *E. coli*: *treF* or *treA*) (Boos *et al.*, 1987; Horlacher *et al.*, 1996; Wera *et al.*, 1999; Argüelles, 2000).

Bioinformatics analyses of the *Arabidopsis* genome reveals that there are about 11 TPS homologues divided into two classes depending on the presence of phosphatase boxes in their TPP domain (C-terminal part). This classification is developed by homology comparison with the yeast TPS and TPP genes. Class I consists of four genes including *AtTPS1* that lack phosphatase boxes and class II consists of seven genes that contain phosphatase boxes (Leyman *et al.*, 2001; Eastmond *et al.*, 2003). The *Arabidopsis AtTPS1* was cloned and characterized as TPS by complementing the yeast mutant lacking *tps1* (Blázquez *et al.*, 1998). *AtTPS1* is expressed in almost all *Arabidopsis* tissues while a mutant disrupted for *AtTPS1* is embryo lethal (Eastmond *et al.*, 2002; Gómez *et al.*, 2005). In *Arabidopsis*, *AtTPS1* positively regulates the cell wall biogenesis and cell division and influences the carbohydrate metabolism during embryo development (Gómez *et al.*, 2006). A new class III of TPP genes has been reported in *Arabidopsis* that consists of ten putative TPP genes containing the TPP domain. Two of the genes *AtTPPA* and *AtTPPB* are already characterized as TPP by complementing the yeast mutant lacking *tps2* gene (Vogel *et al.*, 1998; Eastmond *et al.*, 2003). Trehalase may have a regulatory role in carbohydrate metabolism and allocation in plants and in *Arabidopsis* a trehalase (*AtTRE1*) gene has been identified by

complementation of trehalase-deficient yeast mutant (Müller *et al.*, 2001) and several homologues of TPS, TPP and trehalase genes have now been cloned from a number of other plant species such as tobacco, potato and sunflower (Goddijn & Smeekens, 1998). A list of AtTPS1 orthologues found in different plant species such as sugar beet, sugar cane, tobacco, potato, lotus, tomato, apple, soybean, cotton, sunflower, maize, rice, wheat, barley, sorghum, salt cress (*Thellungiella halophila*) and almond has been presented by Leyman *et al.* (2006).

TPS, TPP and trehalase genes have been characterized in many species. A TPS gene of the resurrection plant *Selaginella lepidophylla* conferred thermotolerance and osmotolerance in yeast (Zentella *et al.*, 1999) and a cotton TPS gene was found to be drought inducible (Kosmas *et al.*, 2006). In rice, TPP genes *OsTPP1* and *OsTPP2* were induced due to cold, drought, salinity and ABA (Pramanik & Imai, 2005; Shima *et al.*, 2007), and in soybean, a trehalase gene *GMTRE1* was constantly expressed at a low level in different tissues (Aeschbacher *et al.*, 1999). Immunogold localization assay showed the existence of an *AtTPS1* homologue in tobacco (Almeida *et al.*, 2007). A tobacco TPP gene *NtTPPL* complemented the yeast mutant lacking *tps2*. *NtTPPL* was induced by heat stress and partially induced by salt and low temperature (Wang *et al.*, 2005). In most of the higher plant species, whether transgenic or not for the trehalose biosynthesis gene(s), the trehalose-accumulation has been found to be merely detectable (Holmström *et al.*, 1996; Romero *et al.*, 1997). However, trehalase inhibitor ValidamycinA treatment enabled the detection of trehalose accumulation in *Arabidopsis* and in transgenic tobacco and potato plants overexpressing trehalose biosynthesis genes (Goddijn *et al.*, 1997; Müller *et al.*, 2001; Karim *et al.*, 2007).

In some organisms alternative trehalose pathways exist. Alternative trehalose biosynthesis genes trehalose synthase (*TSase*) from the basidiomycete *Grifola frondosa* Fr. or trehalose phosphorylase (*PsTP*) gene of edible mushroom *Pleurotus sajor-caju* could catalyze the trehalose synthesis directly from glucose and glucose-1-phosphate in a one step process. Interestingly, *PsTP* could complement the growth of yeast double mutants lacking *tps1* and *tps2* (Han *et al.*, 2005; Zhang *et al.*, 2005). Many pathogens can induce trehalose production in plants during their symbiotic or pathogenic interaction with the plant. For example, a plant pathogen *Plasmodiophora brassicae* could induce trehalose production in *Arabidopsis* roots due to the expression of putative TPS gene *PbTPS1*. The pathogen also induced *Arabidopsis* native trehalase gene *AtTRE1* which indicated that trehalase was induced as a part of the plant defence system to break down excess trehalose that could interfere with the plant defence mechanism through creating a disadvantageous impact on plant sensing and signalling as well as the regulation of the carbon metabolism (Brodmann *et al.*, 2002). Recent studies suggest that trehalose may have a regulatory influence on the expression of different stress related genes and many of these trehalose inducible genes support the JA/ET dependent signal transduction pathways (Bae *et al.*, 2005a,b; Shima *et al.*, 2007). Apart from the role of trehalose or T6P, it could also be speculated that similar to the situation in yeast *Tps1*, plant TPS could exert a regulatory function on sugar signalling pathways (Bonini *et al.*, 2003; Karim *et al.*, 2007).

### ***Drought tolerance with growth aberrations***

The introduction of yeast TPS gene (*ScTPS1*) in tobacco produced enhanced drought tolerant transgenic plants but with multiple altered pleiotropic phenotypes such as lancet-shaped leaves and stunted growth (Holmström *et al.*, 1996; Romero *et al.*, 1997). Yeast *ScTPS1* under the control of the CaMV 35S promoter increased drought tolerance in potato but also caused an abnormal phenotype ranging from dwarfish growth to aberrant root phenotype (Yeo *et al.*, 2000). *E. coli* genes *otsA* and *otsB* have shown earlier to cause enhanced stress tolerance and higher biomass in tobacco and potato along with altered phenotypes such as larger or lancet-shaped leaves and growth defects (Goddijn *et al.*, 1997; Pilon-Smits *et al.*, 1998; Goddijn & van Dun, 1999).

During stress, higher plants possibly use the sucrose as a transport sugar as the best choice (Wingler, 2002). Adding trehalose in the media strongly inhibits the root elongation and leaf growth, which indicates that trehalose, at least exogenously added, may have a toxic effect on plant cell growth (Wingler *et al.*, 2000; Fritzius *et al.*, 2001). In trehalose producing plants most of the studies showed a higher accumulation of many sugars including glucose, fructose, trehalose, sucrose and starch as well as enhanced stress tolerance. It might be possible that excess accumulation of sugars could cause problems in the sugar sensing and signalling which might have an adverse effect on normal physiological development and growth (Brodmann *et al.*, 2002; Eastmond *et al.*, 2002; Avonce *et al.*, 2005). In *Arabidopsis* short root growth was observed in plants grown in trehalose containing media and that could be due to the induced expression of starch synthesis gene and higher accumulation of starch (Ramon *et al.*, 2007). In the *pho3* mutant of *Arabidopsis* which has a non functional SUC2 gene that encodes a sucrose-H<sup>+</sup> symporter, AGPase (ADP-glucose pyrophosphorylase that catalyzes the first step of starch synthesis) activity has found to be higher. The *pho3* mutant plants accumulate huge amounts of sugars such as glucose, fructose, sucrose and starch and also show severe retarded growth (Lloyd & Zakhleniuk, 2004).

### ***Drought tolerance without growth aberrations***

In rice, introducing trehalose bifunctional hybrid gene TPSP from *E. coli* (*otsA* and *otsB*) enhance the trehalose accumulation about 200 fold more than the independent transgenic plants containing *otsA* or *otsB* alone without causing any growth aberrations. Transgenic plants with trehalose bifunctional genes also confer tolerance to drought, cold and salt and increased photosynthetic capacity (Seo *et al.*, 2000; Garg *et al.*, 2002; Jang *et al.*, 2003). Tobacco chloroplasts transformed with *ScTPS1* produce transgenics with enhanced drought tolerance without any growth defect which was obvious in nuclear transformed ScTps1 transgenics. Trehalose accumulation was almost 25-fold higher (approximately 400 µg/g fresh weight) in chloroplast transgenic plants over the nuclear transgenic plants (Lee *et al.*, 2003).

Tobacco plants transformed with *TSase* from *G. frondosa* Fr. showed no negative growth retardation but rather showed enhanced drought and salt tolerance (Zhang *et al.*, 2005). Tobacco plants expressing *PsTP* of *P. sajor-caju* constitutively under the control of CaMV 35S promoter produced drought tolerance without any growth defect. Since *TSase* and *PsTP* catalyzed the trehalose directly from glucose and glucose-1-phosphate it could be hypothesized that the TPS protein or T6P produced by the common trehalose biosynthesis pathway might have the influencing effect on altered pleiotropic phenotypes (Han *et al.*, 2005; Zhang *et al.*, 2005).

It has been shown recently that enhanced drought tolerance is believed to be coupled with growth aberrations due to the overproduction of yeast *Tps1* in tobacco or *Arabidopsis* cytosol. Growth aberration has been observed in transgenic plants having only *ScTps1* overproduction. These growth defects can be overcome through different metabolic approaches (Karim *et al.*, 2007). First of all, constitutive overproduction of both *ScTPS1* and *ScTPS2* in tobacco transgenics under the control of *Arabidopsis* RuBisCO (ribulose-1,5-bisphosphate carboxylase) promoter eliminates the growth problem, while retaining the improved drought tolerance. Moreover, the overexpression of both *ScTPS1* and *ScTPS2* increases the trehalose accumulation in the transgenic tobacco. In another approach the overexpression of *ScTPS1* under the control of a drought inducible *Arabidopsis* promoter *AtRAB18* (Lång & Palva, 1992) showed enhanced drought tolerance and drought inducible trehalose accumulation without having any growth retardation in tobacco (Holmström *et al.*, 1996; Karim *et al.*, 2007). It has been reported that the transcription factor DREB1A that binds to dehydration responsive promoter element plays an important role in drought, cold and salt tolerance. Expressing the gene for DREB1A confers all these stress tolerances. In *Arabidopsis*, overexpression of *DREB1A* cDNA under the control of strong constitutive 35S cauliflower mosaic virus (CaMV) promoter has resulted in an enhanced stress tolerance as well as severe growth retardation while expression from a stress inducible *rd29A* promoter has minimized the negative growth effects while providing a greater tolerance to stress conditions (Kasuga *et al.*, 1999).

### ***Role of trehalose-6-phosphate***

The accumulation of metabolic intermediate sugar phosphate T6P, is believed to have the toxic effect on plant cell growth, which causes altered growth and morphological phenotypes (Schluepmann *et al.*, 2004). Human fungal pathogen *Candida albicans* contains TPS and TPP genes *CaTPS1* and *CaTPS2* respectively, and inactivation of *CaTPS1* resulted in inhibition of hyphae formation and the virulence of the pathogen, on the other hand, disruption of the *CaTPS2* resulted in 50-fold hyper-accumulation of T6P, thermosensitivity and rapid death of the cells at 44°C. This indicates that the T6P is toxic and might itself elicit the stress response even in plants (Van Dijck *et al.*, 2002a). However, in plants T6P is found to be required for sugar utilization in the cell (Goddijn & Smeekens, 1998; Schluepmann *et al.*, 2003). In a recent study in *Arabidopsis* it has been demonstrated that changes in sucrose levels correlate with rapid changes in the

T6P content in cytosol which in turn activates AGPase that catalyzes the first step of starch synthesis in the chloroplasts. Thus T6P might have a signalling or regulatory role in sugar sensing and utilization of carbohydrate in between cytosol and chloroplasts (Kolbe *et al.*, 2005; Lunn *et al.*, 2006; Lunn, 2007).

In the plant, T6P is thought to be controlling the photosynthetic capacity (Paul & Pellny, 2003). Introducing the *E. coli* TPS gene *otsA* into tobacco enhanced the photosynthetic capacity (CO<sub>2</sub> assimilation per unit leaf area under light and RuBisCO activity) and it was correlated with increased T6P content rather than trehalose in the plant. However, plants transformed with the *E. coli* phosphatase gene *otsB* or *trec* which encodes trehalose phosphate hydrolase, produced the opposite effect with T6P reduced to almost half of the amount found in wild type as well as reduced photosynthetic capacity (Paul *et al.*, 2001; Pellny *et al.*, 2004).

When carbohydrates are abundant, plants switch off photosynthesis in a process of efficient use of energy and the carbon metabolism. Glucose plays a prime role in this metabolism by regulating an array of genes where hexokinase, HXK acts as a glucose sensor. In yeast, the T6P producing enzyme ScTps1 controls sugar influx into glycolysis and the synthesis of T6P has been found to have an inhibitory effect on hexokinase activity (Thevelein & Hohmann, 1995). TPS and T6P regulate the hexokinase and glycolysis activity differently in yeast and plants. The yeast TPS system can be substituted partially by the plant TPS system for example the *AtTPS1* can complement the yeast *tps1* null mutant growth in glucose medium but without yeast hexokinase activity. The lower activity of plant TPS enzymes are due to the N-terminal domains, by truncating the N-terminal part of the genes could restore their full functionality in yeast in a glucose medium (Goddijn & Smeekens, 1998; Van Dijck *et al.*, 2002b). In plants three different hexokinase-dependent/-independent glucose signal transduction pathways are involved. In *Arabidopsis* the first pathway is the *Arabidopsis* hexokinase HXK-dependent pathway where the photosynthetic and sugar-regulated gene expression is correlated with AtHXK activity. The second one is the glycolysis-dependent pathway mediated by both plant hexokinase AtHXK and antagonistic yeast hexokinase activity. The last one is AtHXK-independent pathway through which it might be possible that the sugar transporters and sugar sensors such as extracellular sugar-binding proteins might perceive and transmit the glucose signalling as well as AGPase mediated sugar signalling (Xiao *et al.*, 2000). *Arabidopsis* hexokinase AtHXK plays an important role under a wide range of plant development and growth conditions. AtHXK might have dual role in glucose signalling and metabolism and the effect on growth promotion or inhibition is dependent on the glucose concentration and the cell conditions. AtTPS1 expression is AtHXK dependent and AtTPS1 positively regulates AtHXK expression in normal condition but represses AtHXK activity only in the presence of glucose. AtHXK has been found to be involved in glucose responses, cell proliferation, leaf expansion, root and inflorescence growth, reproduction and senescence as well as related gene expressions. All the physiological functions of AtHXK in plant growth and development, as well as the role of AtTPS1 or T6P on glucose and ABA signalling may be the key factors behind the drought tolerance and vegetative development (Moore *et al.*, 2003; Avonce *et al.*, 2004, 2005).

In a microarray analysis of *Arabidopsis* using the Affymetrix ATH1 chip containing more than 22,500 probe sets reveals that both the putative TPS and TPP genes of trehalose biosynthesis pathways are induced by nitrate in roots but not in shoots. Then it could be suggested that nitrate may have an influence on T6P levels. It might be possible that T6P is a regulator of the pentose phosphate pathway involving nitrate reduction and pentose oxidation (Wang *et al.*, 2003b).

## **Biotic stress signalling**

### ***Wound signalling***

Tissue damage in plants is associated most often with insect herbivore infestation. Phloem-feeding whiteflies and aphids cause small wounds in plant foliage that are perceived as pathogens by plant defence system and activate the SA-dependent and JA/ET-dependent signalling pathways. On the other hand, extensive tissue damage caused by chewing insects such as beetles, caterpillars and cell-content feeders such as mites and thrips activate JA -dependent and -independent wound responses and herbivore responsive genes. Mechanical wounding and herbivore feeding is not equivalent and differently regulate the signal transduction pathways (Walling, 2000). For example in a microarray analysis not all genes found to be induced by mechanical wounding, are induced by herbivore feeding (Reymond *et al.*, 2000). Insect herbivore feeding causes wound-induced responses but the feeding damage can even cause direct and extensive responses because of their highly stimulating oral secretions that work as effective elicitors and cause the production of volatiles and hormonal defence cascades indirectly by plants (Walling, 2000). Similar to insect herbivores wound signalling activates induced defences in plants both locally and systemically by signalling cascades involving systemin, jasmonate, oligogalacturonic acid (OGA) and H<sub>2</sub>O<sub>2</sub> (Gatehouse, 2002). Tissue damage usually induces local osmotic stress responses that are often found to be a key component in the response to mechanical wounding (Reymond *et al.*, 2000; Denekamp & Smeekens, 2003).

Higher plants have various defence responses against abiotic and biotic agents including the transcriptional activation of wound responsive genes (Rojo *et al.*, 1999). These genes generally encode wound-induced proteins. The functions of several wound-induced proteins are well known, such as proteases or proteinase inhibitors, proteins involved in the biosynthesis of plant secondary metabolites or defence signalling compounds and their signal transduction pathways (Ryan, 1990; Sticher *et al.*, 1997; Yen *et al.*, 2001). The defence responses include either hypersensitive response (HR) or the production of antimicrobial secondary metabolites, phytoalexins and pathogenesis related (PR) proteins (Penninckx *et al.*, 1998) or wound healing or repair of the damage (Bowles, 1990). Some of the defence responsive genes are active in the site of wound, whereas others are also active systemically in the non-damaged parts and prevent the pest from spreading. The proteinase inhibitor gene family is a well-characterized example of the systemically inducible genes (Ryan, 1990; Titarenko *et al.*, 1997; Ussuf *et al.*, 2001). The proteinase inhibitors play an important role in plants as a part of the natural plant defence system against a wide range of insect pests and pathogens

including bacteria, fungus, virus and nematodes (Pautot *et al.*, 1991; Urwin *et al.*, 1998; Haq *et al.*, 2004). There are four classes of proteinase inhibitors such as cysteine, serine, metallo-proteinase inhibitors and aspartyl proteinase inhibitors. Several insect families possess cysteine proteinases as part of their digestive systems and both natural and synthetic cysteine proteinase inhibitors have shown their inhibitory effect on digestive cysteine proteinases (Ryan, 1990). Seven cysteine proteinase inhibitors are identified in *Arabidopsis* (Martínez *et al.*, 2005). *Arabidopsis* cysteine proteinase inhibitors AtCYS expressed in transgenic poplar (*Populus alba* L.) plants confers resistance to the larval attack of the chrysomelid beetle *Chrysomela populi* L. (Delledonne *et al.*, 2001).

JA-dependent and JA-independent pathways have been proposed for the wound signal transduction in *Arabidopsis*. Small oligosaccharides, such as fungal-derived chitosan or oligogalacturonides are plant cell wall derived signalling molecules that act as elicitors to activate the cascade of defence genes expression during pathogenesis and wounding (Reymond *et al.*, 1995; Zablackis *et al.*, 1995; Norman *et al.*, 1999; León *et al.*, 2001; Moscatiello *et al.*, 2006). In locally damaged leaves, the activation of oligosaccharide-dependent signal transduction pathways trigger the expression of ET production which blocks the expression of JA-responsive genes but allows the JA-independent wound responses and gene expression. However, in the systemic leaves the diffusion of ET allows the activation of JA responsive genes and the wound signal transduction pathways (Rojo *et al.*, 1999). Wound-induced resistance is usually mediated by the products of the octadecanoid pathway. The production of defence-related products such as phytoalexins and proteinase inhibitors requires signals from octadecanoid compounds such as JA. The interaction between ET and the octadecanoid pathways can be either synergistic or antagonistic (Kessler & Baldwin, 2002). In a wound signal transduction cascade, oligosaccharides, defence related peptide systemin, JA, ET, ABA, electrical pulses and hydraulic pressure form a complex of signalling network and the subsequent activation of proteinase inhibitors and wound responsive genes (Hildmann *et al.*, 1992; Boari & Malone, 1993; Peña-Cortés *et al.*, 1995; O'Donnell *et al.*, 1996; Rojo *et al.*, 1999; León *et al.*, 2001). Local tissue damage elicits a cascade of systemic defence responses against herbivore attack and wounding in many plants. JA confers a major module of the systemic wound signal network (Schilmiller & Howe, 2005).

During wound induction or JA treatment, nitric oxide is produced and works as an inter- or intracellular mediator of the signal transduction pathways, which occurs within a short time and in a JA-dependent manner (Orozco-Cárdenas & Ryan, 2002; Huang *et al.*, 2004). Systemin is an 18-amino-acid polypeptide, produced at the wound sites of tomato plants, and systemically regulates the activation of many herbivore and pathogen responsive defence genes. The systemin is a major regulatory element of wound-induced systemic defence responses in tomato (Ryan, 2000).

Transcriptional profiling reveals that about 8% of the 8,200 genes studied in *Arabidopsis* were altered by wounding at steady-state mRNA levels. Many osmotic stress- and heat shock-regulated genes were highly responsive to wounding and a number of genes involved in SA, JA, ET and ABA pathways

were activated by wounding (Dong, 1998; Cheong *et al.*, 2002, Kim *et al.*, 2003). A wound inducible *Arabidopsis* transcription factor gene *AtMYB102* is rapidly induced by osmotic stress, ABA or salinity (Denekamp & Smeekens, 2003). Furthermore, a large portion of wound inducible genes in *Arabidopsis* were also regulated by water stress (Reymond *et al.*, 2000). Approximately 500 mRNAs have been estimated to constitute the insect-responsive transcriptome in tobacco (Hermsmeier *et al.*, 2001). The expression of these genes can be induced by exposure to exogenous JA (Farmer & Ryan, 1992; McConn *et al.*, 1997). In tomato, salt stress activates the wound responsive genes proteinase inhibitor II (*Inh II*), lipoxygenase (*lox*) and prosystemin (*ps*). Salt stress in plants enhances the wound responses locally and systemically. The simultaneous effect of wounding and 100 mM salt treatment almost enhanced the *Inh II* accumulation to double amount of water and wound treatments only. The *Inh II* accumulation was found to be JA-dependent. The results suggested that wounding and salt stress could regulate same genes (Dombrowski, 2003). A new type of tobacco transmembrane protein NtC7 that belongs to the receptor-like protein family is induced by not only wounding but also with salt and osmotic stresses (Tamura *et al.*, 2003).

### ***Plant-pathogen interactions***

In addition to the abiotic stress factors, a large number of crop losses are due to plant diseases caused by plant pathogens. Plant pathogens can be defined as parasitic biotic organism that can cause disease in plants. These include a wide range of organisms, such as bacteria, fungi, viruses, protozoa and nematodes (Baker *et al.*, 1997; Agrios, 2005). The main characteristic of a plant pathogen is that it acts as a plant parasite by feeding, growing or multiplying and sheltering in host plants. Pathogens do not just take up nutrients from the host plant but cause more damage to the plant by secreting different harmful substances from the pathogen itself and also causing the release of compounds from the host plant as a response mechanism. The pathogen attack and host tissues damages result in biochemical and functional changes, metabolic and physiological disorders leading to partial impairment or even complete death of the host plant. The plant parasitic pathogens that can only grow and reproduce in a living host are called biotrophs or obligate parasites. Some biotrophs that live most of their life cycle on living host but under certain conditions can live on dead organic matter as saprophytes are termed as semibiotrophs or hemibiotrophs. Other plant pathogens, those termed as necrotrophs or facultative parasites live and grow well on dead organic matter most of their life cycle but can attack and kill the living plant cells and show parasitism (Agrios, 2005). Pathogens frequently try to manipulate host defence mechanism by switching from biotrophic to necrotrophic growth during the course of pathogenesis (Abramovitch & Martin, 2004).

Plant defence to pathogens results from a complex combination of structural plant characteristics, constitutive and induced defence responses. Plants achieve these inducible protective conditions through both local and systemic regulation of specific genes. Following the pathogen attack and elicitor recognition by specific receptors in the host plants, there initiates a cascade of cytological, molecular and biochemical responses in cell and tissues (Kombrink & Schmelzer, 2001). Plants

recognize the presence of pathogens through the detection of pathogen associated molecular patterns (PAMPs) and Avr proteins using plant specific pathogen recognition receptors (PRRs). The PAMP-triggered and Avr-triggered signal transduction pathways are sometimes overlapping in plants (Asai *et al.*, 2002; Espinosa & Alfano, 2004; Nürnberger *et al.*, 2004). PRRs activate the signal transduction pathways of MAPK cascades that subsequently activate defence related transcription factors. Pathogens produce protease and phosphatase to inhibit defence-related MAPK activity and pathogenic bacteria use type III effectors to suppress the signal transduction pathways activated by PRR surveillance and defence systems (Espinosa & Alfano, 2004; Nürnberger *et al.*, 2004;). Several MAPK cascades, such as wound-induced protein kinase (WIPK) and SA-induced protein kinase (SIPK), have been reported to be involved in the induction of defence responses. Moreover, the first defence response by plant, such as HR, is preceded by the activation of endogenous SIPK and WIPK (Jonak *et al.*, 2002). During pathogenesis the phytopathogen avirulence genes *avr* function as ligands and host plant resistance genes *R* function as receptors in an interaction leading to plant resistance to disease. Avirulence genes *avr* determine the inability of a given bacterial strain to infect a plant carrying the corresponding *R* gene and this resistance reaction is referred to as gene-for-gene resistance (Van den Ackerveken & Bonas, 1997; Abramovitch & Martin, 2004). Several bacterial *avr* genes showed to contribute to virulence on susceptible plants lacking the corresponding *R* gene. Most of the cloned and characterized plant *R* genes contained a specific sequence motif called leucine-rich repeats (LRRs). LRRs were found to mediate protein-protein interactions and act as receptors for elicitors (the Avr or Avr-dependent proteins) of pathogens. Normally massive intracellular and intercellular changes occurred in plants during *R-avr* interaction (Holt *et al.*, 2000; Dangl & Jones, 2001).

### ***Hypersensitive response (HR)***

Plants cope with pathogen attacks by using mechanisms of resistance that rely both on preformed protective defences and on inducible defences. Recognition of a pathogen by the plant often triggers a localized resistance reaction, known as the hypersensitive response (HR), which is characterized by programmed cell death or PCD at the site of infection and that differs from developmental PCD (Greenberg, 1997). HR also triggers the induction of local and systemic defence responses including cross-linking of cell wall proteins (cell walls surrounding the lesion site are reinforced with callose and lignins), the synthesis of signalling molecules such as SA (Chen *et al.*, 1995), JA (Creelman & Mullet, 1995), ET (Ecker & Davis, 1987), production of ROS and NO, antimicrobial compounds phytoalexins (Sticher *et al.*, 1997; Hammerschmidt, 1999; Thomma *et al.*, 1999), secondary metabolites glucosinolates (Brader *et al.*, 2001; Reichelt *et al.*, 2002), PR proteins (producing digestive enzymes chitinases and glucanases) (Kombrink & Schmelzer, 2001) and overall the activation of MAPK cascades (Jonak *et al.*, 2002). In plants the immediate defence response against pest and pathogen attack is an inborn system also termed as innate immunity where the host specific receptors encoded by disease resistance genes interact with microbial effector genes to activate defence responses. The plant innate immune response includes

HR response leading to localized PCD is essential for plant development (Cohn *et al.*, 2001; Nürnberger *et al.*, 2004; Liu *et al.*, 2005). Hypersensitive cell death is commonly controlled by direct or indirect interactions between pathogen avirulence gene products and those of plant resistance genes and it can be the result of multiple signalling pathways. The HR displayed by resistant plants against invading pathogens is a prominent feature of plant-pathogen interactions (Hammond-Kosack & Jones, 1996; Heath, 2000; Holt *et al.*, 2000; Cohn *et al.*, 2001).

### ***Systemic acquired resistance (SAR)***

The induction of HR locally often triggers a non-specific resistance throughout the plant against a broad spectrum of organisms and contributes to a phenomenon known as systemic acquired resistance, SAR (Ryals *et al.*, 1996; Sticher *et al.*, 1997; Métraux *et al.*, 2002; Gozzo, 2003). Once triggered, SAR provides resistance to a wide range of pathogens for days or weeks. Resistance shown by an entire plant species to a specific parasite or pathogen is known as nonhost resistance, and is expressed by every plant towards the majority of potentially pathogenic microbes (Heath, 2000). Induced responses or defences are responsible for limiting pathogen growth and infection. HR is accompanied by an increase in SA biosynthesis, transcriptional activation of various *PR* genes and induction of structural barriers such as cell wall lignification that protect the plant against enzymatic degradation and the establishment of a long-lasting SAR response (Hammond-Kosack & Jones, 1996; Ryals *et al.*, 1996; Sticher *et al.*, 1997). The expression of *PR* genes has served as a reliable marker for the induction of SAR. Therefore, *PR* genes are also referred to as SAR genes (Grüner *et al.*, 2003). However, even if HR may protect the plant against biotrophic pathogens that need to take nutrients from living cells, the HR-induced cell death may facilitate the growth of necrotrophic pathogens that benefit from host cell death (Govrin & Levine, 2000; Glazebrook, 2005). NPR1, a regulatory protein, is essential for the transduction of SA signal and *PR* gene activation. NPR1 is a key determinant in the signal transduction pathway in SAR response. The *npr1* (nonexpressor of *PR* genes) mutants are impaired in *PR* gene expression and are non-responsive to SAR inducers (Cao *et al.*, 1994; Kinkema *et al.*, 2000; Spoel *et al.*, 2003).

### ***Induced systemic resistance (ISR)***

In *Arabidopsis*, nonpathogenic, root-colonizing *Pseudomonas fluorescens* bacteria trigger an induced systemic resistance (ISR) response against infection by the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* (Pieterse *et al.*, 1998; van Wees *et al.*, 2000; Ton *et al.*, 2002). ISR induced by non-pathogenic bacteria follows a novel signalling pathway in which components from the JA and ET responses are engaged successively to trigger a defence reaction. ISR is regulated by NPR1 but independent of SA accumulation and pathogenesis related (*PR*) gene activation. In *Arabidopsis* SA-dependent SAR responses and JA/ET-dependent ISR responses are simultaneously active with an additive effect against bacterial pathogen *P. syringae*, which indicates that SAR and ISR pathways are compatible (van Wees *et al.*, 2000; Pieterse *et al.*, 2001; Ton *et al.*, 2002).

### ***Type III secretion systems (TTSS) in phytopathogenic bacteria***

Gram-negative phytopathogenic bacteria transfer virulence proteins across their outer membrane through a variety of secretion systems that are now classified into four (I-IV) major types and several minor ones. Type III secretion systems (TTSS) are used by plant pathogenic bacteria to suppress plant innate immunity. Bacteria use type III and IV secretion or transfer systems to deliver proteins or protein-DNA complexes into the plant host cell (Baker *et al.*, 1997). TTSS are well documented in *Erwinia* spp., *P. syringae*, and *Xanthomonas* spp. and also encountered in most gram-negative phytopathogenic bacteria. *Agrobacterium* is about the only gram-negative phytopathogenic genus in which this system has not been found (Cornelis & Van Gijsegem, 2000; Gürlebeck *et al.*, 2006).

TTSS consist of 15-20 Hrp (hypersensitive response and pathogenicity) proteins building a secretion apparatus used by virulent bacteria to transfer effector proteins into host plant cells. The primary role of bacterial effector proteins is to suppress the PCD based HR and PR protein production (Espinosa & Alfano, 2004; Jamir *et al.*, 2004). Several conserved *Hrp* genes, designated as *Hrc* (Hrp conserved), encode membrane-associated proteins that are an essential part of the TTSS and direct the effector transport across the bacterial envelop during infection in the host tissue (Alfano & Collmer, 1997, 2004; Baker *et al.*, 1997; Cornelis & Van Gijsegem, 2000).

### **Stress signalling and cross talk**

Plant signalling molecules SA, JA, ET and ABA, are involved in defence signalling in plants during several biotic and abiotic stresses such as wounding, pathogen attack, dehydration, and temperature stress and regulated by a complex network of signalling pathways (Dong, 1998; Grill & Himmelbach, 1998; Pieterse *et al.*, 2001; Thomma *et al.*, 2001; Devoto & Turner, 2003; Thaler & Bostock, 2004; Glazebrook, 2005). The use of different mutant plants either insensitive or deficient for their biosynthesis have revealed the central role of these plant defence signalling molecules and their cross talk in plant defence responses (Koorneef *et al.*, 1984; Guzmán & Ecker, 1990; Cao *et al.*, 1994; Delaney *et al.*, 1994; Feys *et al.*, 1994; McConn & Browse, 1996; Xie *et al.*, 1998; Pieterse *et al.*, 2001; Berger, 2002). It has been speculated that in the absence of stress, the antagonistic interactions among signalling pathways helps to maintain low levels of expression of stress responsive genes. When plants experience a specific stress, one signalling pathway may become dominant over the others. A specific subset of stress responsive genes may be induced through activation of positive regulators of gene expression of one pathway while they are simultaneously suppressed by other negative regulators of gene expression from another pathway (Rojo *et al.*, 2003; Anderson *et al.*, 2004).

SA has a central role in plant defence against pathogen attack (Ryals *et al.*, 1996). JA is involved in defence responses to abiotic and biotic stress factors and regulates wound and insect herbivore induced signalling and senescence (Creelman & Mullet, 1997; Walling 2000; He *et al.*, 2002). There are both

synergistic and antagonistic interactions between SA and JA signalling pathways. The primary interaction between the SA and JA pathways are mutually antagonistic (Peña-Cortés *et al.*, 1993; Norman-Setterblad *et al.*, 2000). However, microarray analysis and other experiments in *A. thaliana* have revealed that many defence related genes are co-activated by both SA and JA pathways (Reymond & Farmer, 1998; Norman-Setterblad *et al.*, 2000; Schenk *et al.*, 2000; van Wees *et al.*, 2000; Karim *et al.*, 2006). The signalling molecule ET that is involved in defence responses and senescence shows positive and negative interactions with SA and JA during different stress responses in plants (Ecker & Davis, 1987; Reymond & Farmer, 1998; Schenk *et al.*, 2000; Lorenzo *et al.*, 2003; Nakano *et al.*, 2006). Sometimes ET can promote the resistance phenotypes in plants similar to SA and JA (Norman-Setterblad *et al.*, 2000) however, it has been also reported that ET can promote disease susceptibility in plants (Lund *et al.*, 1998; Hoffman *et al.*, 1999). Most of the studies have shown the synergistic interaction between JA and ET signalling pathways, but sometime the JA and ET pathways can regulate the defence related genes independently as well (Schenk *et al.*, 2000). Both ET-dependent and -independent SA signalling pathways also exist in plants (O'Donnell *et al.*, 2001; Kunkel & Brooks, 2002). SA and ET were found to act synergistically on defence gene expression and antagonistically on disease resistance in tomato plants (Diaz *et al.*, 2002). JA, SA and ET form a complex defence signalling network rather than linear and independent pathways. The phytohormone ABA is involved in plant metabolism during diverse physiological and developmental processes in every phase of plant growth and also mediates the tolerance and adaptation response to different abiotic stresses (Grill & Himmelbach, 1998; Tamminen *et al.*, 2001; Finkelstein & Rock, 2002; Achuo *et al.*, 2006). ABA has an antagonistic effect on SA or the JA-ET pathways while ET can act as a negative regulator of the ABA pathway (Garcarrubio *et al.*, 1997; Ghassemian *et al.*, 2000; Audenaert *et al.*, 2002; Anderson *et al.*, 2004). However, ABA acts synergistically with jasmonate-induced defences against herbivores and antagonistically with salicylate-based resistance to some pathogens (Thaler & Bostock, 2004).

## **Peptide transporters in plants**

The uptake and allocation of nitrogenous compounds, including amino acids and peptides, is an essential process in living organisms. In eukaryotes, peptide transporters (PTRs) and the oligopeptide transporters (OPTs) are the two families of proteins that transport small peptides. Members of the OPT family can transport tetra- and pentapeptides (Hauser *et al.*, 2001; Koh *et al.*, 2002). Whereas, members of the PTR family transport di- and tripeptides as well as many other molecules (Hauser *et al.*, 2001; Stacey *et al.*, 2002a). PTR family members are also called POT family members for proton-dependent oligopeptide transporter and cause symport of one or more H<sup>+</sup> simultaneously with the transported molecule. They are about 450-600 amino acids long and usually exhibit 12 putative  $\alpha$ -helical transmembrane regions. Some PTR family members show sequence similarity with the protein members of a major facilitator superfamily, MFS (Paulsen & Skurray, 1994; Steiner *et al.*, 1995; Pao *et al.*, 1998; Saier, 2000; Karim *et al.*, 2005). MFS is one of the largest groups of transporters and membrane proteins

and ubiquitously present in prokaryotes, archaea and eukaryotes. These transporters use proton-motive force to drive the transport of a wide range of molecules, almost any substances of biological interest in a uniport, symport or antiport mode (Pao *et al.*, 1998; Abramson *et al.*, 2004). However, the nitrate and peptide transporters of the PTR family belong to prokaryotes and eukaryotes are classified into four broad groups depending on different topologies of transmembrane proteins (Chiang *et al.*, 2004). In yeast (*S. cerevisiae*) a unique di- and tripeptide transporter Ptr2 has been characterized and so far been used as a model system for eukaryotic PTR characterization by complementation (Perry *et al.*, 1994). In yeast, there are also two oligopeptide transporters Opt1 and Opt2 (Hauser *et al.*, 2000).

More than fifty putative PTR-type family members have been identified in *Arabidopsis* through sequence comparison (Stacey *et al.*, 2002a). The putative PTR family members in the *Arabidopsis* genome have been phylogenetically classified into four PTR subfamilies or groups, group I (19 members), group II (nine members including AtPTR1, At3G54140 and AtPTR2, At2G02040), group III (14 members including AtPTR3, At5G46050) and group IV (10 members including CHL1, At1G12110; NTL1, At1G69850 and NTP2, At2G26690) (Waterworth & Bray, 2006). In *Arabidopsis* there are nine putative OPT genes (*AtOPT1* to *AtOPT9*), which have been identified and they show significant sequence similarity with the yeast OPT at the protein level (Koh *et al.*, 2002; Stacey *et al.*, 2002b, 2006).

The first characterized PTR of *Arabidopsis* was AtPTR2-B, later renamed as AtPTR2. It was found to be highly expressed in embryos, germinating seeds, roots, stems, leaves, flowers and siliques and involved in embryo and seed development. It was also designated as NTR1 and identified as a high affinity peptide transporter having weak histidine transport activity (Rentsch *et al.*, 1995; Song *et al.*, 1996, 1997; Stacey *et al.*, 2002a). The second characterized PTR from *Arabidopsis*, AtPTR1 was found to be a plasma membrane-localized transporter expressed in vascular tissues and showed low-affinity histidine transport activity. It also showed transport activity of several di- and tripeptides and phytotoxin phaseolotoxin a modified tripeptide produced by the plant pathogen *P. syringae* (Dietrich *et al.*, 2004). *AtPTR1* was found to be expressed in the hypocotyl, cotyledons, siliques, root tips, leaves and during seed germination (Dietrich *et al.*, 2004). Third *Arabidopsis* PTR, AtPTR3 was shown to be inducible by different abiotic and biotic factors (Karim *et al.*, 2005; Karim *et al.*, 2006).

Besides small peptide transporters, some members of PTR-type transporters have been shown to function as nitrate transporters in *Arabidopsis*. The first of them was a dual affinity nitrate and chlorate transporter CHL1, recently renamed as AtNRT1.1, which was found highly active in the nascent organs of roots and shoots during vegetative and reproductive growth phases (Tsay *et al.*, 1993; Wang *et al.*, 1998; Guo *et al.*, 2001). Later another homologue of CHL1 in the same gene family was identified as a low affinity nitrate transporter and named as AtNRT1.2 (Huang *et al.*, 1999). In total, four PTR-type low-affinity nitrate transporters homologues in the AtNRT1 family have now been identified in *Arabidopsis* (Okamoto *et al.*, 2003).

Several PTRs or PTR-type nitrate transporters have been characterized in different plant species. A low affinity nitrate transporter from *Brassica napus*, BnNRT1.2 showed high homology to CHL1 and was found to be expressed at a high level in the nitrate pre-treated roots and was found to transport nitrate and histidine (Zhou *et al.*, 1998). The barley (*Hordeum vulgare*) PTR, HvPTR1 was found to be able to transport dipeptides and expressed in the scutella of germinating barley grain (West *et al.*, 1998) and localized to the plasma membrane (Waterworth *et al.*, 2000). A faba bean (*Vicia faba*) PTR, VtPTR1 with high homology to AtPTR2 was shown to transport dipeptides and expressed in germinating seeds and developing seedlings (Miranda *et al.*, 2003). A PTR-type nitrate transporter and an AtNRT1.2 homologue from rice (*Oryza sativa*) OsNRT1 showed higher homology to plant PTRs such as AtPTR2. OsNRT1 showed low affinity nitrate transport activity and was found to be expressed in root hair and epidermis as well as in most external layer of the root (Lin *et al.*, 2000). Interestingly, another putative PTR-type rice nitrate transporter OsNRT1.3 that showed lower amino acid sequence similarity with OsNRT1 was found to be induced by drought but not with ABA or salt. OsNRT1.3 was found to be expressed in embryo, aleurone layer of seeds, leaves, roots and flowers (Hu *et al.*, 2006). Soybean (*Glycine max*) nitrate transporters GmNRT1.1, GmNRT1.2 and GmNRT1.3 showed constitutive and nitrate induced expression in the leaves or roots or in both (Yokoyama *et al.*, 2001). The presence of a large number of PTRs in plants suggests that they have many potential roles in physiological growth, development and metabolism (Stacey *et al.*, 2002a). Phylogenetic analysis with PTR sequences indicated the existence of many PTR-type transporters in plants, but it might be more practical that not all of them are transporting small peptides rather transporting non-peptide substrates. Moreover, 20 amino acids can generate thousands of di-/tripeptide substrates in different combinations and therefore, testing and identification of more substrates for PTR family members would help to pinpoint the function of plant PTRs (Waterworth & Bray, 2006).

## **T-DNA mediated transformation as a tool in plant research**

During the last three decades the plant transformation and production of genetically engineered plants, for research and commercial purposes, using a natural genetic engineer bacterium *Agrobacterium* and its T-DNA (transfer DNA) transfer system has become a common practice all over the world (Gelvin, 2003). T-DNA insertion causes foreign DNA insertion into the plant genome either creating a new gene introgression or resulting in gene knockout or null mutation. *Agrobacterium* mediated T-DNA transformation has also been used as the basic delivery system to create overexpression mutants of native genes or to produce transgenic lines where the heterologous genes are introduced into new species to study the function of the respective genes further (Leyman *et al.*, 2006; Karim *et al.*, 2007).

T-DNA mutagenesis has replaced chemical mutagenesis because the T-DNA provides a direct route to identify and characterize the mutated gene *in situ*. In *Arabidopsis* T-DNA insertion mutagenesis is a common and well practiced means

of introducing or disrupting gene function due to the insertion of foreign DNA randomly into the genome (Krysan *et al.*, 1999; An *et al.*, 2005; Karim *et al.*, 2007). The distribution of the T-DNA insertion ranged from the intragenic (exons, introns, 5' and 3' regulatory regions) to intergenic regions (Krysan *et al.*, 2002; Szabados *et al.*, 2002; Stangeland *et al.*, 2005). T-DNA is integrated into the plant genome through illegitimate recombination, but a low frequency of T-DNA integration is possible through homologous recombination if homologous sequences are present in the transformed DNA (Risseuw *et al.*, 1997).

In many cases of T-DNA insertions, single-copy T-DNA insertion has been found to be associated with small or large rearrangements such as deletions and/or duplications of target site sequences, deletions and/or duplications of T-DNA (including left- and right-border sequences) and vector backbone sequences, and gross chromosomal rearrangements such as translocations, duplications or deletions (De Buck *et al.*, 2000; Tax & Vernon, 2001; Krysan *et al.*, 2002; Meza *et al.*, 2002; Forsbach *et al.*, 2003). T-DNA inserts containing several T-DNA regions and binary vector backbone sequences associated with T-DNA sequences are often integrated in insertion lines. Possibly the integration of complete vector backbone sequences into the plant genome is because of a conjugative transfer initiated at the right border and subsequent continued copying at the left and right borders, which is termed as read-through. This indicates that the left border is not frequently recognized as an initiation site and the right border is not efficiently recognized as a termination site for DNA transfer (De Buck *et al.*, 2000; Meza *et al.*, 2002; Stangeland *et al.*, 2005).

The two main ways, to use the T-DNA mutagenesis to study the functions of unknown genes, are the loss-of-function and the gain-of-function mutants of the target genes. In the loss-of-function approach the functions of a gene can be recognized from the phenotype of the knockout mutants. One of the loss-of-function mutagenesis approaches is the promoter-tagging or trapping by T-DNA insertion, in which plant promoters and genes can be identified based on the random insertion and integration of a promoterless reporter gene in the plant genome and its activation by native plant regulatory sequences (Kertbundit *et al.*, 1991; Bade *et al.*, 2003; Svensson *et al.*, 2005; Karim *et al.*, 2006). Another such method, called enhancer trapping, is where the reporter gene is fused to a minimal promoter of the reporter gene (TATA) that is unable to drive reporter gene expression alone but can be activated by a native chromosomal enhancer element resulting in expression of the reporter gene (Springer, 2000; Ko & Kamada, 2002). In a similar method termed as gene-trap systems the delivery vector contains the promoterless reporter gene with splice acceptor sequences in the upstream. Expression of the reporter gene occurs due to the transcriptional fusion of upstream exon sequences of the native gene to the reporter gene upon the insertion of T-DNA into an intron and splicing by the chromosomal splice donor to the splice acceptor sites in the reporter gene (Springer, 2000; Ryu *et al.*, 2004).

However, the repetitive sequences and many closely related gene sequences or genetic redundancy in the plant genome inhibits the proper elucidation of the gene function through the loss-of-function methods. In that case a gain-of-function approach is found to be helpful. In this method the novel T-DNA mutagenesis

vector system is used to introduce transcriptional enhancer sequences of different plant viruses into the plant genome. Upon insertion these viral enhancers cause transcriptional activation of the plant genes in close proximity and thereby activate the genes that are associated with inserted T-DNA. Therefore, this method is termed as activation tagging (Weigel *et al.*, 2000; Chalfun-Junior *et al.*, 2003; Dong & Von Arnim, 2003; Nakazawa *et al.*, 2003; Tani *et al.*, 2004).

## **Aim of the study**

The general aim of this work was to characterize transgenic *Arabidopsis* plants showing altered stress-related phenotypes. The detailed objectives were:

- Identification of the gene responsible for wound and salinity induced reporter gene expression in a promoter tagged *Arabidopsis* knockout mutant line
- Characterization of the T-DNA insert in the identified peptide transporter knockout mutant
- Characterization of the peptide transporter protein with yeast complementation
- Characterization of the regulation of the peptide transporter gene
- Characterization of the phenotypes of peptide transporter mutants
- Generation of transgenic *Arabidopsis* plants, having yeast (*S. cerevisiae*) trehalose biosynthesis enzyme ScTps1 (trehalose-6-phosphate synthase) targeted into chloroplast
- Identification of the transgenic lines, having the ScTps1 protein targeted into chloroplast
- Characterization of drought tolerance and growth phenotypes in transgenic plants with different compartmentalization of the ScTps1

## Results and discussion

### Identification and characterization of *Arabidopsis* peptide transporter AtPTR3 (I and II)

Gene trapping by T-DNA mediated promoter tagging was utilized to identify a knockout mutant and to characterize its phenotype in *Arabidopsis*. A binary vector pMHA2 that contains a promoterless GUS (*uidA*) reporter gene and a kanamycin resistant marker gene in the T-DNA was used to produce transgenic lines in *Arabidopsis* ecotype C24 (I). One of the *Arabidopsis* lines was found to have a wound-induced expression of the reporter gene *uidA*, which suggested that in that line the T-DNA had caused an insertion in a wound-induced gene. This mutant line was further characterized in this study.

### Localization of the insert and identification of AtPTR3 gene

To localize the T-DNA insert in the *Atptr3-1* mutant, Southern analysis of mutant genomic DNA was performed. In the Southern analysis, a 3.8 kb fragment originating from *Hind*III-*Eco*RI double digestion suggested that this fragment might contain plant DNA because the *Eco*RI site is not present in the T-DNA. Cloning and sequencing of this fragment and an homology search with the sequence using BLASTN revealed that the sequence had 100 % identity with *Arabidopsis* genomic DNA in chromosome 5. This result suggests that the T-DNA had caused a knockout-mutation in a putative PTR-type transporter gene *At5g46050*. This gene was renamed as *AtPTR3* because it is the third characterized gene coding for PTR-type peptide transporters in *Arabidopsis* (I). The predicted topology of AtPTR3 matched with the topology of other PTR-type transporters of plants (I, Chiang *et al.*, 2004).

### Characterization of the insert in *Atptr3* mutants

The insert of the *Atptr3-1* mutant was analyzed by Southern using different parts of the binary vector pMHA2 as probes, and the whole insert was amplified by long range PCR and cloned as two large fragments, 9.3 kb and 12.1 kb in size (Fig. 1a, II). Restriction digestion analysis and partial sequencing of the cloned insert revealed two tandem T-DNA inserts, separated by an entire vector backbone, and an additional small fragment of the vector. Neither rearrangements in genomic DNA nor the plant genomic DNA preceding the second *gus* gene were observed. Therefore, in spite of the large size, the *Atptr3-1* mutant was concluded to carry a single insertion in the second intron of the PTR gene *AtPTR3* (I and II). T-DNA inserts containing several T-DNAs and the vector backbone are frequently observed among T-DNA mutants of *Arabidopsis* (De Buck *et al.*, 2000; Meza *et al.*, 2002). The presence of the T-DNA insert in an intron is also common among T-DNA inserts in *Arabidopsis* (Krysan *et al.*, 2002; Szabados *et al.*, 2002; Stangeland *et al.*, 2005).

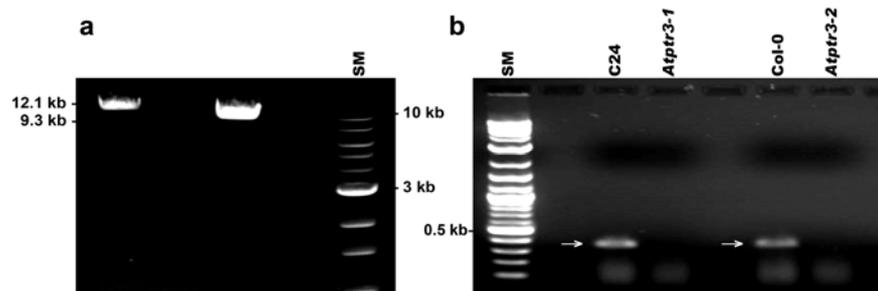
A T-DNA insertion mutant in the *At5g46050* (salk\_003119) in *Arabidopsis* ecotype Col-0 was ordered from NASC, The European Arabidopsis Stock Centre (Alonso *et al.*, 2003) and renamed as *Atptr3-2*. The PCR analysis of the line in subsequent generations revealed a single T-DNA insertion in the second intron of the *At5g46050* gene in a similar location to *Atptr3-1* (II). These two independent mutants for the same *Arabidopsis AtPTR3* gene in two ecotypes, C24 and Col-0, made it possible to verify that the phenotypes observed in the mutants were due to the T-DNA insert (II).

### ***Characterization of the transporter function of AtPTR3 protein***

The ability of AtPTR3 protein to transport peptides was characterized with complementation in a *S. cerevisiae* di-/tripeptide transporter *ptr2Δ* mutant auxotrophic for histidine (His), leucine (Leu) and methionine (Met). Both the previously characterized AtPTR2 (Song *et al.*, 1996) and AtPTR3 supported the growth of the *Ptr2* yeast mutant on minimal media supplemented with 1 mM dipeptide His-Leu, His-Phe (His-phenylalanine) and Leu-Met and tripeptides Leu-His-Leu and Met-Leu-Gly (Met-Leu-glycine). Due to the functional OPT all of the tested yeast strains survived in the media containing a tetrapeptide Met-Gly-Met-Met (II). Two previously characterized PTRs, AtPTR2 and AtPTR1 from *Arabidopsis* have been found to complement the yeast *ptr2Δ* mutants and restore growth on di-/tripeptide media (Song *et al.*, 1996; Dietrich *et al.*, 2004).

### ***Expression of AtPTR3 gene in Arabidopsis wild type and mutant lines***

In RT-PCR analysis no *AtPTR3* transcripts were observed in either *Atptr3-1* or *Atptr3-2* mutants after wound induction, which indicated a complete knockout mutation of the *AtPTR3* gene in the mutant lines (Fig. 1b, I).

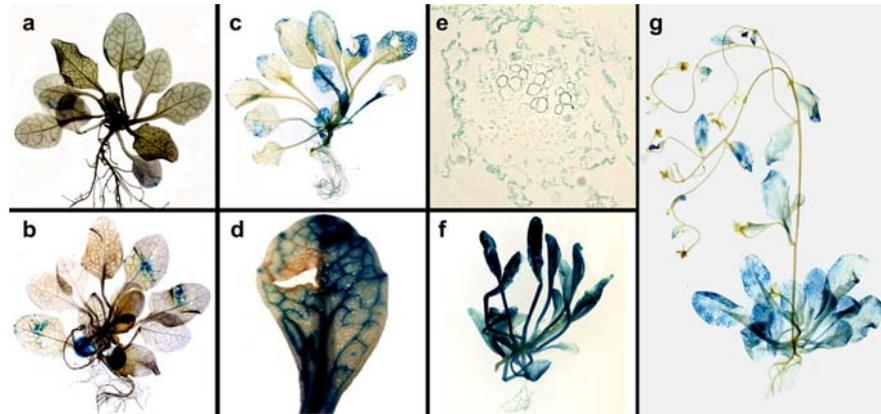


**Fig. 1.** (a) The whole T-DNA insert in *Atptr3-1* mutant was cloned as two large fragments, 9.3 kb and 12.1 kb in sizes by long range XL PCR (II). The PCR products were separated in a 0.5% agarose gel. (b) RT-PCR with *AtPTR3* gene specific primers verified the knockout of *At5g46050* transcripts in wounded *Atptr3-1* and *Atptr3-2* mutants, while transcripts were visible (arrows) only in wild type lines C24 and Col-0, respectively (I). SM indicates DNA size marker.

The activity of the *AtPTR3* promoter was studied by analysing the GUS expression in *Atptr3-1* mutant plants, which were either wounded or inoculated with pathogenic bacterium *Erwinia carotovora* subsp. *carotovora*, or were hyperhydric or senescent. Sometimes in the GUS analysis very weak and constitutive expression of the *gus* gene was observed in leaves of untreated control plants (Fig. 2a). In the wounded, non-inoculated plants the induced *AtPTR3* promoter expression was visible around the wounds, but no GUS activity could be detected in the unwounded systemic leaves, suggesting that wounding does not cause a systemic induction of the *AtPTR3* promoter (Fig. 2b, I). The inoculated leaves resembled the wounded ones at the beginning of the infection, but at later time points the GUS activity was visible in a larger area of the inoculated leaf, especially around the veins as well as in the non-inoculated leaves of the plant, which suggested the systemic induction of *AtPTR3* expression (Fig. 2c, 2d). Microscopic analysis of the GUS stained inoculated leaves showed that *AtPTR3* is expressed in the tissue surrounding vascular bundle (Fig. 2d, 2e).

During cultivation of the wild type C24 and *Atptr3-1* plants on sterile media, some plantlets showed a hyperhydric (vitrified) phenotype, especially if the growth conditions or media composition were not optimal (Delarue *et al.*, 1997; Franck *et al.*, 2004). Hyperhydric plants are brittle and look turgid and translucent (Fig. 2f) and show several abnormal characteristics compared to the normal plants grown in optimal condition (Fig. 2a). GUS staining of the *Atptr3-1* plants suggested that the *AtPTR3* gene was expressed at a high level in hyperhydric plants (Fig. 2f). However, no difference in the frequency of hyperhydric plants was observed between the mutant line and the wild type C24. The result suggests that the *AtPTR3* gene might be induced by the stress leading to vitrification.

To study whether the *AtPTR3* promoter was active in the unstressed plant, the GUS expression in the *Atptr3-1* mutant was followed throughout the life cycle of the plant. GUS staining of the germinating seeds and young plants up to three weeks showed no GUS activity. Whereas, increasing GUS activity was observed in mature and flowering *Atptr3-1* plants grown in media or soil for five to eight weeks. In these old plants GUS expression was visible in leaves, roots and young siliques. These results suggest that the *AtPTR3* promoter is active during flowering or senescence (Fig. 2g). However, no phenotypic differences could be observed at any age between the wild type and the mutant line in the greenhouse or *in vitro* cultivation. Most of the defence signalling molecules such as SA, JA, ET and ABA play regulatory roles in senescence response pathways (Smart, 1994; Fan *et al.*, 1997; Morris *et al.*, 2000; He *et al.*, 2002). It is known that all these molecules induce defence-related genes. Thus, one would expect to see the expression of these genes during senescence (Guo *et al.*, 2004). In *Arabidopsis* a drought inducible gene *erd1* was also induced by senescence (Simpson *et al.*, 2003).

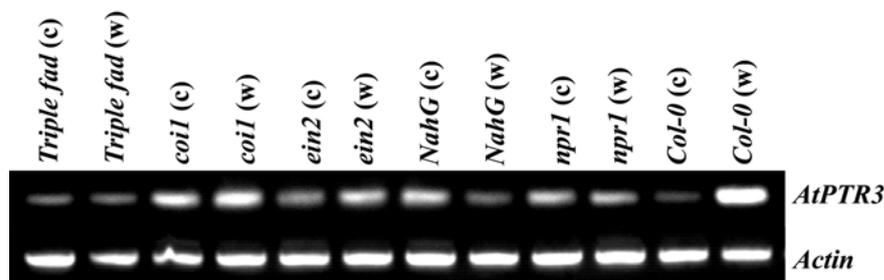


**Fig 2.** GUS analyses on four week old media grown plants (**a-f**). Untreated normally grown *Atptr3-1* mutant control plant (**a**). GUS expression showed the localized *AtPTR3* induction in wounded leaves (**b**) and systemic GUS induction in *Erwinia* inoculated plants (**c**). In an *Erwinia* inoculated leaf (**d**) and in the microscopic view of the GUS stained area (**e**) the staining could be seen around the veins and in the cells surrounding vascular tissue. Induction of *AtPTR3* by GUS expression in hyperhydric plant (**f**). For wounding and inoculation photos were taken 24h after treatment. Expression of *AtPTR3* in senescent plant. Six weeks old media grown senescing plant was subjected to GUS histochemical assay (**g**).

Induction of GUS activity in *Atptr3-1* mutant by salt concentrations ranging from 20 to 200 mM suggested salt-induced *AtPTR3* expression. The salt-containing medium caused strong induction of GUS activity in whole mutant plants, including cotyledons and roots (**I**). GUS activity was also observed, by the amino acids His, Leu and Phe treatments, in cotyledons and lower leaves of *Atptr3-1* mutant plants, indicating that *AtPTR3* might be induced also by amino acids, the building blocks of the small peptides transported by *AtPTR3* (**I**). It has been shown recently in tomato plants that salt stress could cause the activation of wound inducible defence related genes and wounding as well as JA could induce enhanced salt tolerance in plants with the induction of a  $\text{Ca}^{2+}$ -dependent protein kinase. This cross-tolerance between wounding and salt stresses might indicate the overlapping plant responses during biotic and abiotic stresses (Dombrowski, 2003; Capiati *et al.*, 2006). *E. carotovora*-induced potato gene, *Solanum tuberosum*-*Erwinia* induced-2 (*Stei2*) was up-regulated by NaCl, wounding, dehydration and ABA. However, treatment of the leaves with SA, methyl jasmonate (MeJA) and ET did not induce the *Stei2* expression (Sós-Hegedüs *et al.*, 2004). It has been shown in microarray gene expression analysis in *Arabidopsis* that plant defence signalling molecules ABA, JA and ET play regulatory roles during salt stress signalling and adaptation (Ma *et al.*, 2006).

Phytohormones, ABA, methyl ester of JA MeJA and the ET precursor ACC and SA induced *AtPTR3* expression in four week old *in vitro* plants. SA treatment showed an increased level of GUS activity almost in the entire plant while ABA, ACC or MeJA showed GUS activity mostly in cotyledons and 1-2 lower leaves (**II**). Quantitative real-time PCR assays suggested that SA caused the highest

induction, up to 100 times higher than in the mock-treated controls, whereas MeJA and ABA treatments caused moderate induction (**II**). The low induction caused by MeJA and ABA might be due to the localized induction by these treatments and not necessarily lower activities in the cells where the induction has taken place. RT-PCR was also utilized to study the involvement of JA, SA and ET signalling pathways in wound-induced expression of the *AtPTR3* in different signalling and biosynthesis mutant lines of *Arabidopsis* accession Col-0 (Fig. 3). In untreated wild type Col-0 plants the *AtPTR3* gene was expressed at a low background level, which was strongly increased in wounded plants. *Triple fad* mutant, unable to synthesize JA showed a weak constitutive and inducible expression of *AtPTR3* while, *coi1-1* a JA signalling mutant showed a constitutive as well as a wound inducible expression of *AtPTR3*. The ET signalling mutant *ein2-1* resembled the wild type in having induced expression of the *AtPTR3* in wounded plants, but the induced level was lower than that of the wild type. NahG mutant plants unable to accumulate SA, and the *npr1* mutant that lacks a key regulatory protein of SA signalling in the SAR pathway, resembled each other in showing an inversed expression pattern of *AtPTR3*. In these mutant lines the unwounded plants showed higher *AtPTR3* expression levels than the wounded ones. In summary, wounding caused the down regulation of *AtPTR3* in NahG plants distinctly, and no changes in the expression of *AtPTR3* in *Triple fad* mutants as well as a weaker *AtPTR3* expression in *ein2-1* mutant plants compared to wild type plants, which suggested that all the tested pathways might be involved in the regulation of *AtPTR3* gene in Col-0 (Fig. 3).



**Fig 3.** Wound-induced *AtPTR3* expression in signalling mutants. *AtPTR3* expression was studied with RT-PCR in four weeks old JA signalling mutants *triple fad* (defective in jasmonate biosynthesis) and *coi1* (coronatine-insensitive), ET signalling mutant *ein2* (ethylene insensitive), *NahG* plants unable to accumulate SA and *npr1* (non-expressor of PR proteins) and in the corresponding wild type (**Col-0**). Samples were collected 24 hr after wound-induction (**W**) and unwounded control plants (**C**) and amplification of *Actin* gene was used as controls.

The results obtained in RT-PCR were also verified by quantitative real time PCR (**II**). The result suggested that the expression of *AtPTR3* might be SA-dependent as well as JA/ET-dependent and both the SA and JA pathways might be necessary for the induction of *AtPTR3* after wounding. Co-induction of

*Arabidopsis* genes by both of the SA and JA pathways has been observed for many genes (Reymond & Farmer, 1998; Schenk *et al.*, 2000).

### ***Phenotypic characterization of Atptr3 mutant lines***

The phenotypic differences between the *Atptr3* mutants and the corresponding wild type accessions were studied when the plants were grown on salt (NaCl) containing media (**I**). Seeds of the *Atptr3* mutant and the control plants demonstrated no difference in germination on sterile media with salt concentrations up to 100 mM. At salt concentrations ranging from 140 to 200 mM the germination frequency of the *Atptr3* mutant seeds drastically decreased compared to the wild type seeds in both Col-0 and C24 ecotypes, which suggested that the AtPTR3 protein, besides being induced by salt, might be needed for germination under salt stress (**I**).

The involvement of AtPTR3 in defence responses against the necrotrophic bacterial pathogens *E. carotovora* subsp. *carotovora* strain SCC3193 and the hemibiotrophic pathogen *P. syringae* pv. *tomato* strain DC3000 was studied by inoculating the *Atptr3* mutants and wild type plants with these pathogens. Both the *Atptr3-1* and *Atptr3-2* mutants showed increased susceptibility to the infection caused by both the pathogens (**II**). The disease symptoms proceeded faster in the mutant plants than in the wild type plants. A virulent strain of the bacterial necrotrophic pathogen *Xanthomonas campestris* pv. *campestris* and an avirulent necrotrophic pathogen *Alternaria brassicicola* caused symptoms with similar timing and severity in both the mutant and wild type plants. These results suggested that resistance against some pathogens was compromised in *Atptr3* mutant plants (**II**). The glucosinolate and camalexin profiling showed no difference between *Atptr3-1* mutant and the C24 wild type plants suggesting that these secondary metabolites were not compromised in *Atptr3* mutants (**II**).

The *Atptr3-2* mutant line was inoculated with *P. syringae* TTSS mutants *hrpA* and *hrcC*. Both the wild type and the mutant plants showed similar symptoms and survived from the infection caused by *hrpA* and *hrcC*. *AtPTR3* expression in Col-0 plants inoculated with wild type DC3000 and the *hrpA* and *hrcC* mutants was studied with real-time PCR, and the *AtPTR3* gene was shown to be induced at a higher level in plants inoculated with *P. syringae hrpA* mutant than in plants inoculated with *P. syringae* wild type strain DC3000. These results suggested that the Type III secretion system might suppress the expression of the *AtPTR3* gene which is needed for defence in a compatible interaction between *Arabidopsis* and *P. syringae* pv. *tomato* (**II**). Suppression of host genes by wild type *Pseudomonas* but not by the TTSS mutants has been suggested for hundreds of genes in the microarray results (Thilmony *et al.*, 2006; Truman *et al.*, 2006).

Many different stress responses cause changes in plant cell homeostasis and result in the production of ROS. The total ROS production is dependent on the balance between the ROS production and the ROS scavenging mechanism (Mittler *et al.*, 2002; Rizhsky *et al.*, 2002). Salt stress is believed to induce ROS production and PCD in plants (Klessig *et al.*, 2000; Lin *et al.*, 2006). ROS production is also

involved in defence against pathogens. We exposed the *Atptr3-1* mutant and the wild type C24 to the ROS generating agents paraquat and rose bengal, known to generate intracellular superoxide anion and singlet oxygen, respectively that might cause oxidative stress in plant cells (Vicente *et al.*, 2001; Velikova *et al.*, 2004). There was no phenotypic difference observed in the plants between the wild type and the mutant, but the *Atptr3-1* mutant lines accumulated increased levels of oxidative compounds superoxide radical and H<sub>2</sub>O<sub>2</sub> (II). The ROS scavenging mechanism in *Atptr3-1* plants might be suppressed or there was overproduction of ROS molecules by an unknown mechanism in *Atptr3-1* mutants. The ROS substances that accumulated in response to different stresses may have a dual role, either as signalling molecules in defence responses to pathogen attack or produced excess toxic compounds involved in necrosis of plant cells (Király, 1998; Heath, 2000). It is most likely that the overproduction of ROS substances in *Atptr3* mutants may have a detrimental effect on defence responses or SAR, which may lead to pathogen susceptibility and invasion rather than a resistance response.

To study the function of SAR in the *Atptr3-2* mutant, the expression level of the SAR marker gene *PR1* was analysed with real-time PCR. The results showed lower *PR1* expression in the *Atptr3-2* mutant than in the wild type Col-0, suggesting that the *Atptr3* mutants might be affected in the SAR response (II).

## **Drought tolerance in *Arabidopsis* with trehalose-6-phosphate synthase (ScTps1) targeted into chloroplast (III)**

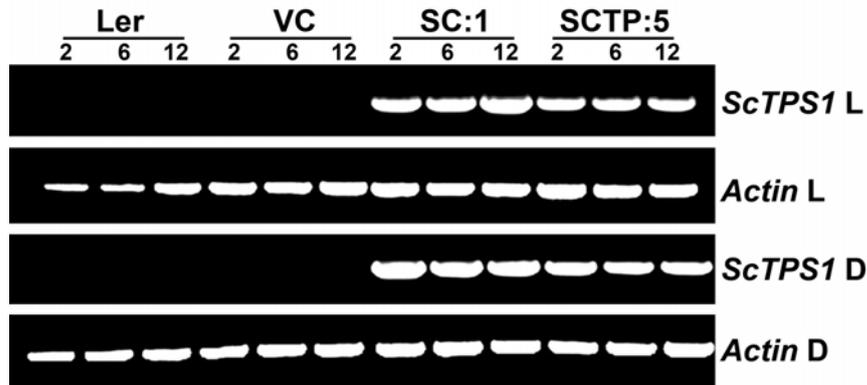
### ***Generation and genetic characterization of transgenic Arabidopsis lines expressing ScTPS1***

To target the yeast (*S. cerevisiae*) Tps1 to chloroplasts a promoter fragment from *Arabidopsis* RuBisCO small subunit gene *AtRBCS1A*, including the transit peptide from the same gene, was fused in front of the coding region of *ScTPS1* (III). The resulting genetic construct *pAtRBCS1A:TP:ScTPS1:3'g7* (pHSK003) was transformed into wild type *Arabidopsis* ecotype *Landsberg erecta* and two transgenic lines, SCTP:5 and SCTP:14, were primarily selected to study the effect of chloroplast-targeted ScTps1 on drought tolerance. For comparison, another transgenic line, SC:1, having a similar construct without a transit peptide for chloroplast targeting, was produced. In the SC:1 transgenic line ScTps1 protein was found to be localized in the cytosol. Plants of the wild type line *L. erecta* (Ler) and an empty vector control (VC) were used as controls (III). Southern analysis of *Arabidopsis* lines transgenic for *ScTPS1* showed that the gene was integrated in the genome of SC:1 in two copies whereas SCTP:5 and SCTP:14 contained one and three copies, respectively. This work (III) was mostly focused on the SCTP:5 transgenic line because of the many inserts in SCTP:14 line.

### ***Characterization of the ScTPS1 expression in transgenic lines***

The chloroplast targeted SCTP:5 line was found to be positive for the localization of the ScTps1 protein into the chloroplast. Western analysis of chloroplast proteins isolated from both SC:1 and SCTP:5 transgenic and wild-type plants, with antiserum raised against ScTps1 showed accumulation of the ScTps1 protein only in line SCTP:5, indicating that the transit peptide conferred importation of ScTps1 into the chloroplasts of SCTP:5 plants (III).

The regulation of *ScTPS1* by the promoter *pAtRbcS1A*, during a 24 hour light cycle of 12 hours of dark period and 12 hours of light period, was monitored in RT-PCR experiments (Fig. 4). It was found that the specific RNA level was the same under the light and dark periods, which indicated a constitutive expression of the *pAtRbcS1A* promoter and consequently the constitutive production of the ScTps1 in the transgenics.



*Fig 4.* Transcription analysis of RuBisCO small subunit promoter *pAtRbcS1A* by RT-PCR. Expression levels of the *ScTPS1* gene under regulation of *Arabidopsis RbcS1A* promoter was studied by RT-PCR. RNA samples were collected from various time points (2h, 6h and 12h) after the onset of light or dark periods of the light cycle. **SC:1 and Sctp:5** represent transgenic lines, **Ler** indicates wild type control and **VC** indicates a vector control line. Numbers below transgenic lines, **2, 6 and 12**, define hours of light (**L**) or dark (**D**) periods. Since the *ScTPS1* primers were used in the RT-PCR analysis no signal was detected in wild type and vector control lines. *Actin* primers were used in all RT-PCR as template RNA quantity control. Samples were visualized on 1 % agarose gels.

### ***Phenotypic characterization and drought tolerance of the ScTPS1 producing transgenic lines***

Two to three weeks old transgenic (SC:1 and Sctp:5) and wild type plants grown in soil or 1% MS media were subjected to drought stress. In both cases the transgenic lines showed improved drought tolerance and recovery after dehydration stress while the wild type could not survive (Fig. 5, **III**). The Sctp:5 plants with ScTps1 protein targeted to the chloroplast performed better than the SC:1 mutant plants in drought stress tests when cultivated in 1% MS media (**III**). When the water status of the three week old unstressed transgenic (SC:1 and Sctp:5) and wild type control *Arabidopsis* plants were compared, similar relative water content (RWC) was observed. However, when the plants were subjected to drought stress at different time points up to four hours, transgenic plants exhibited a better water holding capacity shown by a slower moisture loss as compared to wild type control plants. The results indicated that the enhanced drought tolerance is most likely dependent on a greater retention of water, by a mechanism that still needs to be elucidated.

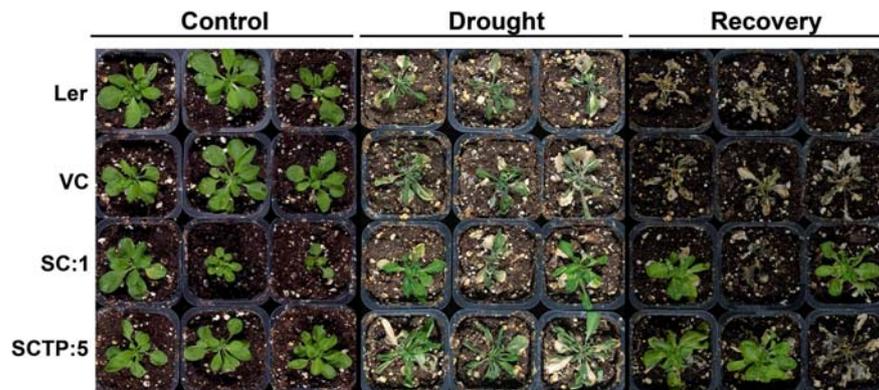


Fig 5. Plants of two week old soil grown *Arabidopsis* wild type **Ler**, vector control (**VC**), transgenic lines **SC:1** and **SCTP:5** were subjected to drought stress in the green house. Panel **Control** shows the plants before the onset of the stress. Watering the plants was stopped for next two weeks (**Drought**). Almost all the plants were wilted at the end of the period. Dehydrated plants were re-watered by adding 10 ml water per pot and plants were kept in covered plastic green houses to keep the moisture high. Three days after re-watering, the recovery of the plants was recorded and photographed (**Recovery**).

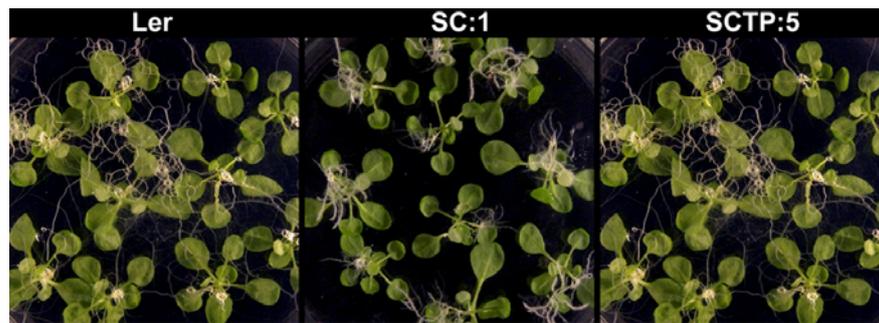


Fig 6. Root phenotype of three week old *in vitro* plants grown on MS media supplemented with 1 % sucrose and photographed through the bottom of the Petri dishes. Wild type, **Ler** plants produced normal roots, whereas the transgenic plants from line **SC:1** produced stunted brushy roots. The plants from transgenic line **SCTP:5**, where the ScTps1 enzyme is directed into the chloroplasts also showed normal root phenotype.

The transgenic *Arabidopsis* line, SC:1, with the ScTps1 protein localized in the cytosol, showed improved drought tolerance but also altered root and shoot phenotypes when grown *in vitro* on MS medium supplemented with 1 % sucrose. In MS medium the roots of the SC:1 plants were about 75 % shorter than the roots of the wild type or Sctp:5 plants (**III**). Retarded brushy roots and somewhat slower growth of the green parts were observed in SC:1 transgenic plants (Fig. 6) that might be due to the localization of ScTps1 and/or the intermediate sugar phosphate T6P in the cytosol. Sctp:5 plants showed normal root and shoot phenotypes similar to wild type plants (Fig. 6). This suggests that the growth

defects can be avoided by targeting the ScTps1 protein into the chloroplast. Lack of aberrant growth effects as well as enhanced drought tolerance in tobacco has also been reported by Lee *et al.*, (2003) in an experiment where the yeast gene *ScTPS1*, regulated by the CaMv35S promoter, was inserted directly into the chloroplast genome of tobacco. The aberrant root phenotype in SC:1 plants growing on sucrose containing media was alleviated when the plants were transferred and cultivated in soil (III), which might indicate the complex role of ScTps1 or T6P as signalling or regulatory molecules in sugar sensing and metabolism influencing plant growth and development. However, it is obvious from the above mentioned results that the growth and developmental aberration are not coupled with the improved drought tolerance.

## Conclusion

AtPTR3 is the first characterized member of the *Arabidopsis* PTR family, which has been found to be induced by different stress factors, such as wounding, salinity, hyperhydricity and pathogens. The induction of the *AtPTR3* gene by different stresses suggests that peptide transport may be needed for relocation or uptake of nutrients needed for stress response and a similar relocation may take place during senescence.

Results showing that *AtPTR3* is predominantly induced by SA suggest that this gene may be involved in the SAR response. This is in accordance with the susceptible nature of the *Atptr3* mutants of both C24 and Col-0 ecotypes against the infection caused by both necrotrophic *E. carotovora* and hemibiotrophic *P. syringae*.

Targeting of the ScTps1 protein, the first enzyme in trehalose biosynthesis pathway, to the *Arabidopsis* chloroplast is found to be sufficient to avoid the developmental aberrations observed in transgenic plants with the enzyme localized in the cytoplasm. Transgenic plants with the ScTps1 targeted to the chloroplasts still exhibit the improved drought tolerance, similarly to the plants with cytoplasmic localization. The data indicate that the improved drought tolerance is most likely caused by improved water retention during drought stress.

## Future perspectives

The expression of *AtPTR3* was observed in *Arabidopsis* by GUS assay and real time PCR. In some online data from microarray analyses *AtPTR3* was found to be induced in developmental and dark-induced senescent leaves and senescing siliques along with many other genes. These preliminary indications could be further verified by real time PCR. The senescence-induced expression of *AtPTR3* can also be verified in different pathway mutants at their senescent stages to observe the dependence of *AtPTR3* expression on SA, JA, ET and ABA pathways during senescence.

It would be tempting to study the localization of the AtPTR3 protein by the expression of AtPTR3-GFP fusion protein in plant cells.

It would be further interesting to study with yeast complementation, what are the other small peptides AtPTR3 could transport. Some PTRs in plants have been found to transport nitrate molecules as well. Therefore, it would be logical to study the role of AtPTR3 as a transporter of other molecules such as nitrate and ammonium.

The GUS analyses and real time PCR results demonstrated the role of all major signalling molecules such as MeJA, SA, ET and ABA as well as salt and wounding on the regulation of *AtPTR3* in *Arabidopsis*. Therefore, comparing the *Atptr3* mutants with wild type plants with several techniques, such as proteomics, metabolomics and microarray profiling would be a very effective way to characterize whether the *Atptr3* mutation affects these pathways.

It would also be interesting to study and compare the influence of the proteins ScTps1 and ScTps2 as well as trehalose and the intermediate sugar phosphate T6P in plants in detail, when the proteins are targeted to different compartments. These studies could include monitoring the metabolome and the photosynthetic capacity as well as the status of the expression of the endogenous *AtTPS1*.

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## Acknowledgements

*All praise to Almighty for blessings and permitting me to explore a bit of the secrets and beauty of the magnificent creation, our green friend plant.*

I know that people are always interested to read this part but I do not know really whether I will be able to express my intense and huge feelings within these pages in a short notice. First of all I would like to pay my heartfelt gratitude to a very special person, Dr. Minna Pirhonen, my learned teacher, my excellent supervisor, my compassionate guide in every single step of my PhD education. How can I show my thankfulness in sentences I do not know? It will take many chapters. Anyway like every people writes I would like to say the same thing “my sincere gratitude and thanks for accepting me as your PhD student and guiding me all the way”. Your guidance and teaching have brightened up my life. My ‘Guru’ my mentor and so called local or should I say “home” supervisor Dr. Kjell-Ove Holmström, my warmest gratitude to you for accepting me as your student and for teaching me the things inside the subject area as well as for all the tips that someone can never find in the manuals and protocols. You taught me the tricky things about computers, softwares, photography and other useful thing in daily life. Thanks for all your concerns and supports during all these years and being a good friend of my family. I would like to wish all the best in your lives because both of you are wonderful persons. I would like to extend my sincere gratitude to my teacher and a member of my supervisory committee Prof. Stefan Hohmann from Göteborg University (GU), without your support I could not make my second manuscript ready.

My earnest gratitude and respect to my examiner, Prof. Hans Ronne for all your support, especially in the beginning of my study that helped me to come to this point. I would like to thank and show my gratitude with sincere appreciation to Dr. Henrik Aronsson from GU for being a good colleague and for his act as my shadow supervisor and helping me a lot especially during the last two critical years, without your support I would not have managed to come to this point too. I acknowledge and pay my cordial respect and gratitude to all my teachers, professors and personnel from my academic department VBSG, SLU: Prof. Christer Jansson, Prof. Christina Dixelius and Prof. Johan Meijer for your kind concern about my PhD education. Specially to Christer for your generous support during my extended period and to Prof. Lisbeth Jonsson and Dr. Per Bergman, ex. prefects for your kind help and support to solve the problems during my PhD study. My warmest gratitude and thanks to Dr. Björn Nicander for your kind support so that I could enjoy the SLU library facilities. Sincere gratitude and thanks to Birgitta Eriksson for your support (long distant) regarding all the official and technical matters. Thanks to Monica Thunberg for all your administrative supports. Cordial thanks to Dimitris Papageorgiou from Repro for your tips and kind help. Gunilla Swärdh, for your excellent work that helped me to produce the third manuscript, I again acknowledge you here with my pleasure. Ulla Pihlgren, thanks for the cytological work, I wish you the best. Dr. Richard Hopkins from SLU, I would like to thank you and show my sincere gratitude for your careful and critical reading and comments on my thesis.

Special thanks to a nice person Peter Dahl from GU for your excellent support and helpful teaching about the yeast work. I would like to acknowledge Prof. Patrick Van Dijk from K.U. Leuven, Belgium for your support in my trehalose work and thanks for your valuable suggestions and discussion. I would like to acknowledge and show my sincere gratitude to the Professors from Helsinki University: Profs. Tapio Palva, Jari Valkonen and Pekka Heino. Special thanks to Dr. Günter Brader for your help and guidance during my stay and work in your lab.