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Dissecting the role of pathogenesis related-10 (PR-10) proteins in abiotic stress tolerance of plants

by

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Abstract

Abiotic stress is one of the major factors that affect food production worldwide and, therefore understanding stress responsive proteins and engineering plants for abiotic stress tolerance is very important. In the present study, the biological role of pea pathogenesis-related 10.4 (PR-10.4; also known as abscisic acid responsive 17; ABR17) in abiotic stress tolerance has been investigated. Our investigation on ribonuclease (RNase) activity of ABR17 suggested that highly conserved histidine-69 and glutamic acid-148 are important for RNase activity. In order to further investigate the biological role(s) of ABR17, transcriptional profiling of pea ABR17-mediated gene expression changes in *ABR17*-transgenic *Arabidopsis thaliana* plants was carried out using microarrays. Our results indicated that pea ABR17 modulates many plant growth/development genes most of which are cytokinin (CK) responsive. These results agree very well with previously reported enhanced endogenous CKs in these transgenic plants. However, no significant changes in transcript abundance of CK biosynthetic genes were observed between transgenic and wild-type plants, suggesting an alternate source of CK in *ABR17*-transgenic plants. It is speculated that ABR17 may act as either a CK reservoir (through its reported CK binding property) or may be responsible for isopentenylated-tRNA degradation (through its demonstrated RNase activity) thereby increasing endogenous CK pools. Furthermore, microarray analysis of salinity stressed *ABR17-Arabidopsis* indicated that ABR17 modulates many stress responsive genes that included four putative AP2 family genes (*RAP2.6*-

At1g43160, *RAP2.6L-At5g13330*, *DREB26-At1g21910* and *DREB19-At2g38340*). Functional characterization of these genes suggested that they are transcription factors and they play very important roles in abiotic stress response in addition to growth and development. Moreover, overexpression of *RAP2.6L* and *DREB19* genes enhanced salinity and drought tolerance in *Arabidopsis*. Taken together, our results suggest that pea ABR17 proteins are important in abiotic stress responses as they may act as source of enhanced CKs and they may also modulate expression of stress responsive genes to enhance stress tolerance in plants. However, additional research aimed at deciphering the links between ABR17 and CK biosynthesis as well as the mechanism of ABR17-mediated gene expression changes should be conducted in order to get more insights into the biological roles of PR10 proteins *in planta*.

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To my dearest grandfather!

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List of Abbreviations

ABA, abscisic acid

ABF, abscisic acid responsive element binding factor

ADP, adenosine-5'-diphosphate

AGI, *Arabidopsis* genome initiative

AMP, adenosine-5'-monophosphate

ANP1, *Arabidopsis Nicotiana* protein kinase-like protein kinase

AP2, apetala-2

APX, ascorbate peroxidase

AREB, abscisic acid responsive element binding factor

At, *Arabidopsis thaliana*

ATHB-7, *A. thaliana* HOMEOBOX 7

ATP, adenosine-5'-triphosphate

BAP, 6-benzylaminopurine

bp, base pairs

bZIP, basic leucine zipper

CaMs, calmodulins

CAMTA, calmodulin binding transcription activator

CaMV, cauliflower mosaic virus

CAT, catalase

CBF, C-repeat binding factor

cDNA, complementary deoxyribonucleic acid

CDPK, calcium dependent protein kinase

CDS, coding sequence

CK, cytokinin

CKX, cytokinin oxidase

CSBP, cytokinin specific binding protein

CSD, copper/zinc superoxidase dismutase

cZ, cis-zeatin

cZR, cZ-riboside

cZRMP, cZ riboside 5'-monophosphate

DAG, diacylglycerol

DAPI, 4', 6-diamidino-2'-phenylindole, dihydrochloride

DAS, days after seeding

DEAE, diethylaminoethyl

DMAPP, dimethylallyl diphosphate

DNA, deoxyribonucleic acid

DRE, dehydration response element

DREB, dehydration responsive element binding factor

DTT, dithiothreitol

DZ, dihydro-zeatin

DZR, DZ-riboside

DZRMP, DZ riboside 5'-monophosphate

ECe, electrical conductivity

EDTA, ethylene diamine tetraacetic acid

EIRE, elicitor-responsive-element

ERE, ethylene response elements

ERF, ethylene responsive element binding factor

ET, ethylene

FAO, food and agricultural organisation

GA, gibberellic acid

GFP, green fluorescent protein;

GH, glycosyl hydrolase

GRP, glycine-rich protein

GST, glutathione S-transferase

GT, glycosyltransferases

HsFs, heat shock transcription factors

HSP, heat shock protein

iP, isopentenyl adenine

IP3, inositol 1, 4, 5-triphosphate

IPR, intracellular pathogenesis related

iPRMP, iP riboside 5'-monophosphate

IPT, isopentenyl transferases

IPTG, isopropyl-beta-D-thiogalactopyranoside

JA, jasmonic acid

LB, Luria-Bertoni

LEA, late embryogenesis abundant

LTP, lipid transfer protein

MAPK, mitogen-activated protein kinase

MeJA, methyljasmonate

MEP, methyl erythritol phosphate

mRNA, messenger ribonucleic acid

MVA, mevalonate

MYB, myeloblastosis

NAA, α-naphthalene acetic acid

NAC, NAM ATAF1, 2, CUC2

NaCl, sodium chloride

NAM, no apical meristem

NDPK, nucleoside diphoshate kinase

NF-Y, nuclear factor Y

Ni-NTA, nickel-nitrilotriacetic acid

NPC4, nonspecific phospholipase C4

NPR1, non-expressor of pathogenesis-related genes 1

ORF, open reading frame

P5CS, Δ^1-pyrroline-5-carboxylate synthetase

PA, phosphatidic acid

PCR, polymerase chain reaction

PDF, plant defensin

PL, pyridoxal

PP2C, protein phosphatases type 2c

PR, pathogenesis related

PRL, pathogenesis related like

PRP, proline-rich protein

PSK2, phytosulfokine precursor 2

qRT-PCR, quantitative real time polymerase chain reaction

RNA, ribonucleic acid

RNAi, RNA interference

RNase, ribonuclease

ROS, reactive oxygen species

RT, room temperature

RT-PCR, reverse transcriptase polymerase chain reaction

S-1-P, sphingosine-1-phosphate

SA, salicylic acid

SAM. Shoot apical meristem

SAR, systemic acquired resistance

SAS, statistical analysis software

SDS, sodium dodecyl sulfate

SI, the international system of units

SIMK, salt stress inducible MAPK

SIPK, salycylic-acid induced protein kinase

SNF1, sucrose-nonfermenting kinase1

SOD, superoxide dismutase

SOS, salt overly sensitive

SRK2C, SNF1-related protein kinase 2

TAIR, the *Arabidopsis* Information Resource

TF, transcription factor

TIGR, the Institute of Genome Research

tRNA, transfer ribonucleic acid

tZRDP, tZ riboside 5'-diphosphate

tZRMP, tZ riboside 5'-monophosphate

tZRTP, tZ riboside 5'-triphosphate

tZ, trans zeatin

UN, United nations

UNEP, United Nations environment programme

USDA, United States department of agriculture

UV, ultraviolet

VC, vector control

WT, wild-type

XET, xyloglucan endo-transglycosylase

X-Gluc, 5-bromo-4-chloro-3-indolyl β-D-lucuronide

CHAPTER 1 Review of literature

1.1 Abiotic stress and food security

Plant stress can be defined as an unfavorable condition that alters the physiological state of the plant and impairs performance of vital functions (Gaspar et al., 2002). Plant stress can be either biotic or abiotic in origin. Biotic stress is caused by living organisms like bacteria, fungi, nematodes, herbivores, weeds and insects while, abiotic stress is caused by environmental factors (like water, light, temperature, metals and salt) and are accountable for significant crop losses. It estimated that abiotic factors can reduce yield by more than 50% in many major crops (Bray et al., 2000). Minimization of crop loss due to plant stress is one of the important strategies to achieve food security for the growing population. The Food and Agriculture Organization (FAO) estimate that the World population will reach 8.3 billion by 2030 and farmers will have to produce 30 percent more grain than they do now to feed the future population (FAO, 2002). Arable land is diminishing at a shocking rate; the per capita cropland area is predicted to shrink in most densely populated countries (Lal, 2007).

Salinity and drought are among the aforementioned abiotic stresses that negatively affect arable land and food production (Ashraf et al., 2009). For instance, nearly 20% of the world's arable land is affected by salinity alone and it is expected to increase up to 50% by 2050 (Wang et al., 2003; UNEP, 2008). Globally, the area affected by drought is also gradually increasing and loss of

agricultural productivity due to drought stress could increase to 20-40% (http://www.globalresearch.ca/index.php?context=va&aid=12252). It is important to note that over 70 Mha of land is already affected by drought stress in rice alone (Ashraf et al., 2009). In addition, the changing climate is expected to worsen soil conditions, increase water shortages, drought, flooding and desertification (Kurukulasuriya and Rosenthal, 2003; FAO, 2010). This reinforces the need to use marginal lands and the need for crop improvement in terms of higher productivity under adverse environmental conditions (FAO, 2010).

Genetically engineered crop plants for abiotic stresses (drought, salinity, extreme temperatures, water-logging) offer a means of improving food security by helping to sustain farming in marginal and degraded lands (FAO, 2002; Wang et al., 2003; FAO, 2010). However, gene manipulations for abiotic stresses are not yet effective in practice because of our limited knowledge of stress-associated metabolism in plants (Vinocur and Altman, 2005). In this context, it is very important to understand plant stress responses as well as the biological significance of genes/enzymes involved in the stress signaling network. In this chapter, the effects of salinity and drought stresses on plants, their signaling pathways and use of genetic engineering to combat abiotic stress are described in detail.

1.2 Salinity

Soil salinity is the accumulation of excessive soluble salts like sodium chloride, magnesium sulfate, calcium sulfates and bicarbonates sufficient to affect plant growth (Munns and Tester, 2008). Among all the salts, sodium chloride (NaCl) is the most soluble and widespread that affects soil quality (Teakle and Tyerman, 2010). Soil salinity is caused by different phenomena including the weathering of parent materials, deposition of oceanic salt carried by wind and rain, irrigation of the crop and insufficient drainage (Proust, 2008). Approximately, 20 percent of the irrigated land and 2.1 percent of the dry land agriculture is affected by soil salinity (Abdelfattah et al., 2009; http://www.fao.org/nr/land/en/). Salt concentration in soil is measured in terms of electrical conductivity (ECe) and the SI unit of ECe is dS/m. The ECe is defined as the electrical conductivity of the saturated paste extract; equivalent to the concentration of salts in saturated soil or in a hydroponic solution (Rhoades et al., 1999). Soil having an ECe of 4 dS/m or more is considered as saline soil, and many crops are sensitive even when soil ECe is below 4 dS/m (USDA-ARS, 2008). An ECe of 4 dS/m is equivalent to roughly 40 mM NaCl and generates osmotic pressure of approximately 0.2 MPa sufficient to reduce the yield of most crops (Munns and Tester, 2008; USDA-ARS, 2008). In this section, the effects of salinity on plant growth, mechanisms of salt tolerance and salt stress signaling pathways are described.

1.2.1 Effects of salinity on plant growth

Salinity affects plant growth in two ways 1) osmotic stress and 2) ionic stress (Läuchli and Epstein, 1990; Munns, 2002). Osmotic stress is caused by the high salt concentration around roots which results in dehydration due to difficulty in absorbing water from the soil. Ionic stress is caused by the toxicity of the ions accumulated in the leaves and shoot, which eventually results in death of the plant or plant organs. Osmotic stress has an immediate effect on plant growth whereas the ionic effects dominate the osmotic effect only at higher salt conditions (Munns, 2002). Interactions between osmotic and ionic stress depend on factors like organ, plant age, genotype, species, ionic strength and salinizing solution (Läuchli and Grattan, 2007).

In the osmotic stress period, the dehydration results in loss of water in cells, decrease in cell volume and reduced cell elongation (Fricke and Peters, 2002). Over time, reduced cell division and cell expansion lead to stunted growth and, as a result, shoot growth and number of tillers/branches will be reduced (Mass and Grieve, 1990; Hernandez et al., 1995). Leaves become smaller and thicker (Longstreth and Nobel, 1979; Volkmar et al., 1997). Reduced shoot growth is considered as a means to preserve carbohydrates for energy requirements and recovery after stress relief (Bartels and Sunkar, 2005). It is also thought to contribute to osmotic adjustment by solute accumulation (Osorio et al., 1998). Early flowering, dying of older leaves, inhibition of lateral branches and

reduced number of flowers will become apparent over time under severe salinity stress (Maas and Poss, 1989; Munns and Tester, 2008). Roots continue to grow under salinity stress in contrast to reduced shoot growth in order to increase surface area for sequestration of toxic ions (Bartels and Sunkar, 2005). Often, salt tolerance is correlated with high root growth rate, rapid plant development and early flowering (Munns et al., 2000 & 2006). In the ionic stress state, toxic ions accumulated in the leaves may dehydrate cells and/or inhibit enzymatic reactions (Lacerda et al., 2003). Toxic ions can also cause membrane damage, disturb solute balances and interfere with nutrient uptake (Volkmar et al., 1997). This results in the death of older leaves resulting in reduced photosynthetic capacity, which affects overall carbon balance that is necessary to sustain growth (Munns and Tester, 2008). In general, salinity affects plant growth by disturbing vital activities such as photosynthesis, protein synthesis, nutrient acquisition, enzyme functions, and causes symptoms like stunted growth, chlorosis, necrosis, and even plant death (Volkmar et al., 1997; Hasegawa et al., 2000).

1.2.2 Mechanisms of salt tolerance

There are different categories of salinity tolerance. One of them is by reducing the response to osmotic stress and avoiding osmotic effects like reduced cell volume, cell elongation and stomatal closure (Munns and Tester, 2008). Reduced response to osmotic stress results in greater leaf growth and increased stomatal conductance. However, this would be beneficial only when plants have sufficient water (Munns and Tester, 2008). The other type of salt tolerance

mechanism is Na^+ ion exclusion and compartmentalization, which is performed by Na^+/H^+ antiporter (Blumwald et al., 2000; Hasegawa et al., 2000; Zhu, 2001). Roots exclude Na^+ ions to reduce accumulation of toxic ions in leaves and protect them from early death during salt stress, and plant cells compartmentalize excessive Na^+ ions into the vacuole to avoid ion toxicity in the cytoplasm (Hasegawa et al., 2000; Zhu, 2001; Munns and Tester, 2008). Achievement of an optimal K^+/Na^+ ratio is also important in addition to maintaining lower Na^+ concentration and, the higher concentration of K^+ stimulates Na^+ efflux and decreases Na^+ influx (Jeschke, 1972; Maathuis and Amtmann, 1990; Lazof and Belnstein, 1999; Hauser and Horie, 2010). In addition to Na^+ ions, reduced absorption, intracellular compartmentalization and efflux of Cl^- is equally important for salinity tolerance (Teakle and Tyerman, 2010). Furthermore, accumulation of osmolytes or compatible solutes like sugars (fructose, glucose, proline, glycine-betaine and alanine betaine), sugar alcohols (glycerol and methylated inositols) and tertiary sulfonium compounds, is one of the salt tolerance mechanisms (Yancey et al., 1982; Delauney and Verma, 1993; Rhodes and Hanson, 1993; Nuccio et al., 1999; Chen and Murata, 2002). The osmoprotectants act as free radical scavengers or as chaperons to stabilize cell membranes and proteins and, also act by lowering cellular osmotic potential and restoring intracellular salt concentrations (Yancey et al., 1982; Hare et al., 1998; Diamont et al., 2001). The relative significance of these salinity tolerance mechanisms vary with species, salt concentration, length of exposure, and local

environmental conditions like soil moisture and air humidity (Munns and Tester, 2008).

1.2.3 Salt stress signaling

The adaptation of plants to any stress depends on the activation of cascades of molecular events required for stress perception to expression of genes related to stress tolerance (Mahajan et al., 2008). Plants respond to salt stress by activating different signaling networks whose products will help them in ion homeostasis, osmotic homeostasis, detoxification, stress damage repair and growth control (Zhu, 2002). Different salt signaling networks including salt overly sensitive (SOS) signaling, oxidative stress and reactive oxygen species (ROS) signaling, and abscisic acid (ABA) dependent gene regulation are described below.

1.2.3.1 SOS signaling

An increase in extracellular concentrations of Na^+ around plant roots elicits an increase in cytosolic free Ca^{2+} levels and activates the salt stress signaling pathway called SOS (Knight et al., 1997; Zhu, 2002; Bertorello and Zhu, 2009; Turkan and Demiral., 2009). Salinity induced changes in the phospholipid composition of plasma membranes activate phopholipases which generate secondary messengers including inositol 1,4,5-triphosphate (IP3), diacylglycerol (DAG) and phosphatidic acid (PA) that may activate protein kinase C and trigger Ca^{2+} release (Xiong and Zhu, 2002; Shilpi and Narendra, 2005;

Hong et al., 2010). Calcium signals are sensed by a myristoylated calcium-binding protein (SOS3) which in turn interacts and activates a serine/threonine

protein kinase called SOS2 (Liu and Zhu, 1998; Halfter et al., 2000; Ishitani et al., 2000; Liu et al., 2000; Bertorello and Zhu, 2009). SOS2 has a regulatory FISL motif in its C-terminal end and SOS3 activates SOS2 via this motif in a calcium dependent manner (Halfter et al., 2000; Albrecht et al., 2001). SOS2 together with SOS3 regulate the expression levels of a plasma membrane Na^+/H^+ antiporter gene *SOS1* and also activate the transport activity of Na^+/H^+ antiporter protein (Shi et al., 2000; Qiu et al., 2002). *SOS1* mRNA is unstable under normal growth conditions and salt stress induces *SOS1* mRNA stability through ROS mediated NADPH oxidase (Chung et al., 2008). During salinity stress, Na^+/H^+ antiporters are involved in Na^+ exclusion and compartmentalization and overexpression of these proteins has resulted in salt tolerance, emphasizing their importance in salt signaling (Apse et al., 1999; Blumwald et al., 2000; Shi et al., 2003; Yang et al., 2009). SOS signaling proteins also interact with other vascular transport Na^+ and H^+/Ca^{2+} proteins and regulate Na^+ movement as well as Ca^{2+} homeostasis (Uozumi et al., 2000; Rus et al., 2001; Zhu, 2002; Cheng et al., 2004). Furthermore, *SOS4* which encodes a pyridoxal (PL) kinase that is speculated to function upstream of ethylene and auxin production in root hair and *SOS5* which encodes a cell surface adhesion protein required for normal cell expansion under salt stress have been reported (Mahajan et al., 2008; Turkan and Demiral, 2009).

1.2.3.2 Oxidative stress and ROS signaling

Oxidative stress is a consequence of ionic and osmotic effects caused by salt stress, where ROS accumulate in cells more than the normal equilibrium for these species (Pang and Wang, 2008; Miller et al., 2010). Reduction of molecular oxygen (O_2) results in ROS such as, superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), and during salinity the formation of ROS increase (Asada and Takahashi, 1987; Apel and Hirt, 2004; Pang and Wang, 2008). At lower concentrations, ROS act as signaling molecules while at higher concentrations they damage lipids, proteins and nucleic acids (Gomez et al., 1999; Hernandez et al., 2001; Pang and Wang, 2008). Stress-induced ROS triggers ROS scavenging systems consisting of antioxidants like carotenoids, ascorbate, glutathione, tocopherol and antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) in order to help plants cope with the oxidative stress damage (Asada, 1999; Miller et al., 2010). ROS sensors sense salt stress-induced ROS and activate mitogen-activated protein kinase (MAPK) cascades (Knight and Knight, 2001; Miller et al., 2010). Through the process of phosphorylation MAPK cascades transfer information from sensors to cellular responses and regulate the expression of transcription factors (TFs) and stress-related genes (Tena et al., 2001; Nakagami et al., 2005; Pang and Wang, 2008). In plants, SIMK (salt stress inducible MAPK), SIPK (salycylic-acid induced protein kinase), ANP1 (*Arabidopsis Nicotiana* protein kinase-like protein kinase), MPK3, MPK6, NDPK2 (nucleoside diphosphate kinase 2) have been implicated in

osmotic stress signaling (Munnik et al., 1999; Kovtun et al., 2000; Mikolajczyk et al., 2000; Moon et al., 2003). Although no ROS sensor has been unambiguously identified in plants, redox-sensitive TFs such as NPR1 (non-expressor of pathogenesis-related genes 1), heat shock TFs (HsFs) and phosphatase inhibitors are speculated to be involved in stress induced ROS perception (Mittler et al., 2004; Pang and Wang, 2008). Identification of ROS sensors and an understanding of how different cellular signaling networks are linked to the ROS response are the future challenges to be addressed.

1.2.3.3 ABA dependent signaling

ABA is an important phytohormone that is involved in various aspects of plant physiology and development including, seed development, seed dormancy and synthesis of seed storage proteins and lipids (McCarty, 1995; Bentsink and Koornneerf, 2002; Finkelstein et al., 2002). In addition, it plays a crucial role during osmotic stress and is therefore referred to as a stress hormone (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002; Raghavendra et al., 2010). ABA regulates the expression of important ion transporters like Na^+/K^+ antiporter during salinity stress (Shi and Zhu, 2002; Yokoi et al., 2002). Proteins from the SOS salt signaling pathway interact with ABA regulators and ABA regulators also interact with K^+ channel (Vranova et al., 2001; Cherel et al., 2002; Qiu et al., 2002; Quintero et al., 2002; Ohta et al., 2003; Kuhn et al., 2006; Yoshida et al., 2006). The K^+ channel plays an important role in stomatal opening and closing and, disturbed K^+ homeostasis may contribute to salt sensitivity (Zhu et al., 1998;

Rus et al., 2004). In addition, ABA and its biosynthetic genes have been found to increase during salt stress and therefore ABA is speculated to be involved in ion homeostasis during salinity (Xiong and Zhu, 2003; Raghavendra et al., 2010).

1.3 Drought

Drought is one of the major environmental factors that affect crop productivity and it can be defined as extended period of water deficiency resulting in severe damage to crops and yield loss (Boyer, 1982; Heim, 2002; Ashraf et al., 2009; Farooq et al., 2009). Plant water deficit develops when the water demand is more than the supply. The supply and demand depends on the available soil moisture and crop evapotranspiration rate, respectively. Prevailing weather conditions like air temperature, relative humidity and wind determine the rate of crop evapotranspiration (Heim, 2002). Drought is classified into four distinct types based on the duration and intensities of water shortages (Dracup et al., 1980; Wilhite and Glantz, 1985; Heim, 2002). Meteorological drought is described as a temporary period of dry weather and hydrological drought is associated with the shortfalls of water supply from ground water, streams, lakes and rivers, while socioeconomic drought is a situation when drought begins to affect people and their livelihood. Finally, agricultural drought occurs when a low precipitation adversely affects crop production (Dracup et al., 1980; Wilhite and Glantz, 1985). In general, in the context of crop production drought is defined as inadequate plant-available water over a period of time (Hounan et al.,

1975). In this section, the effects of drought on plant, mechanisms of drought tolerance and drought signaling are described.

1.3.1 Effect of drought on plant

Water is a universal solvent that constitutes 80-90% of the fresh weight of plants and maintains the turgidity and temperature of cells (Kirkham, 2005). Water deficit results in loss of cell turgidity and affects plants' physiological processes (Vaadia et al. 1961; Reddy et al., 2004). Water stress affects plant growth and development by affecting cell division, cell expansion, membrane integrity, protein synthesis, lipid synthesis, chlorophyll and carotenoid production (Hsiao, 1973; Zwiazek and Blake, 1990; Fan et al., 1994; Riccardi et al., 1998; Farooq et al., 2009; Jaleel et al., 2009). The impact of drought stress is greater when it occurs during early vegetative growth, flowering and reproductive stages (Trippi and Thimann 1983; Farooq et al., 2009). In general, drought stress results in leaf wilting, leaf desiccation, leaf area reduction, leaf abscission, photosynthesis reduction and poor yield (Winston, 1990; Golakiya and Patel, 1992; Reddy et al., 2004).

1.3.2 Drought tolerance mechanisms

Plants adopt various adaptive mechanisms to cope with drought conditions, which include drought escape, drought avoidance and drought tolerance (Farooq et al., 2009). Drought escape involves strategies like early maturation that helps plants avoid drought conditions (Kumar and Abbo, 2001;

Araus et al., 2002). In the case of drought avoidance, plants maintain cell turgidity either by saving water or by absorbing water more efficiently, and different strategies include stomatal closure, cuticular barrier, reduced leaf area and root adaptations (Ludlow and Muchow, 1990; Ball et al., 1994; Sandquist and Ehleringer, 2003; Kavar et al., 2007). Drought tolerance mechanisms include the accumulation of osmoprotectants, antioxidants, dehydrins, late embryogenesis abundant (LEA) proteins and cell wall related proteins (Kramer and Boyer, 1995; Turner et al., 2001; Farooq et al., 2009).

1.3.3 Drought stress signaling

Drought stress elicits effects in plants that are similar to those caused by the osmotic stress component of salt stress and both are integrated by many signaling networks (Zhu, 2002: Shi, 2007). Different integrating networks include Ca^{2+} signaling, oxidative signaling, kinase cascades and ABA signaling (Zhu, 2002). The integration of salt and drought signal transduction is illustrated in Figure 1-1. While plant cells maintain low cytoplasmic levels under normal conditions by Ca^{2+} ATPases and Ca^{2+}/H^{+} antiporters, the Ca^{2+} concentration increases in response to drought in a manner similar to that in salt stress as previously discussed (Sze et al., 2000; Hirsch, 2001; White and Broadley, 2003). Although calcium signals are considered to be universal in response to stress, their stress-specific responses are not yet completely understood (Shi, 2007; Kim et al., 2009). It has been suggested that different

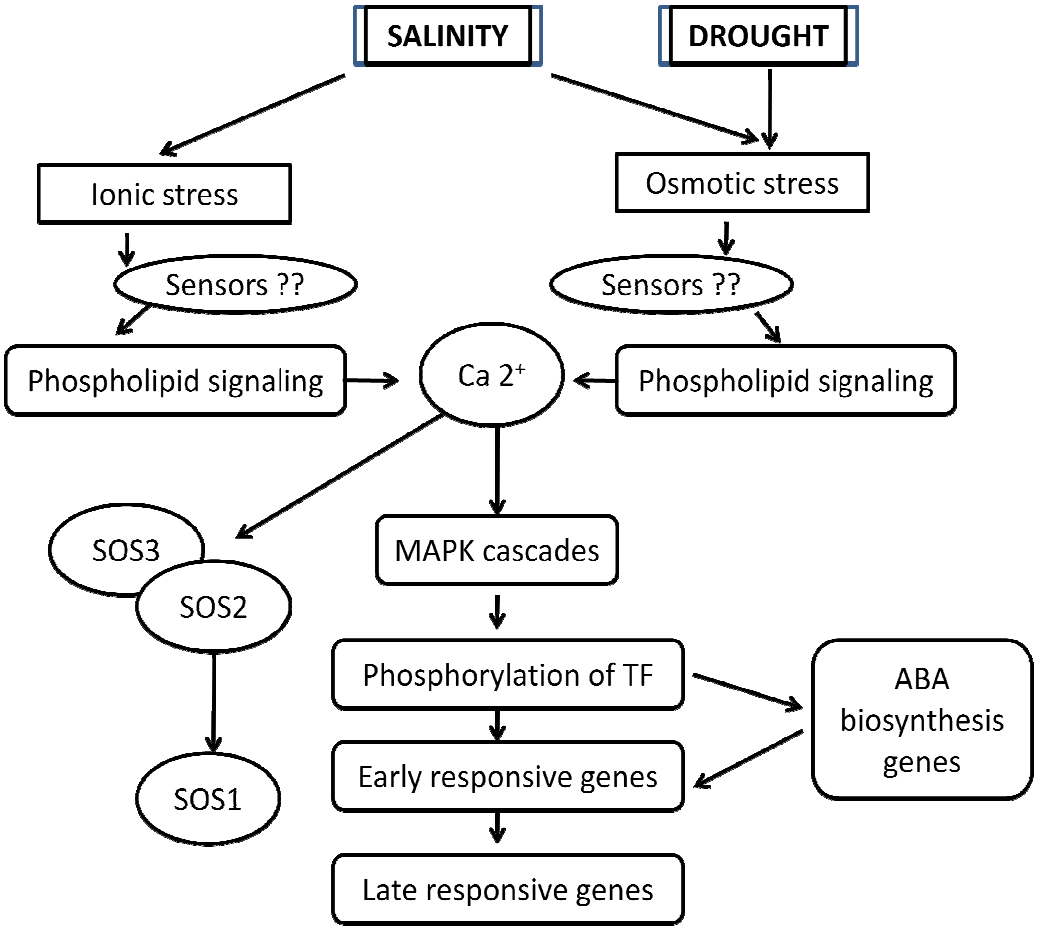

Figure 1-1 Integration of salt and drought signaling network

(based on Zhu, 2002)

stimuli activate different Ca^{2+} permeable channels localized in distinct locations within a cell and generate location specific spatial Ca^{2+} elevation with varying frequency, period and amplitude with different stimuli (Evans et al., 2001; Shi, 2007). For example, under drought stress, Ca^{2+} signals are important for stomatal closure to reduce water loss and Ca^{2+} signals are activated by ABA induced H_2O_2 production (Pei et al., 2000; Kim et al., 2010). In addition, it has also been demonstrated that drought-induced cytoplasmic Ca^{2+} oscillations are sphingosine-1-phosphate (S-1-P) dependent (Ng et al., 2001). The Ca^{2+} signals are perceived by different proteins including calmodulins (CaMs), calcium dependent protein kinases (CDPKs), Ca^{2+} and CaM binding TFs and the targeted downstream effectors include proteins involved in various cellular metabolic processes and physiological adjustment (Day et al., 2002; Shi, 2007; Kim et al., 2009). Therefore, $Ca2^{+}$ signals are important regulators of the plant response to osmotic stress conditions.

The involvement of ABA in cellular development and in the salt response has been previously discussed (section 1.2.3.3). ABA biosynthesis is regulated both by developmental cues as well as by stress stimuli (Xiong and Zhu, 2003; Raghavendra et al., 2010). ABA is a very important signaling molecule in drought stress and it is involved in the regulation of stomatal closure to minimize water loss (Zhu, 2002; Xiong, 2007; Kim et al., 2010). Based on promoter studies, it has been suggested that, during drought stress, ABA is synthesized in vascular tissues and guard cells (Christmann et al., 2005). ABA regulates

stomatal closure either by increasing cytosolic Ca^{2+} levels as mentioned earlier or by recently understood Ca^{2+}-independent routes, and many kinases and phosphatases assist in ABA regulation of guard cells (Levchenko et al., 2005; Xiong, 2007; Kim et al., 2010). In the Ca^{2+}-dependent route, elevated Ca^{2+} levels inhibits plasma membrane proton pumps and inward K^+ channels and activate anion outward channels, which in turn leads to K^+ efflux and reduced osmolarity in guard cells leading to stomatal closure (Schroeder et al., 2001; Fan et al., 2004; Pei et al., 2005). In Ca^{2+}-independent regulation, protein phosphatases type 2c (PP2C) ABA negative regulators (such as ABI1, ABI2 and HAB1) will interact with SNF1 (Sucrose-Nonfermenting Kinase1)-related protein kinases (OST1/SnRK2.6/SnRK2E, SnRK2.2/SnRK2D and SnRK2.3/SnRK2I) that act as positive regulators of ABA and stomatal opening (Raghavendra et al., 2010). In the presence of ABA, the recently identified ABA receptors RCARs/PYR1/PYLs that belong to the Betv1 super family, bind with ABA and ABA negative regulators to form the RCAR–ABA–PP2C complex to release negative regulation and to activate SNF1 kinases (Klingler et al., 2010). The SNF1 kinase OST1 activates the anion channel SLAC1 and inhibits cation channel KAT1 through phosphorylation which results in release of osmotically active compounds and deflation of guard cells and stomatal closing (Geiger et al., 2010; Kim et al., 2010). In addition to regulation of stomatal closure, ABA also regulates expression of many genes that code for products like antioxidants, compatible solutes and signal transduction components that are involved in physiological adjustment under drought stress (Leung and Giraudat, 1998; Finkelstein and

Rock, 2002; Raamanjulu and Bartels, 2002; Raghavendra et al., 2010). Few examples of these antioxidant encoding genes include genes for glutathione S-transferases, superoxide dismutase, catalse, ascorbate peroxidase, glutathione reductuase, proline, sugars, proteases and LEA proteins (Hoth et al., 2002; Jiang and Zhang, 2002; Zhu, 2002; Xiong, 2007; Miller et al., 2010). In addition, ABA also activate the expression of ABFs/AREBs (ABA-responsive Element Binding Factor/Protein) type TFs, basic region/leucine zipper (bZIP)-type TFs like ABI5, apetala-2 (AP2)-type TFs like ABI4, and MYC/MYB-type TFs that are key regulators in expression of stress responsive genes under osmotic stress (Raghavendra et al., 2010).

1.4 Genetic engineering for abiotic stress tolerance

As discussed previously, physiological and biochemical changes in response to different abiotic stresses include membrane disorganization, generation of ROS, accumulation of osmolytes, antioxidants, chaperonines, dehydrins, PR and LEA proteins, expression of TFs and stress related genes, changes in phytohormone profile, inhibition of photosynthesis and changes in nutrient acquisition (Mahajan et al., 2008; Munns and Tester, 2008; Ashraf et al., 2009; Farooq et al., 2009; Teakle and Tyerman, 2010). Genes and gene products induced/synthesized during the stress response function both in stress tolerance as well as stress signaling (Yamaguchi-Shinozaki and Shinozaki, 2006). For example, detoxification enzymes, antioxidants, LEA proteins, molecular chaperones help plants to tolerate stresses, while kinases and TFs help in inducing

stress responsive signal cascades in response to stress (Yamaguchi-Shinozaki and Shinozaki, 2006). Genomics, proteomics, metabolomics and transgenic technologies have been very helpful in identifying gene products involved in plant defense signaling response pathways and in introducing such genes into crop plants to get stress tolerant phenotypes. Some successful examples of abiotic stress tolerant transgenic plants are discussed in this section.

1.4.1 Genes involved in ion exclusion, ROS signaling and osmotic adjustment

Ion homeostasis is an important process in plant salinity tolerance that is obtained by proteins called antiporters (see section 1.2.3.1) and genetic manipulation of antiporters has been useful in generating salt tolerant plants. For example, salinity tolerant *Arabidopsis* and canola plants have been developed by overexpressing the Na^+/K^+ antiporter transport genes, *AtNHX1, SOS1* and *SOS3* (Aspe et al., 1999; Zhang et al., 2001; Shi et al., 2003; Yang et al., 2009). *Arabidopsis* plants overexpressing *GmCAX1*, a putative antiporter accumulated less Na^+, K^+ and Li^+ ions and were tolerant to higher levels of Na^+ and Li^+ ions during germination (Luo et al., 2005). Furthermore, *Arabidopsis* overexpressing the wheat Na^+/H^+ antiporter *TNHX1* and H^+ pyrophosphatase *TVP1* showed salt and drought stress tolerance (Brini et al., 2007). Similarly, potato (*Solanum tuberosum*) plants overexpressing the barley antiporter gene *HvNHX2* had greater salinity tolerance compared to their wild type counterparts (Bayat et al., 2010). Antioxidant enzymes have also been helpful in generating abiotic stress tolerant plants. For example, *Brassica* species overexpressing glutathione synthetase

showed cadmium tolerance (Zhu et al., 1999), *N. tabacum* overexpressing pea Cu/Zn SOD genes showed enhanced resistant to ozone-induced foliar necrosis and oxidative stress (Gupta et al., 1993; Pitcher and Zilinskas, 1996) and rice plants transformed with wheat catalase exhibited enhanced cold tolerance (Matsumura et al., 2002). Furthermore, simultaneous expression of superoxide dismutase (*SOD*), ascorbate peroxidase (*APX*) and nucleoside diphoshate kinase (*NPDK*) conferred greater tolerance to oxidative stress and high temperature stress (Kim et al., 2010).

Similarly, manipulation of plants for increased levels of osmotic agents (glycine betaine, osmotin, fructan, trehalose, proline, etc.,) has resulted in abiotic stress tolerance. Abiotic stress tolerance in transgenic plants engineered for elevated glycine betaine and proline content are best examples. For instance, transgenic cotton (*Gossypium hirsutum* L.) expressing *AhCMO* that encodes choline monooxygenase, a major enzyme in the synthesis of glycine betaine, conferred salinity tolerance due to accumulation of a high level of glycine betaine (Zhang et al., 2009). Furthermore, wheat (*Triticum aestivum* L.) expressing *betA* encoding choline degydrogenase from *Escherichia coli* conferred salinity tolerance by accumulating higher levels of glycine betaine (He et al., 2010). Similarly, transgenic *Medicago truncatula* expressing *Δ^1-pyrroline-5-carboxylate synthetase (P5CS)* from *Vigna aconitifolia* conferred osmotolerance by accumulating higher levels of proline content (Verdoy et al., 2006). Other examples of gene manipulation for increased osmolytes include improved

tolerance to water and salt stress in transgenic wheat overproducing mannitol (Abebe et al., 2003), freezing tolerance in transgenic tobacco plants with higher fructan content (Li et al., 2007) and enhanced multiple abiotic stress tolerance in transgenic rice plants with increased trehalose content (Garg et al., 2002). In addition to manipulating transport enzymes and osmotic agents, the manipulation of other abiotic stress response intermediate proteins like LEA and heat shock proteins (HSPs) has resulted in tolerance to temperature extremes, salinity and drought. For example, expression of barley LEA protein *HVA1* has resulted in enhanced tolerance to water deficit and salt stress in rice and wheat (Xu et al., 1996; Sivamani et al., 2000; Chandrababu et al., 2004). Similarly, overexpression of HSP, *DnaK1* from a halotolerant *Cyanobaterium* resulted in enhanced salt tolerance in *N. tabacum* (Sugino et al., 1999) and overexpression of *Athsp101* in rice resulted in enhanced heat tolerance (Katiyar-Agarwal et al., 2003). These examples demonstrate that the intermediates of stress signalling pathway that help plant to adapt to the extreme environmental conditions can be utilised in engineering plants for stress tolerance.

1.4.2 Genes encoding kinases and phospolipases

Kinases (MAPKs and CDPKs) are an important class of enzymes that are part of signal transduction pathways activated by various abiotic stress factors, and manipulation of these enzymes has resulted in cold, light, drought and osmotic stress tolerance. For instance, overexpression of *OsCDPK7* enhanced salt, cold and drought tolerance (Saijo et al., 2000). Similarly, overexpression of

OsMAPK5 increased tolerance to drought, salt and cold stresses (Xiong and Yang, 2003). In addition, the overexpression of MAPKKK gene *DSM1* was shown to regulate ROS and enhance tolerance to dehydration in rice (Ning et al., 2010). Furthermore, overexpression of *AtNDPK2* (NDP kinase 2) and *SRK2C* (a SNF1-related protein kinase 2) in *A. thaliana* resulted in multiple abiotic stress tolerance (Moon et al., 2003; Umezawa et al., 2004). More recently, a calcium/calmodulin-regulated member of the receptor-like kinase, CRLK1 has been shown to be a positive regulator of cold tolerance through knock out mutant analysis (Yang et al., 2010). In addition, it was suggested that *CRLK1* plays a role in bridging calcium/calmodulin signaling and cold signaling (Yang et al., 2010) and therefore it may be worth overexpressing *CRLK1* to see whether it enhances cold tolerance in plants. Similar to kinases, protein phospholipases which are involved in membrane lipid hydrolysis are also important in plant defense signaling and some have been used in engineering plants for abiotic stress tolerance (Katagiri et al., 2001; Hong et al., 2010). For example, *Arabidopsis* overexpressing *phospholipase D* has demonstrated enhanced freezing tolerance (Li et al., 2004) and maize overexpressing *ZmPLC1* enhanced drought tolerance (Wang et al., 2008). Similarly, overexpression of phosphatidylinositol-phospholipase C2 in canola enhanced drought tolerance (Georges et al., 2009) and overexpression of *nonspecific phospholipase C* (*NPC4*) in *Arabidopsis* enhanced hyperosmotic stress tolerance (Peters et al., 2010). These examples suggest that signaling molecules like kinases and phopholipases have potential utility in enhancing abiotic stress tolerance in crop plants.

1.4.3 Genes encoding TFs

TFs are DNA binding proteins that bind to specific *cis*-elements present in promoters of the genes they regulate to control the expression of mRNA from DNA (Latchman, 1997). They regulate gene expression by activating or by repressing the recruitment of the enzyme RNA polymerase to the specific genes they regulate (Karin, 1990; Nikolov and Burley, 1997). It is not surprising that 5% of the *Arabidopsis* genome encodes TF genes, given the importance of TFs in plant growth, development and stress responses (Riechmann et al., 2000). In signal transduction net work, TFs play important roles from perception to expression of stress responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006). To date, the biological roles of many TFs and their *cis*-acting elements have been identified and transcriptional regulatory networks for abiotic stresses in *Arabidopsis* are represented in Figure 1-2 (Yamaguchi-Shinozaki and Shinozaki, 2006; Saibo et al., 2009). In conclusion, TF encoding genes have been very helpful in generating abiotic stress tolerant plants and a few specific examples are discussed below.

1.4.3.1 AP2 family genes

AP2 family is one of the major TF families in *Arabidopsis* comprising 147 genes (Feng et al., 2005). In addition to their important function in plant growth and organ development, AP2 TFs play critical roles in stress signaling (Saleh and Pages, 2003; Guo et al., 2005). The ERF (ethylene responsive element binding

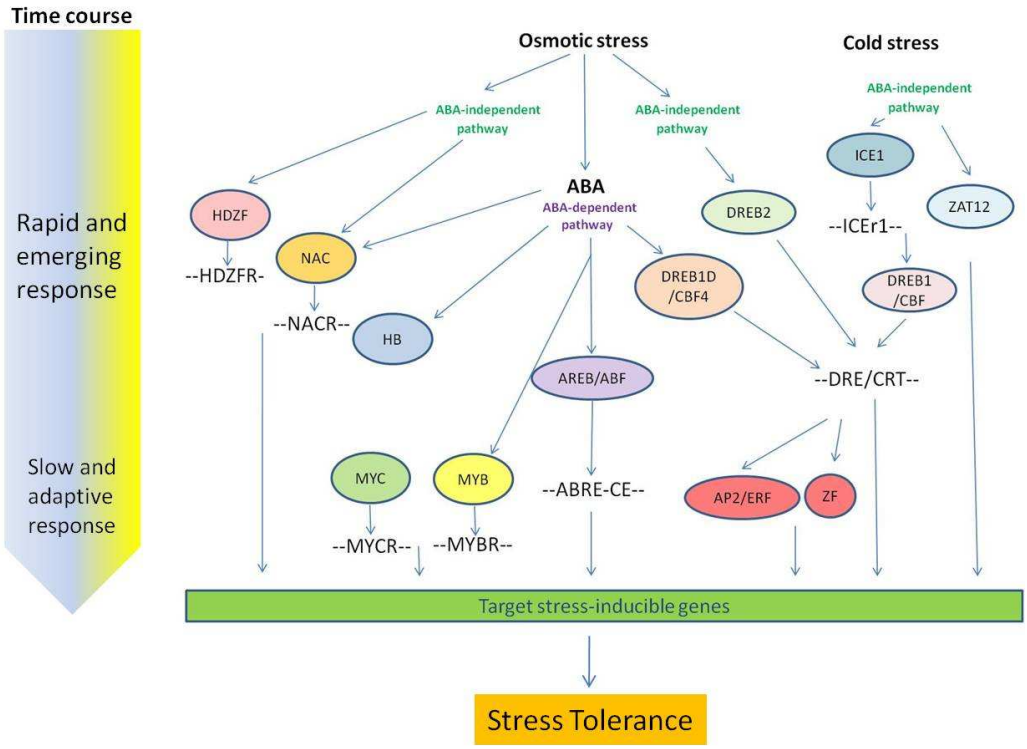

Figure 1-2 Transcriptional regulatory networks involved in abiotic stress response in *Arabidopsis*

(Adapted from Yamaguchi-Shinozaki and Shinozaki, 2006)

TFs are shown in colored ellipses/circles and their *cis*-acting elements are shown as –*cis* elements--.

factor) subfamily proteins regulate expression of ethylene inducible pathogenesis related genes and other stress related genes by binding to ethylene response elements (ERE) or GCC boxes found in their promoters (Ohme-Takagi and Shinshi, 1995). On the other hand, the DREB (dehydration responsive element binding factor) subfamily proteins regulate the expression of low temperature and / or water deficit responsive genes by binding to C-repeat or dehydration response elements (CBF/DRE) in their promoters (Stockinger et al., 1997; Gilmour et al., 1998).

Among DRE binding proteins, DREB1A and DREB2A are the most studied genes and they are induced in *Arabidopsis* in response to low temperature and dehydration, respectively (Liu et al., 1998). The overexpression of *DREB1A* enhanced freezing and dehydration tolerance in transgenic *Arabidopsis* plants but affected plant growth and development (Liu et al., 1998). However, the expression of *DREB1A* with the stress inducible rd29A promoter resulted in minimal effects on plant growth and improved tolerance to multiple abiotic stresses (Kasuga et al., 2004). Furthermore, over-expression of *Arabidopsis CBF3*/*DREB1A* and *ABF3* in *Oryza sativa* enhanced abiotic stress tolerance (Oh et al., 2005). In addition, over-expression of rice *OsDREB1A* in *Arabidopsis* induced stress inducible genes and improved tolerance to drought, high-salt, and freezing stresses demonstrating the regulatory role of DREB proteins in stress responses and suggesting the potential utility of *OsDREB1A* in producing stress tolerant, transgenic crops (Dubouzet et al., 2003). Similarly, heterologous

expression of *Arabidopsis CBF1*/*DREB1* cDNA in *Lycopersicon esculentum* resulted in enhanced tolerance to chilling and oxidative stresses (Hsieh et al., 2002). In case of DREB2A, posttranslational modification is required for activation and overexpression of constitutive active *DREB2A* resulted in significant drought stress tolerance in transgenic *Arabidopsis* (Sakuma et al., 2006). Furthermore, a DREB gene from soybean *GmDREB2* when overexpressed in tobacco caused accumulation of higher levels of free proline compared to the wild type plants under drought conditions and conferred drought and high-salt tolerance (Chen et al., 2007). Similarly, another DREB gene *GmDREB3* from soybean enhanced tolerance to cold, drought, and high salt stresses with minimum effects on plant growth when overexpressed using the Rd29A promoter in *Arabidopsis* (Chen et al., 2009). Furthermore, overexpression of the drought responsive gene *CBF4* in *Arabidopsis* resulted in the activation of a C-repeat/dehydration-responsive element containing downstream genes involved in cold and drought adaptation, and enhanced freezing and drought stress tolerance (Haake et al., 2002). These examples illustrate that DREB genes have critical roles in regulating stress responsive genes that are required for plant survival under drought, cold and salinity stress.

Similar to DREB genes, ERF genes also regulate stress responsive genes and have been useful in developing stress tolerant plants. For example, overexpression of the soybean *GmERF3* gene resulted in enhanced salt and drought tolerance in addition to disease resistance in tobacco (Zhang et al., 2009).

Similarly, tobacco plants expressing the sugarcane gene *SodERF3* encoding an ERF-AP2-type TF had increased tolerance to drought and osmotic stress without any affects on plant growth and development (Trujillo et al., 2009). Furthermore, overexpression of *Arabidopsis RAP2.2* resulted in improved plant survival under hypoxia stress, while T-DNA knockout lines of this gene had poorer survival rates than the wild type (Hinz et al., 2010). In addition, an example of successful AP2 TF transgenic under field condition include overexpression of *AP37* with the OsCc1 promoter in rice that enhanced tolerance to multiple abiotic stresses including drought, high salinity and low temperature at the vegetative stage (Oh et al., 2009). Moreover, *AP37* transgenic plants also demonstrated significantly enhanced drought tolerance at the reproductive stage with a higher seed set of 16–57% over controls under field conditions (Oh et al., 2009). These examples demonstrate that the ERF subfamily AP2 TF has potential for engineering crop plants with abiotic stress tolerance.

Other examples of AP2 TF genes in abiotic stress tolerance include transgenics of *CAP2, CaPF1 SHN* and *WXP1genes.* The 35S promoter-driven expression of *CAP2* (from *Cicer arietinum*) in tobacco resulted in an increase in leaf surface area and number of lateral roots (Shukla et al., 2006). Furthermore, it upregulated abiotic stress and auxin response genes, and enhanced dehydration and salt tolerance in transgenic plants (Shukla et al., 2006). Similarly, *CaPF1* (from *Capsicum annuum*) affected expression of genes that contain either a GCC or CRT/DRE box in their promoter regions and enhanced freezing tolerance in

Arabidopsis (Yi et al., 2004). Furthermore, overexpression of the AP2 family gene *Tsi1* (for Tobacco stress-induced gene1) in tobacco induced many PR proteins and enhanced osmotic and biotic stress tolerance (Park et al., 2001). Similarly, the *SHN* (shine) genes enhanced drought tolerance by altering cuticle properties when overexpressed in *Arabidopsis* (Aharoni et al., 2004) and overexpression of *WXP1* (from *Medicago truncatula*) enhanced drought tolerance in transgenic *Medicago sativa* by increasing cuticular wax accumulation (Zhang et al., 2005). These examples illustrate that AP2 family TF genes are very important in abiotic stress signaling cascades and that they regulate the expression of many stress responsive genes whose products are essential for stress tolerance.

1.4.3.2 bZIP (basic leucine zipper)-type TFs genes

The bZIP-type TF family proteins are known to play important roles in ABA-dependent gene expression by binding to ABRE (ABA responsive elements) of ABA-responsive genes (Choi et al., 2000) and have been useful in engineering abiotic stress tolerant phenotypes in plants. For example, the ABRE-binding proteins AREB1, AREB2 and ABF3 activate many downstream genes including LEA genes, PP2C genes, rd29B and TFs, and regulate ABA signaling under water stress (Uno et al., 2000). Furthermore, the *AtWRKY63* T-DNA insertion mutant called *abo3* was hypersensitive to ABA, and had reduced expression of ABA-responsive TF gene *ABF2*/*AREB1* and stress-inducible genes *RD29A* and *COR47*, suggesting a role for *AtWRKY63* as well as *AREB1* in ABA signaling and drought stress response (Ren et al., 2010). A wheat bZIP-type TF

encoding gene *Wlip19* acts as a transcriptional regulator of *Cor*/*Lea* genes, and when expressed in transgenic tobacco demonstrated a significant enhanced freezing and other abiotic stress tolerance (Kobayashi et al., 2008). Similarly, another stress inducible bZIP TF gene *OsbZIP23* when overexpressed in rice improved tolerance to drought and high-salinity stresses and sensitivity to ABA (Xiang et al., 2008). In contrast, a null mutant of *OsbZIP23* showed significantly decreased sensitivity to ABA and decreased tolerance to high-salinity and drought stress (Xiang et al., 2008). Furthermore, the mutant phenotype was complemented by transforming *OsbZIP23* back into the mutant clearly demonstrating its function and utility in abiotic stress tolerance (Xiang et al., 2008). These examples illustrate the importance of bZIP-type TF family genes in abiotic stress response and ABA sensitivity, and also their utility in genetic engineering of crop plants for abiotic tolearance.

1.4.3.3 MYB (MYB for MYeloBlastosis) family genes

MYB TF family proteins accumulate in response to ABA during abiotic conditions and regulate the expression of stress responsive genes like *RD22* by binding to MYB cis-element recognition sites (Abe et al., 2003). Overexpression of these TF genes has resulted in ABA sensitivity and abiotic stress tolerance. For instance, *CpMYB10* from the dehydration resistant plant *Craterostigma plantagineum,* when overexpressed in *Arabidopsis,* resulted in desiccation and salt tolerance of transgenics lines in addition to glucose-insensitivity and ABA hypersensitivity (Villalobos et al., 2004). Similarly, *AtMYB44* overexpressing

Arabidopsis showed enhanced abiotic stress tolerance and down-regulation of PP2C (serine/threonine protein phosphatases 2C) genes, while *atmyb44* knockout lines demonstrated reduced abiotic stress tolerance and upregulation of PP2C genes compared to the wild type plants (Jung et al., 2008). Therefore, it was suggested that *ATMYB44* confers salinity and drought tolerance by increasing ABA sensitivity and by down regulation of genes encoding PP2Cs, which have been described as negative regulators of ABA signaling (Jung et al., 2008). Furthermore, the overexpression of *OsMYB3R*-2 from *Oryza sativa* in *Arabidopsis* resulted in up-regulation of cold-related genes (like *DREB2A*, *COR15a*, and *RCI2A*) and tolerance to freezing, drought, and salt stress (Dai et al., 2007). Similarly, rice *Osmyb4*, *OsMYB3R*-2, *OsMYBS3* overexpressed *Arabidopsis* and Rice plants demonstrated enhanced cold and freezing tolerance (Vannini et al., 2004; Ma et al., 2009; Su et al., 2010). Furthermore, the gain and loss-of function analyses of ABA-inducible MYB gene *SlAIM1* from *Solanum lycopersicum* has suggested its role in regulating trans-membrane ion fluxes and genetic control of crosstalk between biotic and abiotic stress responses in plants (Abuqamar et al., 2009). The ectopic expression of *SlAIM1* resulted in ABA sensitivity, less accumulation of Na^+ and salinity and oxidative stress tolerance, while tomato *SlAIM1* RNA interference (RNAi) plants were less sentitive to ABA and more sensitive to salinity and oxidative stresses (Abuqamar et al., 2009). These examples show the importance of MYB TFs in regulating the expression of stress responsive genes and their use in enhancing abiotic stress tolerance in crop plants.

1.4.3.4 NAC [an acronym for NAM (No Apical Meristem)] family genes

The NAC TF family is one of the largest TF families and genes from this family have been known to function in developmental process as well as in stress response (Souer et al., 1996; Olsen et al., 2005; Nakashima et al., 2007; Yoo et al., 2007). For instance, *AtNAC2* acts downstream of ethylene and auxin pathways and is involved in lateral root development and the salt stress response (He et al., 2005). In addition, *SNAC1*, a drought inducible NAC gene in guard cells, when overexpressed in rice, significantly enhanced percent seed set and drought resistance under field conditions (Hu et al., 2006). In addition, these *SNAC1* transgenic rice plants showed drought resistance and salt tolerance at the vegetative stage as well (Hu et al., 2006). The stress tolerance response in *SNAC1* transgenic plants has been correlated with induction of the MYB TF gene and genes encoding proteins related to osmotic adjustment, cell membrane stability and stomatal closing (Hu et al., 2006). Similarly, the constitutive overexpression of *OsNAC6* in rice plants improved tolerance to dehydration and high-salt stresses in addition to blast-disease tolerance (Nakashima et al., 2007). More recently, it has been reported that root specific expression of a NAC-type TF *OsNAC10* enhanced drought tolerance and improved grain yield in rice under field drought conditions (Jeong et al., 2010). Furthermore, *ANAC019* (another NAC family gene) has been identified as a new positive regulator of ABA signaling and ectopic expression of *ANAC019* results in ABA hypersensitivity and expression of stress responsive genes like *COR47*, *RD29b* and *ERD11* (Jensen et al., 2010).

These examples demonstrate that NAC family TF genes are involved in the abiotic stress response and have the potential to enhance stress tolerance of crop plants when overexpressed.

1.4.3.5 Others

There are many TFs whose *cis*-elements and function are not clear. However, transgenic plants obtained by manipulation of these TFs have resulted in abiotic stress tolerance. For example, the Alfin TF family gene *Alfin1* overexpressing *M. sativa* plants demonstrated salinity tolerance and enhanced endogenous levels of NaCl-responsive gene *MsPRP2* (Winicov and Bastola, 1999). The constitutive overexpression of C2H2-type zinc finger TFs *SCOF*, *ZPT2-3*, and *ART1* induce stress responsive genes and confer abiotic stress tolerance in plants (Kim et al., 2001; Sugano et al., 2003; Yamaji et al., 2009). Furthermore, a nuclear factor Y (NF-Y) TF gene *NFYA5* is strongly induced by ABA and drought stress and *NFYA5* overexpressing *Arabidopsis* displayed reduced leaf water loss and drought stress tolerance compared to the WT plants (Li et al., 2008). Similarly, heat shock TFs (HsFs) are the essential regulators of the heat stress response and overexpression of the *Arabidopsis HsfA2* gene confers increased thermo-tolerance, and also salt and osmotic stress tolerance (Ogawa et al., 2007). In addition, the GRAS TFs are plant specific and play important role in plant development and stress responses. A poplar GRAS TF gene *PeSCL7* overexpressing *Arabidopsis* showed enhanced drought and salinity tolerance (Ma et al., 2010). Similarly, abiotic stress inducible tri-helix TF genes

GmGT-2A and *GmGT-2B* from soybean, when overexpressed in *Arabidopsis*, altered many stress related genes and enhanced salt, freezing and drought tolerance (Xie et al., 2009). Furthermore, CAMTA (calmodulin binding transcription activator) TF family protein CAMTA3 has been demonstrated to be a positive regulator of *CBF2* expression and possible integral link between calcium/calmodulin signaling and cold-regulated gene expression (Doherty et al., 2009). These examples suggest that the biological function of many TF proteins in abiotic stress tolerance is still to be explored, and many of these TF genes may be very useful in genetic engineering of plants for abiotic stress tolerance.

1.5 Pathogenesis related 10 proteins

1.5.1 PR proteins

Pathogenesis related (PR) proteins are a group of proteins expressed in plants in response to different biotic and abiotic stress stimuli and they play important roles in plant adaptations to stress conditions (van Loon, 1994; Jwa et al., 2001). They are ubiquitous throughout the plant kingdom and the occurance of PR proteins has been established in all the plant organs (Tahiri-Alaoui et al., 1990; Del Campillo and Lewis, 1992; Eyal et al., 1993; Van Loon, 1999). These protease resistant low-molecular (6-45kDa) proteins (Van Loon, 1999; Edreva, 2005) localize in the vacuole or apoplast (Bol et al., 1990; Buchel and Linthorst, 1999). Originally, PR proteins were defined as “proteins encoded by the host plant but induced only in

pathological or related situations" and related proteins occurring in the absence of pathological or related situations were referred to as "PR-like" proteins (PRLs) (Antoniw et al., 1980; Van Loon and Van Strien, 1999). The criteria to be included in the PR protein families was "pathogen induced expression of the protein in tissue that normally does not express the protein must have been shown in at least two different plant pathogen combinations or expression in a single plant pathogen combination must have been established in independent laboratories" (Van Loon and Van Strien, 1999). However, some PR proteins were constitutively expressed and certain pre-existing PRLs accumulated in higher levels after pathogen infection, therefore the "pathogenesis related proteins" became a popular term for all microbe-induced proteins (Van Loon et al., 2006). Based on the amino acid sequence and/or biological activity PR proteins have been classified into 17 different families (Van Loon et al., 2006). The PR families include chitinases, glucanases, endoproteinases, peroxidases, defensins, thionins, lipid transfer proteins, thaumatin-like proteins and ribonucleases, germin-like proteins (Table 1-1) which function in wide range from cell wall rigidification to signal transduction (Van Loon et al., 1994; Van Loon et al., 2006). PR proteins are involved in plant defense mostly as antifungal, antibacterial, antiviral, insecticidal and nematicidal proteins and these functions are attributed to their hydrolytic, membrane-permeabilizing, ribonuclease or proteinase-inhibitor activities (Edreva, 2005). PR proteins are associated with SAR (systemic acquired resistance) and their induction is mediated by different signaling molecules like salicylic acid, methyl jasmonate, ethylene, reactive

Table 1-1 Families of pathogenesis related proteins

Family	Type member	Properties
PR-1	Tobacco PR-1a	Unknown
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco 'R'	Chitinase type I, II
PR-5	Tobacco 'S'	Thaumatin-like
PR-6	Tomato Inhibitor I	Proteinase-inhibitor
PR-7	Tomato P_{69}	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase
PR-10	Parsley "PR1"	Ribonuclease-like
PR-11	Tobacco "class V" chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	*Arabidopsis* THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate-oxidase-like
PR-17	Tobacco PRp27	Unknown

(Adapted from Van Loon, 2006)

oxygen species, kinetins and auxins (Ryals et al., 1996; Tamas et al., 1997; Paakkonen et al., 1998; Buchel and Linthorst, 1999; Kitajima and Sato, 1999; Van Loon, 1999; Fujibe et al., 2000; Schaller et al., 2000; Schultheiss et al., 2004). In addition to their induced expression in response to external stimuli (bacteria, fungi, nematode, wounding, virus, UV-B radiation, water stress, temperature stress), PR protein expression can be stimulated by internal plant developmental cues during flowering, senescence, seed germination and embryogenesis, suggesting a possible role in preformed defense barriers (Tahiri-Alaoui et al., 1990; Vigers et al., 1991; Del Campillo and Lewis, 1992; Eyal et al., 1993; Buchel and Linhorst, 1999; Ekramoddulah et al., 2000; Edreva, 2005). The salicylic acid (SA) and jasmonic acid (JA) dependent pathways of PR induction have been established and *cis*-regulatory elements mediating PR expression are identified as GCC box, G box, MRE-like sequence and SA-responsive elements (Mitsuhara et al., 1998; Fidantsef et al., 1999; Zhou et al., 1999). Resistance of transgenic plants overexpressing PR genes to biotic and abiotic stresses, constitutive expression of PR genes in plants with naturally high level of disease resistance and stronger expression of PR genes in pathogen resistant compared to susceptible plants suggest that PR proteins are among major players in plant defense signaling (Bachmann et al., 1998; Datta et al., 1999; Wubben et al., 1996; Lawrence et al., 2000; Tonon et al., 2002; Gau et al., 2004; Edreva, 2005; Srivastava et al., 2006a; Srivastava et al., 2007).

1.5.2 PR-10 proteins

1.5.2.1 Occurrence and expression of PR-10 proteins

Among PR proteins, members of the PR-10 family are small (15 to 18 kDa), acidic and intracellular pathogenesis related proteins that have demonstrated ribonuclease activity and ligand-binding properties (Van Loon et al., 1994**).** PR-10 proteins were first described in pea resistance to fungi (Riggleman et al., 1985) and cultured parsley cells in response to elicitor treatment (Somssich et al., 1988). Now, there are number of studies reporting the occurrence of PR-10 proteins throughout the plant kingdom in monocots, dicots and gymnosperms (Liu and Ekramoddoullah, 2006). PR-10 proteins are induced in response to fungal pathogens (Jwa et al., 2001), parasitic higher plants (Borsics and Lados, 2002), and bacterial pathogens (Richard-Molard et al., 2004). They are also expressed in response to drought (Hashimoto et al., 2004) and salinity (Kav et al., 2004). Some of the PR-10 proteins are induced by both abiotic and biotic stressors (Hashimoto et al., 2004). Promoter analysis of different PR-10 proteins has revealed *cis*-regulatory elements like W-box, G-box, H-box, elicitor-responsive-element (EIRE)-like sequence, MeJA (methyl jasmonate) responsive motifs, ethylene-responsive enhancer element (ERE)-like motif which are involved in PR-10 expression (Warner et al., 1994; Shah and Klessig, 1996; Rouster et al., 1997; Liu et al., 2005). In addition to the inducible expression in response to stress, the constitutive expression of PR-10 genes has been detected during growth and development of vegetative organs, flowers, pollen grains and

fruits, suggesting a role of PR-10 proteins in defense, growth and development of plants (Apold et al., 1981; Breiteneder et al., 1989; Crowell et al., 1992; Mylona et al., 1994; Warner et al., 1994; Vanek-Krebitz et al., 1995; Biesiadka et al., 2002; Liu and Ekramoddoullah, 2003). PR-10 proteins display different responses to various stresses and therefore it has been difficult to characterize their role in the plant stress response (Iturriaga et al., 1994; Tewari et al., 2003).

1.5.2.1 Structure of PR-10 proteins

Despite the fact that different PR-10 proteins exhibit different responses to abiotic and biotic stresses, they have demonstrated a strikingly conserved amino acid sequence and three dimensional structures (Liu and Ekramoddoullah, 2006). PR-10 proteins are usually of low molecular mass ranging from 15-18 kDa, encoded by genes with open reading frames (ORF) of 456-489 base pairs and often multiple members of the PR-10 family occur in the same plant genome (Liu and Ekramoddoullah, 2006). Amino acid sequence alignment has demonstrated that the phosphate binding (P)-loop motif (GXGGXGXXK) is highly conserved among members of PR-10 proteins from different species (Gajhede et al., 1996; Hoffman-Sommergruber et al., 1997; Wu et al., 2003). X-ray diffraction and NMR spectroscopy studies have demonstrated very similar three dimensional structures for different PR-10 proteins (Gajhede et al., 1996; Neudecker et al., 2001; Biesiadka et al., 2002; Markovic-Housley et al., 2003). In general, they consist of seven stranded anti-parallel β-sheet and three α-helices connected by nine short loops and a hydrophobic cavity (Gajhede et al., 1996; Biesiadka et al.,

2002). A highly conserved P-loop motif and hydrophobic cavity have been suggested to have important roles in the biological activity of PR-10 proteins (Gajhede et al., 1996; Hoffman-Sommergruber et al., 1997; Wu et al., 2003).

1.5.2.2 Ribonuclease (RNase) activity of PR-10 protein

RNase and ligand binding are the two activities that have been attributed to PR-10 proteins based on a number of research studies, although the significance of these activities in the biological roles of PR-10 proteins in defense, plant growth and development is unclear (Moiseyev et al., 1994; Mogensen et al., 2002; Markovic-Housely et al., 2003; Park et al., 2004). RNase activity for PR-10 proteins in the early 90's was proposed due to high amino acid sequence similarity of two ginseng RNase peptides with parsley PR-10 proteins (Moiseyev et al., 1994) and similarities between spatio-temporal expression pattern of PR-10 genes and RNase genes (Walter et al., 1996). RNase activity has been demonstrated in many *in vitro* studies of PR-10 proteins in different species including *Betula* spp. (Bufe et al., 1996; Swoboda et al., 1996), white lupin (Bantignies et al., 2000), *Pachyrrhizus erous* (Wu et al., 2002), cotton (Zhou et al., 2002), *Capsicum annum* (Park et al., 2004), *Solananum* spp. (Liu et al., 2006), *Arachis hypogaea* (Chada and Das, 2006), and *Pisum sativum* (Srivastava et al., 2006a; Srivastava et al., 2007). The P-loop binding motif has been suggested to be involved in the RNase activity of PR-10 proteins and mutations in that region have demonstrated reduced RNase activity (Zhou et al., 2002; Wu et al., 2003). Although there are many studies on the *in vitro* RNase activity of PR-10 proteins,

only a few reports are available those describe the significance of the known RNase activity of PR-10 proteins in *planta*. For example, the importance of RNase activity in the antiviral and antifungal activities of PR-10 proteins has been demonstrated for CaPR-10 and AhPR10 (Park et al., 2004; Chada and Das, 2006). The role of the RNase activity of PR-10 proteins in protection of plants during programmed cell death around infection sites and self-incompatibility during fertilization has also been suggested (Huang et al., 1994; Swoboda et al., 1996; Liu and Ekramoddoullah, 2006). The phosphorylation status of CaPR10 has been demonstrated and the role of phosphorylation in regulation of RNase activity has been suggested (Park et al., 2004). However, not all PR-10 proteins possess RNase activity suggesting that RNase activity, could be mere incidental and of no biological significance (Biesiadka et al., 2002; Liu and Ekramoddoullah, 2004).

1.5.2.3 Ligand binding property of PR-10 protein

The hydrophobic cavity present in PR-10 proteins binds to many types of molecules including cytokinins (CKs), brassinosteroids, fatty acids and flavonoids (Fujimoto et al., 1998; Mogensen et al., 2002; Markovic-Housely et al., 2003). CK specific binding proteins (CSBPs) have also been included in the PR-10 class based on weak sequence homology and secondary-structure prediction (Fujimoto et al., 1998). PR-10 proteins are also described as a general plant hormone carrier during the plant defense response to pathogens, as well as during normal growth and developmental processes based on structural studies (Mogensen et al., 2002; Markovic-Housley et al., 2003; Pasternak et al., 2005). Expression of PR-10

genes is activated by hormones like ABA and CK and PR-10 proteins also have been found to interact with other proteins. Therefore, it has been suggested that PR-10 proteins might be involved in phytohormone signaling (Iturriaga et al., 1994; Carpin et al., 1998; Mogensen et al., 2002).

Research in our laboratory has accumulated information on the involvement of pea (*P. sativum*) PR-10 proteins in abiotic stress tolerance. Proteomic investigation in pea under salinity stress revealed significant up-regulation of PR-10 proteins, which led to the speculation that PR-10 proteins are important in salinity tolerance (Kav et al., 2004). Enhanced germination and early seedling growth under various stress conditions has been demonstrated in pea *PR-10* transgenic models, supporting this hypothesis (Srivastava et al., 2004: Srivastava et al., 2006b). Elevated levels of CKs were also observed in *PR-10* transgenic lines (Srivastava et al., 2006a; Srivastava et al., 2007) which led to the hypothesis that PR-10 proteins may mediate the plant responses to abiotic stress through CKs. Additional evidence supporting this hypothesis has been demonstrated in our laboratory that exogenous application of CK mediates effects that are similar to that mediated by PR-10 (Srivastava et al., 2007). Pea PR-10 proteins have also been over expressed in *E. coli* and the RNase activity of recombinant proteins has been demonstrated (Srivastava et al., 2006a & 2007). Therefore, from our previous experiments it is clear that pea PR-10 proteins are RNases and their constitutive expression elevates CK levels. However, the precise mechanisms through which PR-10 proteins increase CK concentration and

enhance stress tolerance are not clear. One possibility is that the CK moieties present in tRNA (transfer ribonucleic acid) molecules (Hall, 1970) are released by the RNase activity of PR-10 proteins in tRNA dependent CK biosynthesis. The different models of CK biosynthesis will be described in the following section.

1.6 Cytokinins (CKs)

CKs are group of phytohormones that play very important roles in various developmental and physiological processes in plants including cell division (Soni et al., 1995), *de novo* shoot formation (Skoog and Miller, 1957), delay of leaf senescence (Richmond and Lang, 1957; Gan and Amasino, 1995), chloroplast differentiation (Chen et al., 1993; Crowell and Amasino, 1994), seed germination (Gidrol et al., 1994), breaking apical dormancy (Phillips, 1975), root proliferation (Werner et al., 2001), phyllotaxis (Giulini et al., 2004), fruit development (Atkins and Pigeaire, 1993) and nutritional signaling and stress response (Takei et al., 2002). The natural cytokinins are adenine derivatives and include *trans*-zeatin (tZ), *cis*-zeatin (cZ), dihydrozeatin and N^6-(Δ^2-isopentenyl) adenine (Letham 1963; Shaw and Wilson 1964; Mc Gaw and Burch 1995; Prinsen et al., 1997; Emery et al., 2000). These CKs differ in stereo-isomeric position and saturation of the isoprenoid side chain and hydroxylation at the side chain terminus (Figure 1-3) but the physiological significance of these differences are not yet clear (Cedzich et al., 2008).

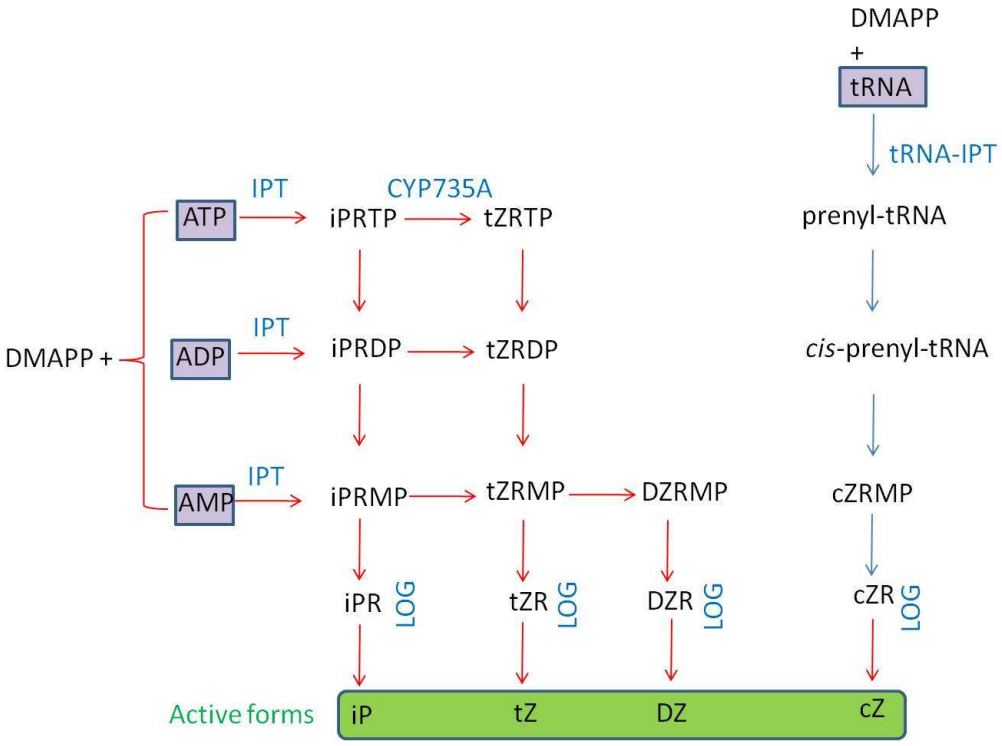

Figure 1-3 Cytokinin biosynthesis

(Adapted from Hirose et al., 2008)

DMAPP- dimethylallyl diphosphate; **iPRMP**- iP riboside 5'-monophosphate; **tZRTP**- tZ riboside 5'-triphosphate; **tZRDP**- tZ riboside 5'-diphosphate; **tZRMP**- tZ riboside 5'-monophosphate; **DZRMP**- DZ riboside 5'-monophosphate; **cZRMP**- cZ riboside 5'-monophosphate; **DZR**- DZ riboside; **cZR**- cZ riboside; **iP**- N^6-Δ^2-isopentenyl) adenine; **tZ**- trans zeatin; **DZ**- dihydro-zeatin; **cZ**- cis-zeatin.

1.6.1 Cytokinin biosynthesis

There are two possible CK biosynthetic pathways in plants: tRNA-independent and tRNA-dependent (Kasahara et al., 2004; Figure 1-3). The methyl erythritol phosphate (MEP) pathway in plastids supplies the isoprenoid precursor dimethylallyl diphosphate (DMAPP) for tRNA-independent CK biosynthesis, while the mevalonate (MVA) pathway in the cytosol supplies the isoprenoid precursor DMAPP for tRNA-dependent CK biosynthesis (Lichtenthaler 1999; Rohmer 2003). The CKs tZ, dyhydrozeatin and isopentenyladenine are synthesized through the tRNA-independent pathway by isopentylation of AMP, ADP or ATP by adenosine-phosphate-isopentenyl transferases (IPT). In *Arabidopsis*, seven IPT genes (*AtIPT1*, *AtIPT3-AtIPT8*) have been characterized, which isopentylate ATP and ADP to synthesize iP and tZ-type CKs (Kakimoto 2001; Takei et al., 2001; Sakakibara 2006). tZ derivatives are derived from hydroxylation of isopentenyl adenine (iP) nucleotides by trans-hydroxylases CYP735A1 and CYP735A2 (Takei et al., 2004). Furthermore, phosphoribohrdrolase LOG also activates CK nucleotides (Kurakawa et al., 2007). In tRNA-dependent biosynthesis IPTs isopentylate adenine of tRNA molecules, and isopenetenylated tRNAs may be further modified by hydroxylation reaction to yield cZ (Miyawaki et al., 2006). The degradation of isopentylated tRNAs has been suggested as a possible source of CKs (Miyawaki et al., 2006). In *Arabidopsis*, two IPTs (*AtIPT2* and *AtIPT9*) have been found which are involved in tRNA isopentylation and synthesis of cZ type CKs

(Golovko et al., 2002; Miyawaki et al., 2006). Furthermore, plants have CK oxidases which metabolize CKs, and so far seven CK oxidase genes have been found in *Arabidopsis* genome (Houba-Herin et al., 1999; Morris et al., 1999; Bilyeu et al., 2001; Werner et al., 2003).

1.6.3 tRNA degradation and CK accumulation during stress

CKs are very important phytohormones that are involved in plant developmental process, and roles in response to environmental stresses have also been suggested. For example, an elevated level of CKs along with proline and osmotin was observed in *ipt*-transgenic tobacco lines similar to salt stressed plants (Thomas et al., 1995). Plants stressed with salt and Al had higher CK ribosides (Massot et al., 1994; Bjork et al., 1987). A recent study has suggested that CKs play a crucial role in photo-protective acclimatization during drought stress (Shao et al., 2010). Furthermore, disturbed RNA stability and higher levels of isopentenylated nucleotides in RNA was observed in salt stressed pea plants (Peterson et al., 1988, Atanassova et al., 1996). Studies from our lab have demonstrated that abiotic stress tolerant transgenic *Brassica* and *Arabidopsis PR-10* plants have higher levels of CKs, and that pea PR-10s (PR-10.1 and PR-10.4) are RNases (Srivastava et al., 2004, 2006a, 2006b & 2007). It was speculated that PR-10 might impart the stress tolerant phenotype by modulating CKs, and supporting results were obtained when exogenous application of CKs on wild type *Arabidopsis* plants resulted in a stress tolerant phenotype similar to *PR-10* transgenic plants that had higher CKs (Srivastava et al., 2007). Based on these

studies, it was suggested that during abiotic stress, PR-10 might modulate the CK pool by tRNA degradation through its RNase activity (Srivastava et al., 2006a & 2007). However, there is no direct evidence that establishes the relationship between tRNA degradation by PR-10 proteins and CK enhancement.

1.7 Research objectives

The broad objectives of the research study and hypotheses are as follows:

1. Identification of important amino acids involved in the RNase activity of pea ABR17 protein. The hypothesis was "highly conserved amino acid residues histidine 69 and glutamic acid 148 are important for catalysis during the RNase activity of pea ABR17 (PR-10.4)".

2. Transcriptional profiling of pea ABR17 mediated changes in gene expression in *A. thaliana.* The hypothesis was "pea ABR17 enhances stress tolerance in *ABR17*-transgenic *Arabidopsis* by modulating the expression of stress responsive genes".

3. Functional characterization of genes identified in salt-treated *ABR17*-transgenic *Arabidopsis* microarray study. The hypothesis was "some of the putative TFs whose transcript abundance was high in salt treated-*ABR17*-transgenic *Arabidopsis* are important for the observed stress tolerance in *ABR17* plants".

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CHAPTER 2 Site-directed mutagenesis of histidine 69 and glutamic acid 148 alters the ribonuclease activity of pea ABR17 (PR-10.4)

2.1 Introduction

Pathogenesis related (PR) proteins are part of the multi-component defense signaling mechanism in plants and are induced when plants are exposed to biotic and abiotic stresses (Edreva, 2005). PR proteins have been classified into 17 families based on their amino acid sequence, serological relations, and/or enzymatic or biological activity (Van Loon et al., 2006). Among them, the PR-10 family consists of low molecular weight (16-19kDa) proteins that are acidic, cytosolic and protease resistant (van Loon et al., 1994). PR-10 proteins were first identified in *Pisum sativum* and parsley during the disease response to fungus (Riggleman et al., 1985; Somssich et al., 1986). Since then a number of studies have reported their constitutive expression as well as their accumulation in response to biotic and abiotic stress in a wide variety of plant species of both angiosperms and gymnosperms (Liu and Ekramoddoullah, 2006).

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As mentioned above, PR-10 proteins are ubiquitous, having been reported throughout the plant kingdom; however their biological functions are unclear. PR proteins have been implicated as general plant hormone carriers since PR-10 proteins and their homologues Bet v 1 and cytokinin-specific binding protein (CSBP) were found to interact with cytokinins, brassinosteroids and flavonoids (Biesiadka et al., 2002; Mogensen et al., 2002; Markovic-Housely et al., 2003; Pasternak et al., 2006; Fernandes et al., 2008). In addition, ribonuclease (RNase) activity has been demonstrated for various PR-10 proteins (Wu et al., 2003; Park et al., 2004; Srivastava et al., 2006a & 2007). Structural studies also support the RNase and ligand binding properties of PR-10 proteins; for example, a glycine rich loop (GxGGxGxxK) similar to the "P" loop motif observed in nucleotide binding proteins, together with the presence of a long forked cavity that may assist in ligand binding, have been observed in PR-10 proteins (Biesiadka et al., 2002; Mogensen et al., 2002; Markovic-Housely et al., 2003; Pasternak et al., 2006; Fernandes et al., 2008).

Abscisic acid responsive 17 (ABR17) is also referred to as PR-10.4 and is a member of the PR-10 family of proteins from *Pisum sativum* (Iturriaga et al., 1994). Other pea PR-10 proteins include PR-10.1 (Pi49), PR-10.2 (Pi176), PR-10.3 (DRRG49-C) and PR-10.5 (ABR18) (Fristensky et al., 1988; Chiang and Hadwiger, 1990; Iturriga et al., 1994; Culley et al., 1995). RNase activity associated with both ABR17 and PR-10.1 has been previously demonstrated (Srivastava et al 2006a & 2007). The present study was carried out in order to

investigate the catalytically important amino acids involved in the RNase activity of pea ABR17. Two variants of ABR17 protein, His69Leu and Glu148Ala have been generated using site-directed mutagenesis which shows an altered RNase activity. In addition, a structural model was deduced and the possible effects of these mutations on substrate binding and catalysis by ABR17 were analyzed and our findings discussed.

2.2 Materials and methods

Site-directed mutagenesis

ABR17 cDNA was retrieved from the pKYLX-71-ABR17 construct (Srivastava et al., 2007) and inserted into pKF19k (TaKaRa Bio Inc., Otsu, Japan) using restriction enzymes *Hind*III and *Xba*I (New England Biolabs, Toronto, Canada). The variants histidine 69 to leucine (H69L) and glutamic acid 148 to alanine (E148A) were made in the pKF19k-ABR17 construct using the polymerase chain reaction (PCR) with mutagenic oligonucleotide primers as per the manufacturer's instructions (Mutan^{TM}-Super Express Km Kit, TaKaRa Bio Inc., Otsu, Japan). Mutagenic primers used for creating the H69L and E148A variants were 5'-TATGTGCTACTCAAACTAGAC-3' and 5'-GAAAGTTTAGCGAAAGTTGCA-3', respectively. The thermo cycling parameters were as follows; 94^{o}C, 3min; 30 cycles for 1 min, 94^{o}C; 1 min, 55^{o}C; 2 min, 72^{o}C; and a final extension step for 10 min at 72^{o}C. The PCR products were transformed into *Escherichia coli* (MV1184 sup^{0} strain, TaKaRa Bio Inc.,

Otsu, Japan) and the variants were selected on Luria-Bertoni (LB) medium with kanamycin (50µg/mL). The clones were sequenced to confirm the desired substitutions, and to verify that no undesired substitutions were introduced, using vector specific primers 5'-TGTGGAATTGTGAGCGG-3' and 5'-GTTTTCCCAGTCACGAC-3'.

Expression and purification of recombinant proteins

H69L and E148A ABR17 cDNAs were amplified from recombinant pKF19k containing variant ABR17 using the gene specific primers 5'GTGGTCGCATATGGAAAATTTGTACTTTCAAGGTATGGGTGTCTTTG TTTTTGATGATGAATAC-3' and 5'-TATATAGCTCGAGTTAGTAACCAGGATTTGCCAAAACGTAACC-3'. The thermo cycling parameters were similar to those given above. Amplified substituted *ABR17* cDNAs were cloned into a pET28a bacterial expression vector (Novagen, California, USA) using *Nde*I and *Xho*I (New England Biolabs, Toronto, Canada) for the expression of an N-terminal poly-histidine-tagged fusion protein. The clones were sequenced using T7 universal promoter sequence primer and transformed into *E. coli* Rosetta (DE3) expression cells (Novagen, California, USA). Wild type (WT) and variant ABR17 (H69L and E148A) proteins were overexpressed and purified using the methods previously described (Srivastava et al., 2006a). Briefly, WT, H69L and E148A cDNAs were induced by 1 mM IPTG, at RT for 3h. Bacterial cells were harvested (8300g for 10min at 4^0C) and the pellets were lysed in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, 15 mM

imidazole, 1% Triton X100, complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany; pH 7.5) using a SONIC 300 Dismembrator (Artek Systems Corporation, New York, USA). Samples were centrifuged (13800g for 10min at 4^0C) and the supernatant was applied on to Ni-NTA agarose (Qiagen Inc., Ontario, Canada) column. The column was washed first with wash buffer 1 (50 mM NaH_2 PO_4, 300 mM NaCl, 5 mM β-mercaptoethanol, 1% Triton X100, 10% glycerol, 15 mM imidazole, complete EDTA-free protease inhibitor cocktail, pH 7.5) and then with wash buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 30 mM imidazole, complete EDTA-free protease inhibitor cocktail, pH 7.5) to remove weakly bound proteins. ABR17 and its variants were eluted in eluting buffer (50 mM NaH_2PO_4, 300 mM NaCl, 150 mM imidazole, complete EDTA-free protease inhibitor cocktail, pH 7.5). The eluted fraction was dialyzed in dialysis buffer (50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, and pH 8.0) at 4^0C. The dialyzed samples were passed through a DEAE sepharose column saturated with dialysis buffer and the weakly bound proteins were removed using wash buffer (50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, 50 mM NaCl, and pH 8.0). ABR17 and its variant proteins were eluted using elution buffer (50 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, 100 mM NaCl, and pH 8.0) to get nearly homogenous recombinant proteins and the eluted fractions were dialysed in dialysis buffer (see above).

RNase activity assays

In-solution RNase activity assays were performed with purified, recombinant proteins (ABR17 WT, ABR17-H69L and ABR17-E148A) as described by Srivastava et al. (2006a) with modifications. Reaction mixtures containing 3.25µg of total RNA from *Arabidopsis* tissue and 6, 9, 12 and 15 µg of recombinant protein (ABR17 WT, ABR17-H69L and ABR17-E148A) were incubated at 37^0C for 3h. The reaction mixture was extracted with 1:1 phenol-choloroform, and the aqueous layer was analysed on a 1.2% agarose gel. RNA was isolated from two week old *Arabidopsis* seedlings as described earlier (Srivastava et al., 2004).

Homology modeling

Modeling of pea ABR17 (Swiss-Prot: Q06931.1, Iturriaga et al 1994) was performed by using the program Modeller-9v4 (Sali et al., 1993). A NCBI BLAST (http://www.ncbi.nlm.nih.gov/) search of pea ABR17 was carried out over the protein data bank in order to find homologous protein structures. The structure of *Lupinus* Llpr10.1b (PDB ID: 1IFV, Swiss-Prot: P52779.1; Biesiadka et al., 2002) was found to possess a maximum sequence similarity of ~66% with pea ABR17, therefore this structure was used as the template for building the ABR17 model. Sequence alignment of the pea ABR17 and *Lupinus* Llpr10.1b proteins was carried out by using the dynamic programming method implemented in MODELLER. For the target sequence of ABR17, 10 final models were created and the model with lowest objective function was selected for further analysis.

Subsequently, the stereochemical quality of the model was evaluated by using the program PROCHECK (Laskowski et al., 1993) and the three dimensional structure was viewed by using the PyMol molecular viewer (www.pymol.org).

2.3 Results and Discussion

ABR17 (PR-10.4) is a PR-10 family member from *P. sativum* (Swiss-Prot: Q06931.1; Iturriaga et al., 1994) and RNase activity for this protein has been demonstrated previously (Srivastava et al., 2007). The important amino acids involved in the RNase activity of ABR17 have been investigated in this study using site-directed mutagenesis. PR-10 protein sequences from different species along with pea ABR17 were aligned (Figure 2-1), and amino acids H69 and E148 of ABR17 were chosen for site-directed mutagenesis. Residue H69 was selected for the mutagenesis study as this residue is conserved in all pea PR-10 proteins (Figure 2-1) and, in addition, histidine residues are often involved in RNase catalytic reactions by acting both as proton donors as well as proton acceptors (Mosimann et al., 1994). E148 was selected for mutagenesis because it is conserved among pea PR-10 proteins as well as among PR-10 proteins from other species (Figure 2-1). In addition, Glu147 of SPE16 (a PR-10 protein from *Pachyrrhizus erosus*) has been demonstrated to be important for RNase activity (Wu et al., 2003). In this study, H69 and E148 were substituted by L69 (leucine 69) and A148 (alanine 148) respectively, using site-directed mutagenesis. Residues leucine and alanine were chosen for substitution as both of the selected

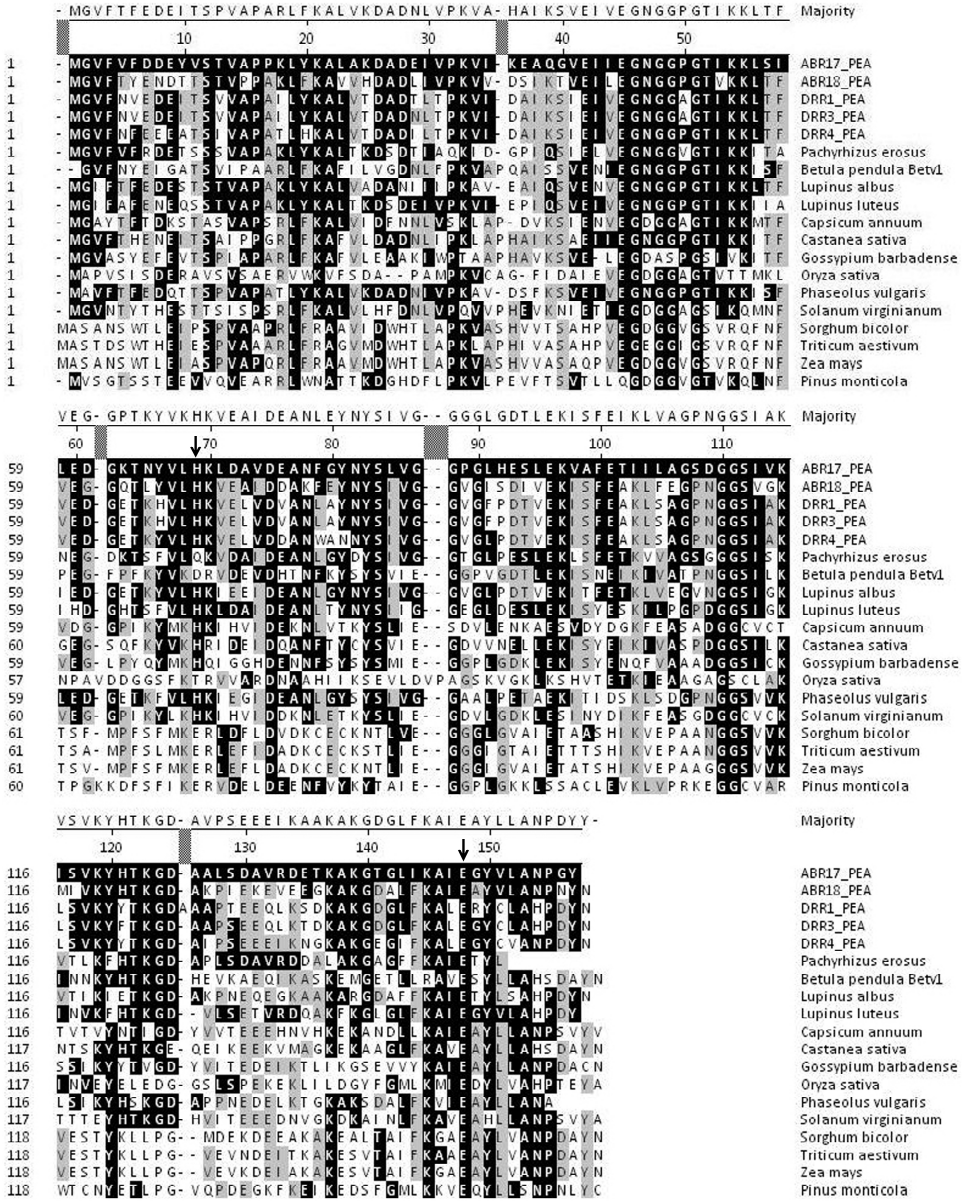

Figure 2-1 Sequence alignment of PR-10 proteins from different species

Residues that match exactly pea ABR17 are shaded in solid black and residues that match with the consensus are shaded in solid light gray. Arrows indicate the amino acids selected for site-directed mutagenesis. Sequence alignment was performed using ClustalW in MegAlign (DNASTAR Lasergene8) software.

residues possess neutral properties unlike the chemical properties exhibited by histidine and glutamic acid.

ABR17 and its H69L and E148A variants were expressed in *E. coli* as N-terminal histidine-tagged fusion proteins after induction of expression with 1 mM IPTG at room temperature. The native ABR17 and the H69L variant proteins were found equally in the soluble as well as in the insoluble fraction, whereas most of the E148A variant protein was present in the insoluble fraction making it extremely difficult to purify (Figure 2-2). All three proteins were purified to near homogenity from the soluble fractions using Ni-NTA and DEAE ion exchange column chromatography (Figure 2-2). RNase activity assays were performed with the uncleaved His-tagged proteins because the histidine tag did not interfere with the RNase activity of the native ABR17 protein as observed in our previous studies (Srivastava et al., 2007). As can be seen from the results presented in Figure 2-3, RNA alone in nuclease free water or in dialysis buffer was intact, while RNA with 12 and 15 µg of WT-ABR17 was degraded. RNA with H69L-ABR17 was not degraded even when the protein amount was 15µg. RNA in the presence of E148-ABR17 was degraded when the protein concentration was as little as 6µg (Figure 2-3). RNA with 12µg denatured WT-ABR17, H69L-ABR17 and E148A-ABR17 proteins was not degraded, suggesting that the RNA

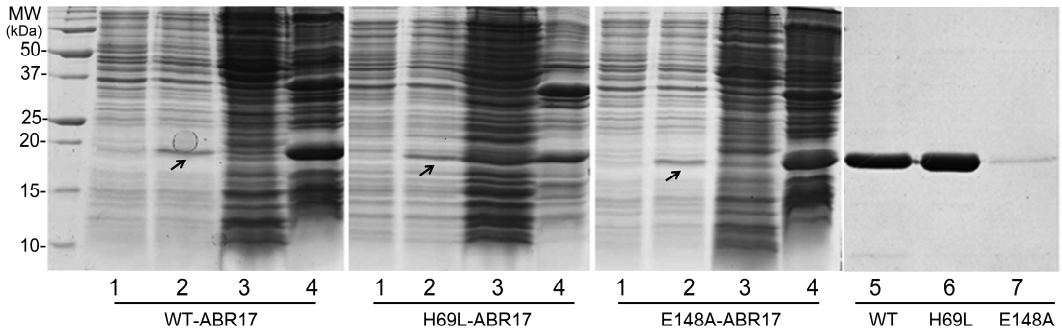

Figure 2-2 Overexpression and purification of pea ABR17 and its variants H69L and E148A in *E. coli*

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) image showing different fractions during overexpression and purification. Lanes 1 and 2 are cell-free extracts from uninduced and induced *E. coli* cultures, respectively. Lanes 3 and 4 are the soluble and the insoluble fractions of induced *E. coli* cultures. Lanes 5, 6 and 7 are purified WT-ABR17, H69L-ABR17 and E148A-ABR17 proteins. Proteins were purified from the soluble fraction.

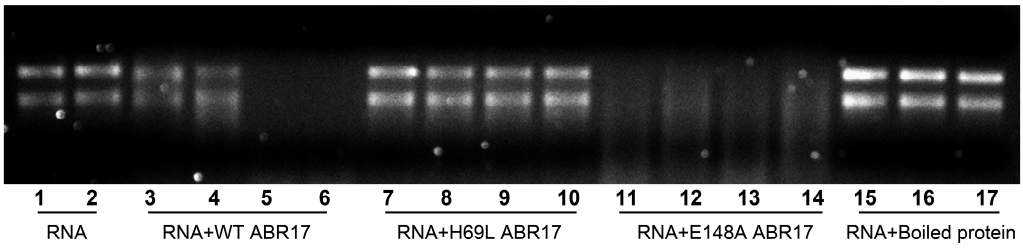

Figure 2-3 Ribonuclease activity assay with recombinant WT-ABR17, H69L-ABR17 and E148A-ABR17 proteins

(1.2% agarose gel electrophoresis image) Lane 1 is 3.25µg total RNA from *Arabidopsis* in nuclease free water. Lane 2 is 3.25µg of total RNA from *Arabidopsis* in dialyzing buffer. Lanes 3 to 6 are reactions with 3.25µg of total RNA from *Arabidopsis* and 6, 9, 12 and 15µg of recombinant WT-ABR17 protein. Lanes 7 to 10 are reactions with 3.25µg of total RNA from *Arabidopsis* and 6, 9, 12 and 15µg of recombinant H69L-ABR17 protein. Lanes 11 to 14 are reactions with 3.25µg of total RNA from *Arabidopsis* and 6, 9, 12 and 15µg of recombinant E148A-ABR17 protein. Lanes 15, 16 and 17 are reactions with 3.25µg of total RNA from *Arabidopsis* and 12µg of boiled/denatured recombinant WT-ABR17, H69L-ABR17 and E148A-ABR17 protein respectively.

degradation was not due to any contaminants present in the reaction mixture. The results from the RNase activity assays indicated altered RNase activity in the ABR17 variant proteins: reduced RNase activity in H69L-ABR17 but enhanced RNase activity in E148A (Figure 2-3). This implies both residues H69 and E148 are indeed important residues for the RNase activity of pea ABR17 protein.

We carried out homology modeling of ABR17 in order to investigate further the structural implications of the site directed mutagenesis of H69L and E148A and to gain additional insights into the catalytic mechanism. The three dimensional structural model of ABR17 was created based on the experimentally determined structure of PR protein llpr10.1b from *Lupinus luteus* (Swiss-Prot: P52779.1; Biesiadka et al., 2002). ABR17 and llpr10.1b proteins posses high sequence similarity (66%) and, therefore, the modeled structure of ABR17 should be reliable for accurate structure function predictions. Similar to llpr10.1b (Biesiadka et al., 2002), the modeled three dimensional structure of ABR17 has a long C-terminal α-helix wrapped by a seven-stranded anti-parallel β sheet and two N-terminal short α-helices, with nine connecting loops and a hydrophobic cavity that was described earlier as a putative ligand binding site (Gajhede et al., 1996; Biesiadka et al., 2002) (Figures 2-4 & 2-5). The highly conserved glycine rich loop is located in loop 4 (L4) and the putative ligand binding site is located between the glycine rich loop and the α (α1& α2) helices towards interface of the β sheet (Figure 2-5). Structural studies have demonstrated that an internal cavity and a glycine rich loop are highly conserved in sequence as well as in

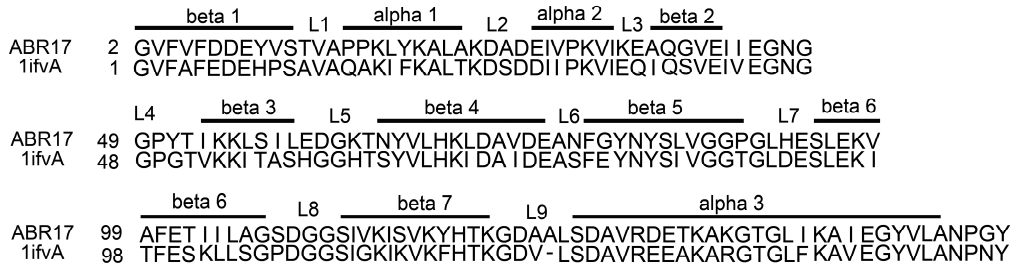

Figure 2-4 Secondary structure of pea ABR17 protein and chain A of *Lupinus luteus* PR-10 protein (lIFVA)

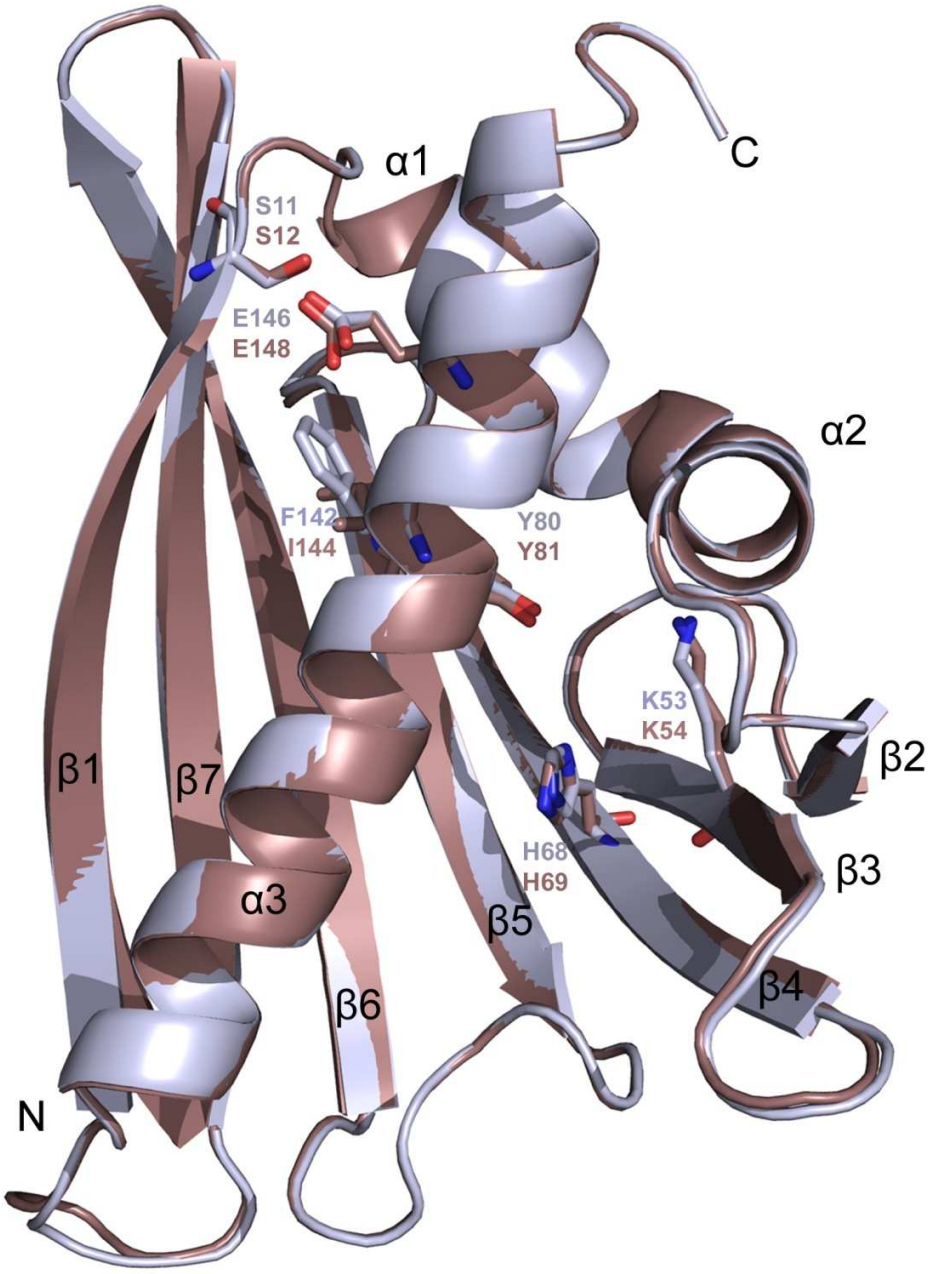

Figure 2-5 Comparative modeling showing the 3-D structure of pea ABR17 and chain A of *Lupinus luteus* PR-10 protein (lIFVA) superimposed

Blue colour indicates lIFVA whereas magenta indicates the pea ABR17 protein.

conformation amongst the different PR-10 proteins and therefore these regions have been suggested to be important for the biological activity of PR-10 proteins (Gajhede et al., 1996; Hoffman-Sommergruber et al., 1997; Wu et al., 2003). Our own structural predictions confirm the conservation of these structural features in pea ABR17. Residue His68 in *Lupinus* PR-10 is located on strand β4 and makes polar contacts with residue Tyr80 and also participates in the hydrogen bonding network with some neighboring polar residues like Lys53 and Asp27 (Figure 2-5). Molecular docking experiments of zeatin on *Lupinus* PR-10 proteins have indicated that residue His68 stabilizes the zeatin molecule through hydrogen bonding interactions (Biesiadka et al., 2002). Along with His68, other two residues Asp27 and Tyr80 were also found interacting with the docked zeatin molecule (Biesiadka et al., 2002). Since zeatin is a nucleoside analog, the putative ligand binding site is compatible with RNA binding (Biesiadka et al., 2002) and, therefore, H69 in pea ABR17 protein might also be involved in substrate stabilization during RNase activity. In another co-crystalization study of *Lupinus* PR-10 with synthetic N, N”-diphenylurea, the His68 was found to adopt two different conformational states (PDB ID 3E85) (Fernandes et al., 2009). In one conformational state the imidazole ring of His68 points towards the substrate binding cavity facilitating substrate binding, whereas in its other conformational state the imidazole ring adopts an outward projection and participates in the hydrogen bonding network with residues Tyr80 and Lys53 (Fernandes et al., 2009) (Figure 2-6). In addition, along with His68 an adjacent residue Tyr80 is observed having multiple conformational states, which can be seen on

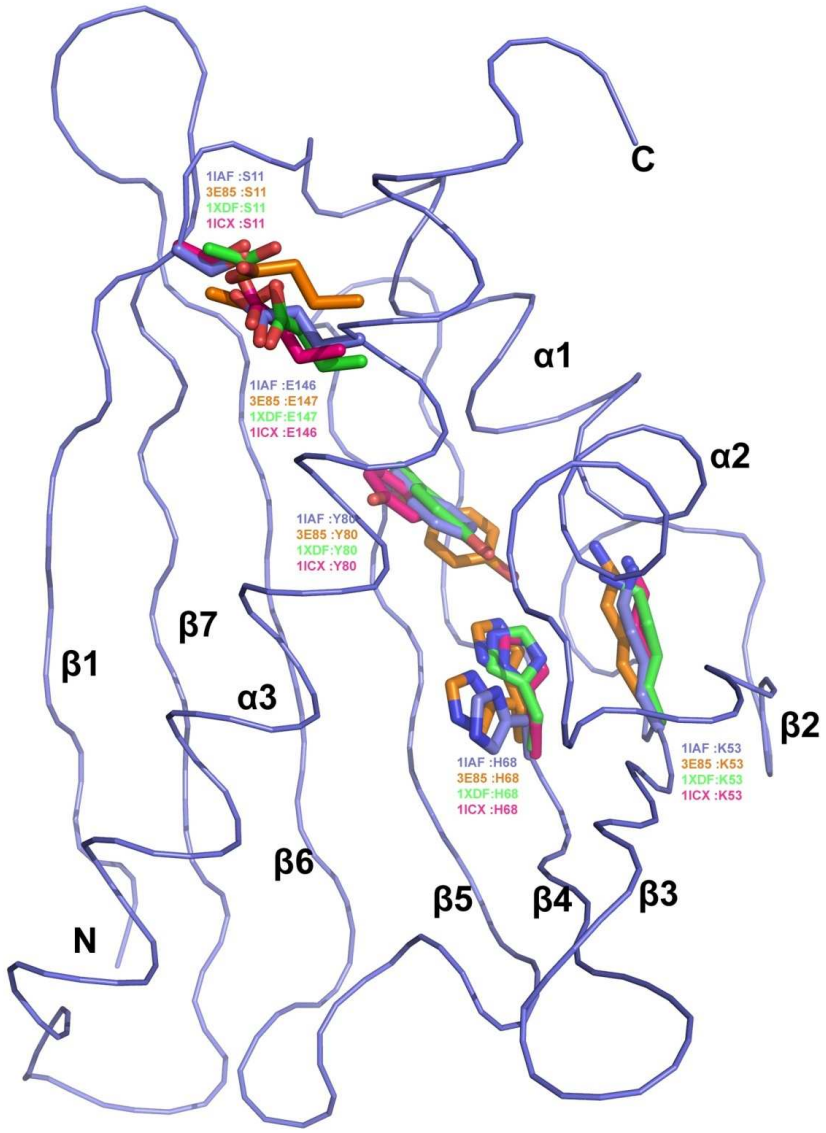

Figure 2-6 Structural superpositions of all PR-10 proteins (PDB ID 1IFV, 3E85, 1XDF, 1ICX)

The c-alpha backbone of PDB ID 1IFV is shown as representative structure whereas those important side chains investigated in this study are shown in different colors.

superimposed structures of substrate free as well as substrate bound PR-10 proteins (Figure 2-6). In order to accommodate substrates in the active site of crystal structure PR-10 (PDB ID 3E85), a noticeable change of volume is observed. This volume change of the substrate binding pocket from 2200Å^3 to 4500 Å^3 is possible because of the helical kink present at Phe142 of the α3 (Fernandes et al., 2009). In addition to the major conformational changes because of α3 helical structure, some side chain conformational changes were also observed for His68 and Tyr80 at the entrance to the substrate binding pocket, possibly facilitating substrate binding as well as the reaction kinetics. In ABR17, the homologous H69L variant, the leucine residue substitution will result in the polar interactions with the substrate being lost and hence this variant will likely have a decreased RNase activity, as was indeed observed in our activity assays. Glu146 in *Lupinus* PR-10 is part of the α3 helical structure and it forms polar interactions with Ser11 of sheet β1 as well as with the backbone amide group of β1 main chain (Figure 2-5). In the E148A variant protein of pea ABR17, when E148 is replaced with alanine, these polar interactions are lost and this could facilitate the widening of the C-term helix (α3) and thus enhance substrate accessibility as well as RNase activity. However, replacement of homologous Glu147 with alanine in *Pachyrrhizus* PR-10 results in reduced RNase activity (Wu et al., 2003) and this could be due to different structural conformation. However, confirmation of this suggestion must await structural characterization of the *Pachyrrhizus* PR-10 protein.

RNase activity has been demonstrated in many *in vitro* studies of PR-10 proteins from different species (Zhou et al., 2002; Wu et al., 2003). Some studies also have demonstrated the relevance of the RNase activity on biological function of PR-10 proteins like antiviral or antifungal activity of PR-10 proteins (Park et al., 2004; Chadha and Das, 2006). Our laboratory has demonstrated that purified recombinant pea ABR17 and PR-10.1 possess RNase activity and *PR-10* transgenic plants exhibit enhanced germination, early seedling growth and stress tolerance and have elevated cytokinin levels (Srivastava et al., 2004, 2006a, 2006b & 2007). However, the relevance of the RNase activity of PR-10 proteins in mediating the observed effects (i.e. enhanced germination, early seedling growth, multiple stress tolerance and higher cytokinin levels) have not been investigated. Both H69L and E148A variants with altered RNase activity will be useful in studying the importance of RNase activity in mediating the observed effects on *PR-10* transgenic plants. Currently, effects of the altered RNase activity on stress tolerance and cytokinin biosynthesis are being studied in our laboratory by transforming H69L and E148- ABR17 mutant cDNA into *Arabidopsis* and characterization of the phenotypes as well as the cytokinin profiles of these transgenic plants.

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CHAPTER 3 Transcriptional profiling of pea *ABR17* mediated changes in gene expression in *Arabidopsis thaliana*

3.1 Introduction

Pathogenesis-related (PR) proteins are part of the plant defense responses that are induced by pathogens as well as by abiotic stresses (Van Loon et al., 1994; Kav et al., 2004). To date, 17 different families of PR proteins have been identified, based on their specific structural and functional properties (Liu and Ekramoddoullah, 2006). Among the PR proteins, the PR-10 family is composed of intracellular acidic proteins with molecular masses ranging from 15-18 kD and are encoded by multiple genes (Van Loon et al., 1994; Liu and Ekramoddoullah, 2006). PR-10 genes were first described in *Pisum sativum* inoculated with *Fusarium solani* (Riggleman et al., 1985), but have been subsequently described in many species (Liu and Ekramoddoullah, 2006). In addition to their inducible expression in response to stresses, PR-10 genes also exhibit constitutive high expressed levels in roots, flowers and pollen during normal growth and development, suggesting additional roles beyond pathogenesis responses (Biesiadka et al., 2002).

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Based on sequence similarities, PR-10 proteins have been suggested to be ribonucleases (RNases) (Moiseyev et al., 1994). Indeed, PR-10 proteins from a variety of species including two pea PR-10 proteins have been demonstrated to possess RNase activity (Srivastava et al., 2006a & 2007). Although RNase activities have been detected for many PR-10 proteins, they have also been shown to interact with molecules such as cytokinins (CKs), brassinosteroids, fatty acids, and flavonoids (Fujimoto et al., 1998; Mogensen et al., 2002; Markovic-Housely et al., 2003). These observations have led to the suggestion that all PR-10 proteins may not be RNases and that PR-10 proteins may be involved in normal plant growth and development as hormone/ligand carriers (Mogensen et al., 2002; Markovic-Housely et al., 2003; Pasternak et al., 2005). This suggestion is further supported by the fact that CK-specific binding proteins (CSBPs) exhibit amino acid sequence and predicted secondary-structure similarities with PR-10 proteins and, for this reason, have been included in the PR-10 family (Fujimoto et al., 1998).

The pea abscisic acid-responsive protein ABR17, induced by the exogenous application of abscisic acid (ABA), is classified as a member of the PR-10 family in pea (Iturriaga et al., 1994). ABR17 is produced late in seed development, and is homologous to dehydrins and late embryogenesis abundant (LEA)-related proteins (Skriver and Mundy, 1990; Goday et al., 1994). ABR17 is also significantly homologous to intracellular pathogenesis related (IPR) proteins and major birch pollen allergen Betv1 proteins (Fristensky et al., 1988;

Breiteneder et al., 1989). Our previous research has demonstrated the expression of ABR17 protein in pea under salt stress (Kav et al., 2004) and the RNase activity of two members of pea PR-10 proteins (PR-10.1 and ABR17) (Srivastava et al., 2006a & 2007). Furthermore, we have also demonstrated that the constitutive expression of pea *PR-10.1* and *ABR17* cDNAs enhance germination and early seedling growth under abiotic stress conditions in *Brassica napus* and *Arabidopsis thaliana*, respectively (Srivastava et al., 2004 & 2006b). In addition, the transgenic plants also exhibited phenotypic differences when compared to their wild type (WT) counterparts, which included precocious flowering, a higher number of lateral branches, and increased numbers of seed pods (Srivastava et al., 2007). Many of these characteristics of *ABR17*-transgenic *A. thaliana* are suggestive of a role for CKs in ABR17 action, particularly increased lateral branching and early flowering (Bonhomme et al., 2000; Tanaka et al., 2006). Our suggestion was further supported by the elevated concentrations of endogenous CKs in *PR-10.1* transgenic *B. napus* as well as *ABR17*-transgenic *A. thaliana* (Srivastava et al., 2006a & 2007).

These observations led us to hypothesize that PR-10 proteins, including ABR17, may mediate the observed phenotypic effects through modulation of endogenous CKs. Additional evidence supporting this hypothesis has been provided by the demonstration that exogenous application of CKs enhances germination under abiotic stress conditions (Srivastava et al., 2007). In order to further investigate the *ABR17*-mediated changes in *A. thaliana*, we investigated

global changes in gene expression using microarrays. Microarray analysis was carried out in an *ABR17*-transgenic line compared to its WT, salt treated *ABR17*-transgenic line compared to untreated *ABR17*-transgenic line, and salt treated WT compared to untreated WT seedlings. Our current findings reveal that, even in the absence of stress, the expression of genes involved in plant growth and development are significantly (and approximately 2-fold) increased in the transgenic line. Salt treated *ABR17*-transgenic *A. thaliana* seedlings showed general salt response comparable to that of the WT counterpart used in this study. However, both the trend as well as the degree of changes in gene expression of many defense related genes, including plant defensins and heat shock proteins, was different providing additional insights into the possible ways in which ABR17 may mediate plant responses to stress.

3.2 Materials and Methods

Plant material and growth conditions

Transformation of *A. thaliana* with the pea *ABR17* cDNA and the generation of homozygous ABR17-transgenic *A. thaliana* (line 6.9) have been previously described (Srivastava et al., 2006b). This line (6.9) was one of the three independently derived transgenic lines that were characterized in that earlier study. The WT (ecotype WS) and transgenic *A. thaliana* plants were grown in the greenhouse for observations as previously described (Srivastava et al., 2007). Lateral branches were counted on plants from three independent biological

replicates with at least 72 plants per replicate. The average number of days required for the opening of the first flower was also recorded on plants from three biological replicates with 36 plants in each replicate.

In order to measure root lengths of seedlings, seeds of *A. thaliana* (line 6.9) and the WT were surface-sterilized (Srivastava et al., 2007) and placed on half strength Murashige & Skoog (MS) medium (Murashige and Skoog, 1962) with or without salt (75 mM or 100 mM NaCl) in square dishes with grids. These dishes were placed vertically in a growth chamber (at 21°C and with light intensity of 250 µmol m^{-2} s^{-1}) and root lengths were measured after 10 days. The seeds of *ABR17* and WT seeds were also grown on half strength MS medium with 0 or 100 mM NaCl to determine their fresh weight and chlorophyll and carotenoid contents, in order to assess their ability to grow in the presence of salt. The average length of the primary roots of 10-day-old seedlings from three independent biological replicates with at least sixty seedlings per replicate was calculated using the Image J software (Image J, NIH, MD, USA).

Chlorophyll and carotenoids were extracted from the pooled 2-week-old tissue grown on MS media, using the procedure described by Srivastava et al. (2006a). Total chlorophyll was estimated using a nomogram (Kirk, 1968) and total carotenoid was measured using the formula:

$$\Delta ACAR_{480} = \Delta A_{480} + 0.114\Delta A_{663} - 0.638\Delta A_{645}$$

where *A* is the absorbance and CAR is the carotenoid content (Kirk and Allen, 1965). The fresh weight, chlorophyll and carotenoid contents were calculated using pooled tissue from three independent biological replicates. Percent germination after one week for *ABR17*-transgenic and WT seeds in the dark and in the presence of light (fluorescent light, 30 µmol m^{-2} s^{-1}) was compared in Petri dishes at RT. This experiment included three independent biological replicates with at least 45 seeds per replicate. All statistical analyses were performed using the Student's *t*-test procedure in SAS version 8e (Statistical Analysis System, 1985).

Tissue for microarray analysis was obtained by placing surface-sterilized seeds of *A. thaliana* (line 6.9) and the WT on half strength MS medium in Petri dishes with or without 100 mM NaCl at RT (21 ± 2°C) under continuous fluorescent light (30 µmol m^{-2} s^{-1}) for 14 days. Seedlings (14-day-old) from three independently grown biological replicates in all three set of experiments (comparison of *ABR17*-transgenic with WT without any stress; comparison of salt treated WT with untreated WT; comparison of salt treated *ABR17*-transgenic with untreated transgenic) were removed from the MS plates, flash frozen in liquid nitrogen and stored at –80°C until used for RNA extraction.

RNA extraction, cDNA synthesis and microarray analysis

In order to investigate the ABR17-induced gene expression changes under normal and salinity stress conditions, we conducted microarray analysis in three separate hybridization experiments. The first set (set I) consisted of comparison of

cDNA samples prepared from *ABR17*-transgenic and WT seedlings, which were grown in the absence of any stress. Set II consisted of cDNA obtained from salt-treated samples of WT and untreated WT; and set III, cDNA samples of salt treated *ABR17*- and untreated *ABR17*-transgenic seedlings for hybridization to the oligonucleotide arrays. Each microarray experiment consisted of six hybridizations according to the principles of dye-swap design (Martin-Magniette et al., 2005) on tissues across three biological replicates of the experiments.

RNA was isolated using the QIAGEN RNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada) from 2-week-old WT and *ABR17* seedling tissue from all three sets of experiments and the integrity of all RNA samples assessed by agarose gel (1.2%) electrophoresis. For microarray hybridization, 6 µg of total RNA was used to synthesize cDNAs using SuperScript® II RT (Invitrogen Inc., Burlington, ON, Canada) with RT polyA-capture primers in 3D Array 900TM (Genisphere Inc., Hatfield, PA, USA). In these microarray experiments, 70-mer oligonucleotide arrays were used which contained 26,090 probes (Array-Ready Oligo Set for *Arabidopsis* genome Version 1.0, Qiagen Operon, Alameda, CA, USA), plus additional probes for quality control. Oligonucleotide arrays were spotted on superamine aminosilane-coated slides (TeleChem International Inc., Sunnyvale, CA, USA). Each pair of samples within each of the three biological replicates was labeled in a reciprocal dye-swap design, for a total of 18 hybridizations (overnight/ at 52°C) in all three sets of experiments. Slides were scanned using ArrayWoRx ^{e} (Applied Precision, Issaquah, WA, USA) and spot

intensities were measured, quantified, normalized and analyzed using TM4 (Saeed et al., 2003). Spots with intensity ratios that differed significantly from 0 (log2 scale) were identified by Student's *t*-test. This procedure highlights the spots that demonstrated statistically significant differential expression between the different samples. The raw microarray data of 18 hybridizations as well as the protocols used to produce the data were deposited in the ArrayExpress database (ArrayExpress: E-MEXP1024 and E-MEXP1566).

Quantitative real-time PCR (qRT-PCR) validation of microarray data

Primers for qRT-PCR were designed using the Primer Express software (Applied Biosystems Inc., Foster City, CA, USA) to ensure that PCR products of approximately 70-80 bp were generated (Table AI-1). cDNA synthesis and qRT-PCR analysis of gene expression of 19 genes were performed using the Taqman system as described previously (Srivastava et al., 2007) on an ABI Prism 7700 Sequence detector (Applied Biosystems Inc., Foster City, CA, USA) and the SNP RT template program, while the SYBR green system as described by Yang et al., (2007) was used to validate the expression of 8 genes. In both cases, the delta-delta method (Livak and Schmittgen, 2001) was used to calculate relative gene expression using actin as the endogenous control. The relative transcript abundance in the controls was normalized to 1 and was used as a basis for comparison to the treatments. Plant tissue from three biological replicates was used in qRT-PCR experiments and reactions for each biological replicate were performed in duplicate (n=6).

3.3 Results and Discussion

Characterization of ABR17-transgenic plants/seedlings

The appearance of 2-week-old WT and *ABR17*- transgenic *A. thaliana* seedlings grown in soil as well as on MS medium (1.5% sucrose, 0.8% agar with pH 5.7) (Murashige and Skoog, 1962) plates are shown in Figure 3-1. At all growth stages investigated, the *ABR17*-transgenic line was considerably more developmentally advanced compared to its WT counterpart. For example, in the 5-day-old transgenic seedlings (Figure 3-1A), cotyledons were more developed than in their WT counterparts and at 14 days the transgenic seedlings possessed more rosette leaves (Figure 3-1A). Similar developmental differences were also observed at 21 days where many transgenic seedlings had started to bolt whereas very few (if any) WT seedlings had advanced to this developmental stage (Figure 3-1B). At 28 days, the transgenic seedlings also possessed more lateral branches (Figure 3-1C, Table 3-1). The transgenic seedlings also flowered earlier than WT with an average difference of at least 2.5 days (Table 3-1). Seedlings for microarray experiments were grown on semi-solid MS media in order to maintain sterility and it was evident that under these growth conditions also the transgenic seedlings were more developmentally advanced (Figure 3-1D). These results are consistent with our previous observations of seedlings from this and other independently derived *ABR17*-transgenic lines grown on semi solid MS media (Srivastava et al., 2006b). In addition, the *ABR17*-transgenic seedlings grown on

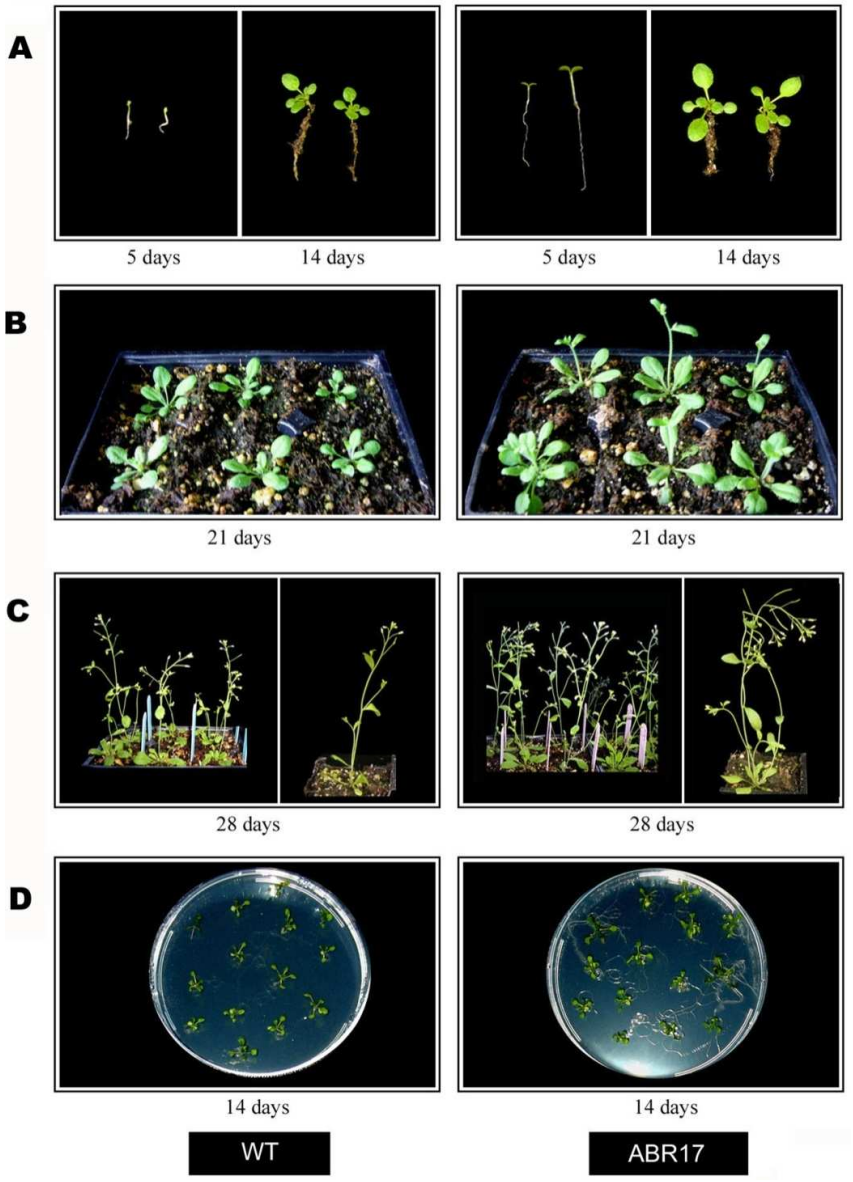

Figure 3-1 Appearance of WT and *ABR17*-transgenic *A. thaliana* at various growth stages

Seedlings at 5, 14 days (A), 21 days (B), 28 days (C) and MS-grown 14-day-old seedlings (D) are shown.

MS medium with 100 mM NaCl were greener and their roots appeared to be longer compared to the WT seedlings grown under similar conditions (Figure 3-2A). Characteristics like root length, fresh weight, chlorophyll content and carotenoid content were measured in salt treated- *ABR17* and WT seedlings. Roots of *ABR17*-transgenic seedlings were relatively longer in the absence of salt whereas upon salt treatment, the differences in lengths were not that obvious (Table 3-1). The fresh weight of *ABR17*-transgenic seedlings was not significantly different from its WT counterpart in the absence of stress. However, in the presence of 100 mM NaCl, the fresh weights of the transgenic seedlings were significantly ($p<0.05$) higher than their WT counterparts (Table 3-1). Although the chlorophyll and carotenoid contents were almost similar in *ABR17* and WT seedlings without any stress, upon NaCl treatment the transgenic seedlings had significantly ($p<0.05$) higher levels of chlorophyll (Table 3-1). Our results indicate that the NaCl treatment had less deleterious effects on the *ABR17*-transgenic seedlings compared to the WT.

In order to further characterize the differences between the WT and *ABR17*-transgenic lines, the ability of both WT and *ABR17*-transgenic seedlings to germinate in the presence or absence of light at RT was compared. In the dark, 85% of *ABR17*-transgenic *A. thaliana* had germinated after one week, whereas only 10% of the WT seeds had germinated under the same conditions (Table 3-1, Figure 3-2B). In contrast, in the presence of light, 100% of both *ABR17*-

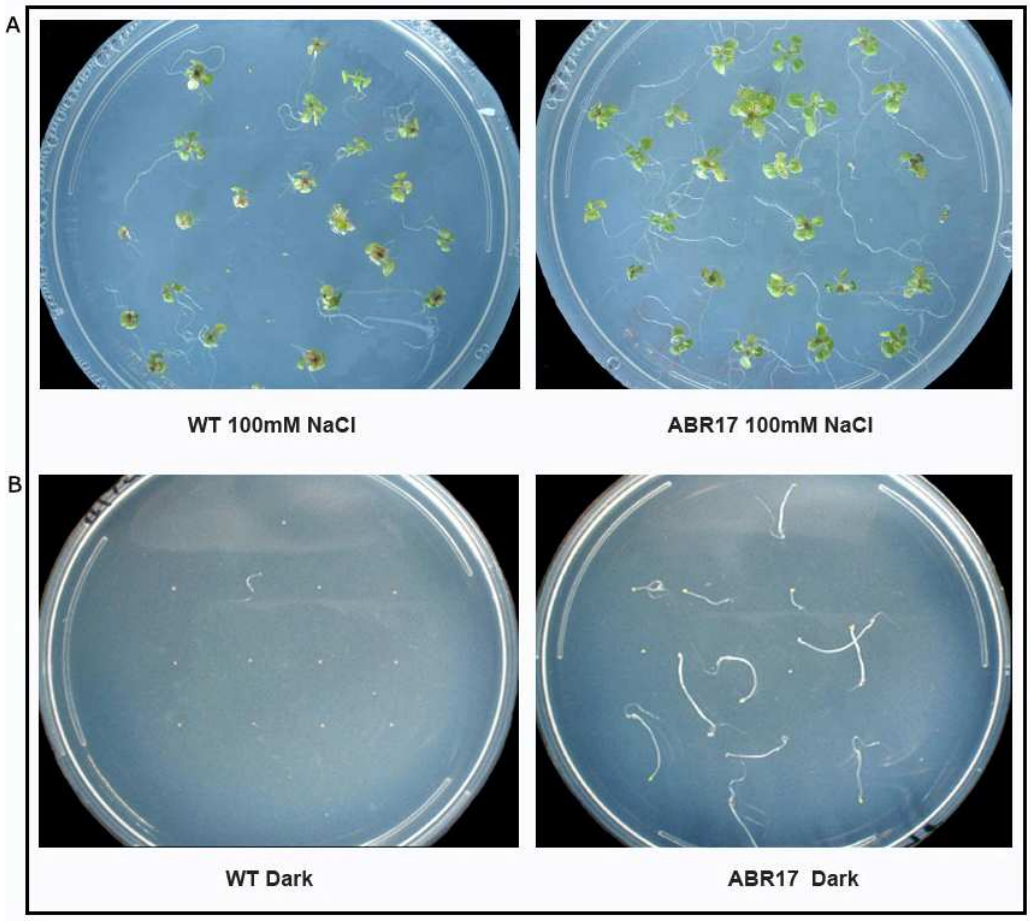

Figure 3-2 Appearance of WT and *ABR17*-transgenic *A. thaliana* in response to treatments

(A) Appearance of WT and transgenic *ABR17 A. thaliana* seedlings grown on MS media with 100 mM NaCl (B) Appearance of 7-day-old WT and *ABR17* transgenic *A. thaliana* seedlings grown under dark

Table 3-1 Morphological and physiological differences between WT and *ABR17*-transgenic *A. thaliana* lines

Morphological and pigment characteristics	**WT** **(Mean ± SE)**	***ABR17*** **(Mean ± SE)**	**p Value**
Number of lateral branches (average)	3 ± 0.3	4.1 ± 0.2	0.025
Days to flower (average)	24 ± 0.1	21.6 ± 0.3	0.002
Germination in dark (Percent)	9.6 ± 3	84.4 ± 2	< 0.001
Root length (cm)			
0 mM NaCl	1.6 ± 0 .1	2.3 ± 0.2	0.003
75 mM NaCl	0.7±0.01	0.8±0.02	0.012
100 mM NaCl	0.5±0.03	0.6±0.02	NS
Fresh weight (g per 21 seedlings)			
0 mM NaCl	0.10±0.003	0.11±0.005	NS
100 mM NaCl	0.014±0.003	0.020±0.0008	0.027
Chlorophyll (µg/g of FW)			
0 mM NaCl	32.3±1.26	33±2.58	NS
100 mM NaCl	8±0.93	13±2.44	0.045
Carotenoid (µg/g of FW)			
0 mM NaCl	2.2±0.063	2.3±0.29	NS
100 mM NaCl	1.0±0.207	1.4±0.014	NS

(NS: Non-significant)

transgenic and WT seeds had germinated in the same period (data not shown). Most *Arabidopsis* ecotypes require light for germination, which is primarily controlled by a reversible red light dependent equilibrium of the photoreceptors (Bentsink and Koorneef, 2002). It is also known that exogenous CKs can substitute for red light and enhance the germination of certain light-requiring species in the dark (Miller, 1956 & 1958; Skinner et al., 1958; Khan and Tolbert, 1965). Furthermore, *A. thaliana* detiolated (*det*) mutants exhibits many characteristics of seeds germinated in the presence of light even when germinated in dark (Chory et al., 1989), a phenotype that has been attributed to CKs because of the fact that even WT seedlings exhibit the same phenotype when germinated in the dark following exogenous CK application (Chory et al., 1994). A role for CKs can also be inferred from the observation that coumarin or far-red light, both of which prevent the formation of CK-nucleosides from storage forms of CKs, inhibit germination of lettuce seeds in the dark (Pietraface and Blaydes, 1982). Interestingly, *amp1 A. thaliana* mutants, that possess higher endogenous CKs, also exhibited a photomorphogenic response similar to our *ABR17*-transgenic seedlings (Chaudhury et al., 1993). Taken together, all these results seem to suggest that endogenous CKs play an important role in the germination of light-sensitive seeds and the elevated endogenous CKs in *ABR17*-transgenic seedlings previously reported (Srivastava et al., 2007) may be responsible for the enhanced germination of this genotype in the dark (Table 3-1, Figure 3-2B).

Transcriptional profiling using microarrays

In order to characterize the molecular changes brought about by the expression of pea *ABR17* cDNA in *A. thaliana* that resulted in the observed phenotypes, we analyzed gene expression by profiling the transcripts of *ABR17*-transgenic plants in the absence and presence of 100 mM NaCl. As described earlier, the first set of microarray analysis was the investigation of the differences in gene expression between *ABR17*-transgenic and WT *A. thaliana* in the absence of NaCl (*ABR17*/WT). The second set of microarray analysis was between 100 mM NaCl-treated WT and untreated WT *A. thaliana* (100 mM NaCl treated WT/WT). The third set of microarray analysis was between 100 mM NaCl treated *ABR17*-transgenic versus untreated *ABR17*-transgenic *A. thaliana* (100 mM NaCl treated *ABR17*/*ABR17*).

Microarrays (70-mer oligonucleotide microarrays) consisting of probes presenting 23,686 unique genes identified by *Arabidopsis* genome initiative (AGI) locus identifiers were used. We identified transcripts as those with mean signal intensities that differed significantly from 0 at $\alpha = 0.05$ in a Student's *t*-test in each set of microarrays. The transcripts were categorized based on shared structural elements and/or inferred function. We selected 12 genes representing different functional categories, which according to our microarray analysis showed enhanced or reduced levels of transcript abundance to validate our microarrays. The results from microarrays and qRT-PCR analysis are discussed below.

First set of transcriptional profiling: genes responsive to ABR17

Of the significantly responsive transcripts due the expression of pea ABR17 in *A. thaliana*, 124 were observed to be modulated in the transgenic line at least 1.5-fold compared to WT with 83 increasing and 41 decreasing in transcript abundance (Table AI-2). Many of these genes had annotations that were associated with either defense or plant growth and development, or both. A total of 16 genes showed significant differences in transcript abundance about 2-fold, where 13 genes exhibited increased transcript abundance and 3 genes showed a decrease in transcript abundance (Table 3-2).

Among the highly induced transcripts in transgenic seedlings that were putatively related to defense responses (Table 3-2), we detected 5 members of the plant defensin (PDF) family which exhibited an increased abundance ~2-3-fold in the transgenic line. PDFs are small (45- 54 amino acids), highly basic cysteine-rich peptides belonging to the large defensin family, and are present throughout the plant kingdom. These proteins are known for their involvement in ancestral non-specific innate immune defense system (Huffaker et al., 2006). In addition to being involved in mediating plant responses to pathogens, defensins may also play an important role in plant growth and development. For example, the constitutive expression of *AtPep1* induced the expression of *PDF1.2* which resulted in better root development in *A. thaliana* suggesting that plant defensins may regulate root development (Huffaker et al., 2006).

Table 3-2 Genes exhibiting nearly 2-fold changes in transcript abundance in *ABR17*-transgenic *A. thaliana* seedlings

AGI [a]	Operon annotation	log 2 ratio	SE [b]	p Value
At5g20230	*Arabidopsis* blue-copper-binding	1.55	0.14	1.57E-03
At4g36060	BHLH family protein	1.49	0.19	4.33E-03
At5g44420	PDF1.2 (Plant defensin 1.2)	1.40	0.38	1.48E-02
At5g42040	Putative 26S proteasome non-ATPase regulatory subunit	1.38	0.41	2.04E-02
At4g22450	Unknown protein	1.37	0.17	3.96E-03
At3g45970	ATEXLA1 (Expansin)	1.32	0.16	1.08E-03
At5g01920	STN8 (State transition 8); KINASE	1.24	0.22	4.47E-03
At2g26010	PDF1.3 (Plant defensin 1.3)	1.17	0.35	1.97E-02
At5g10040	Unknown protein	1.04	0.31	2.79E-02
At1g75830	PDF1.1 (Plant defensin 1.1)	1.04	0.3	1.72E-02
At2g26020	PDF1.2B (Plant defensin 1.2B)	0.96	0.26	1.47E-02
At1g07135	Glycine rich protein	0.95	0.19	7.89E-03
At1g01560	MPK11 putative	0.94	0.1	1.08E-02
At5g48850	Male sterility MS5 family protein	-0.99	0.17	9.96E-03
At1g56430	Putative, nicotianamine synthase	-1.13	0.08	8.78E-04
At3g56980	ORG3 (OBP3-responsive gene 3)	-1.36	0.13	1.91E-03

Foot note: All expression ratios are significant (α=0.05) and are in a log2 scale where fold change is *ABR17*/WT.

AGI [a] – *Arabidopsis* Genome Initiative

SE [b] - Standard error

Another interesting transcript that exhibited increased abundance (2-fold; Table 3-2) in *ABR17*-transgenic plants was a putative mitogen-activated protein kinase (*MAPK*). MAPK cascades are known to play crucial roles in physiological processes such as cell growth, cell cycle regulation and developmental control as well as plant defense signaling (Tena et al., 2001). They are also known to activate WRKY type transcription factors that are involved in transcriptional activation of disease resistance genes (Kim and Zhang, 2004). Indeed, we have observed a modest, but elevated expression of four genes belonging to the *WRKY* family and disease resistance proteins (Table AI-2).

We also observed increased transcript abundance for several genes involved in plant growth and development (Table 3-2). For example, expansins were detected as highly induced transcripts in *ABR17*-transgenic *A. thaliana* (Table 3-2). Expansins are cell wall proteins that are known to induce pH-dependent plant cell wall extension and stress relaxation (McQueen-Mason and Cosgrove, 1995). The expansins have been related to cell differentiation in tissues such as xylem, leaf primordia and root hairs (Reinhardt et al., 1998; Cho and Cosgrove, 2000; Reidy et al., 2001). Previous studies on transgenic plants expressing expansin genes have demonstrated precocious leaf development, longer petioles and larger leaf blades (Cho and Cosgrove, 2000; Pien et al., 2001).

Glycine-rich proteins (*GRP*s) were also detected among growth related genes whose transcripts increased in abundance in *ABR17*-transgenic plants (Table 3-2). GRPs consist of quasi-repetitive glycine-rich domains, most

commonly GGGX, GGXXXGG or GXGX repeats (Sachetto-Martins et al., 2000). Some GRPs have been reported as structural components of the plant cell walls based on their localization pattern (Cassab, 1998). GRPs have also been reported to be activated by osmotic stress (Xu et al., 1995), cold shock (Carpenter et al., 1994) and wounding (Showalter et al., 1991).

The genes that exhibited significant enhanced expression in *ABR17*-transgenic plants also included genes for proline-rich protein (*PRP*) family, xyloglucon endotransglycosylase (*XTH*), glycosyl hydrolase (GH), phytosulfokine precursor 2 (*PSK2*), No Apical Meristem (*NAM*) protein family and glutaredoxins (Table AI-2). PRPs represent a family of structural cell wall proteins that have been implicated in various plant developmental processes (Chen and Varner, 1985; Bernhardt and Tierney, 2000). Similarly, XTH and GH family genes are involved in structuring xyloglucan cross-links in plant cell wall and plant development (Reidy et al., 2001; Goujon et al., 2003; Vissenberg et al., 2005). The *PSK2* gene is also involved in cell growth and differentiation (Yamakawa et al., 1998; Matsubayashi et al., 1999; Igasaki et al., 2003). Similarly, the *NAM* gene product is required for shoot apical meristem (SAM) formation during embryogenesis as well as for normal flower development (Souer et al., 1996; Aida et al., 1997; Sablowski and Meyerowitz, 1998). Glutaredoxins have also been demonstrated to be involved in flower development, probably by mediating post-translational modifications of target proteins required for normal petal organ initiation and morphogenesis (Xing et al., 2005). Our current

observations that the significantly (albeit modest) higher expression of the above mentioned genes related to growth and development including flowering correlates well with the observed phenotypes, which include early flowering, increased lateral branching and seed pods as observed in *ABR17*-transgenic *A. thaliana* (Figure 3-1C).

A role for cytokinins in ABR17-induced changes in gene expression?

Interestingly, members of many of the gene families described above (defensin, expansin, *MAPK*, *NAM*, *WRKY*, *GRP*, *PSK2* and Glutaredoxin) that are involved in plant defense as well as growth and development, have been previously reported to be regulated by CKs. For example, genome-wide expression profiling of immediate-early and delayed CK- response genes of *A. thaliana*, has led to the identification of many genes that are up- regulated by CKs including members of expansin (*At1g69530*), *GRP*s (*At2g21060*), *NAM* (*At4g27410*), F-box protein (*At3g61060*), *ERBF*s, putative ring zinc finger protein (*At1g76410*), a member of the *bHLH* family (*At2g18300*), blue copper binding protein (*At5g20230*) and *PSK2* (Brenner et al., 2005). The blue copper binding protein (*At5g20230*) and putative ring zinc finger protein (*At1g76410*) identified by Brenner et al. (2005) as CK-induced were observed to be up-regulated in our microarrays analysis. Similarly, gene expression analysis of transgenic *A. thaliana* seedlings transformed with a bacterial isopentenyl transferase (*IPT*) (Hoth et al., 2003) gene revealed increased transcript abundance for many members of the *MAPK* and *WRKY* gene families, which included the specific *WRKY* gene -

At1g80840 that has been detected in our microarray studies as being induced by *ABR17* expression (Table AI-2). Another investigation into CK action in *Arabidopsis* has demonstrated increased expression of genes for cytochrome P450, *PDF*, expansin, patatin, *WRKY* members and putative disease resistance protein in response to CKs (Rashotte et al., 2003). Therefore, it is apparent that several genes whose transcript levels were modulated by *ABR17* expression in *A. thaliana* have been previously reported in the literature as being CK-responsive, thereby suggesting an important role for CK-mediated gene expression in ABR17 action *in planta*.

Second set of transcriptional profiling: genes responsive to salt stress in WT A. thaliana.

Microarray- based analyses of the salt responses in *Arabidopsis* have been published in several reports. However, most of these studies have investigated responses to very short-term exposure to salt. In this study, we report the transcriptional changes in *A. thaliana* as a result of long-term, continuous exposure to 100 mM NaCl. Here, we allowed *A. thaliana* seeds to germinate and grow on semi-solid medium in the presence of 100 mM NaCl for 2 weeks, and the RNA extracted from whole seedlings were used for cDNA synthesis and subsequent microarray analysis. The results from microarray analysis of salt treated WT *Arabidopsis* seedlings (Table AI-3) were consistent with previous studies using similar approaches (Jiang and Deyholos, 2006; Ma et al., 2006). We identified 163 genes that showed more than four fold changes in transcript

abundance, which have been previously reported as being responsive to salt. Our results, therefore, indicate that both short-term "shock" treatments with NaCl as well as long-term treatment used in this study elicit similar responses in *A. thaliana* at the transcript level (Table AI-3).

Members of protease inhibitor/lipid transfer protein (LTP) family were seen among highly up regulated and/ or down regulated genes. At least five members showed increase in transcript abundance and 1 member showed decrease in transcript abundance of more than 4 fold (Table AI-3). LTP genes contain ABA-responsive (ABRE) element (GTACGTGG) and are induced by abscisic acid (ABA) (Hughes et al., 1992; White et al., 1994). It has been reported in the literature that NaCl, mannitol or ABA treatments induce the expression of a gene encoding an LTP-like protein in tomato (Hughes et al., 1992; Torres-Schumann et al., 1992). In addition, the changes in the expression of LTP genes during salt stress have been previously reported (Jiang and Deyholos, 2006; Ma et al., 2006). Although most of the LTP genes were up regulated after short term treatment with salt, they were found to be down regulated after 24h of salt treatment (Jiang and Deyholos, 2006; Ma et al., 2006). From our studies, it appears that many of the LTP genes will be up regulated in response to long term stress, as a result of the expected increase in ABA levels.

Other major groups of genes with increase in transcript abundance following NaCl treatment included two members of glycosyltransferases (GTs) and five members of glycoside hydrolases (GHs). GTs and GHs are major

families that play a primary role in structuring xyloglucan cross-links in the plant cell wall (Goujon et al., 2003; Vissenberg et al., 2005). They have been previously reported to be induced by salinity stress in plants and this has been implicated in drought and salt tolerance in *A. thaliana* (Vissenberg et al., 2005; Jiang and Deyholos, 2006). Other genes exhibiting increased transcript abundance included ribonuclease RNS1, osmotin-like protein, hydroxycinnamoyl benzoyltransferase-related, oxidoreductase, 2OG-Fe(II) oxygenase, glutathione transferase and zinc finger (C3HC4-type RING finger) protein (Table AI-3). Similarly, the genes which showed decrease in transcript abundance of more than 4 fold included many photosynthetic genes, plant defensins, heat shock proteins, auxin-induced proteins, disease resistance protein, Bet v I allergen family and bHLH protein. These results are once again consistent with the previously reported results from microarray-based investigation into salinity stress responses (Jiang and Deyholos, 2006; Ma et al., 2006).

Third set of transcriptional profiling: genes responsive to salt stress in presence of ABR17

The results from microarray analysis of salt treated *ABR17* transgenic *A. thaliana* seedlings are presented in Tables 3-3 and 3-4. We identified 129 genes showing either increase or decrease in transcript abundance of more than 4-fold, which included transcription factors (15), stress responsive genes (16), carbohydrate and cell wall metabolism (8), electron transport and oxidoreductases (6), lipid metabolism (3), protein and amino acid metabolism (9), proteins

Table 3-3 Genes exhibiting >4-fold change in transcript abundance in 100 mM NaCl treated *ABR17*- transgenic seedlings

AGI[a]	Operon annotation	GM	SE [b]	p Value
Transcription factors				
At5g43650	bHLH protein family	4.67	0.51	1.22E-02
At1g43160	AP2 TF, RAP2.6	4.52	0.5	1.22E-02
At3g15500	ATNAC3	3.8	0.28	8.39E-04
At1g10585	Transcription factor	3.32	0.2	4.55E-04
At3g43180	zinc finger protein family	3.06	0.6	7.05E-03
At1g21910	AP2 TF, putative	3.01	0.04	2.66E-07
At1g52890	ANAC019	2.96	0.46	7.40E-03
At5g13330	AP2 TF, RAP2.6L	2.86	0.11	1.33E-05
At4g05100	ATMYB74	2.53	0.29	3.21E-03
At2g38340	AP2 TF , putative	2.4	0.27	3.08E-03
At2g46680	ATHB-7 (*A thaliana* HOMEOBOX 7)	2.16	0.02	9.75E-07
At2g38470	WRKY family TF	2.05	0.21	1.75E-04
At4g17460	HAT1	-2.15	0.26	3.96E-04
At2g33810	SPL3	-2.22	0.54	2.55E-02
At1g62360	STM	-2.76	0.35	1.44E-03
Stress response				
At2g03760	Steroid sulfotransferase	3.71	0.1	3.35E-06
At5g43570	Serine protease inhibitor family protein	3.57	0.09	2.85E-06
At4g04220	Disease resistance family protein	3.43	0.16	2.40E-04
At4g37990	Mannitol dehydrogenase, putative	2.85	0.48	1.96E-03
At4g11650	Osmotin-like protein (OSM34)	2.36	0.17	3.95E-05
At5g39580	Peroxidase, putative	2.32	0.32	5.27E-03
At2g33380	RD20 (Responsive to dessication 20)	2.22	0.27	4.30E-04
At5g59820	Zinc finger protein	2.19	0.52	8.55E-03
At2g02990	Ribonuclease, RNS1	2.13	0.1	2.94E-05
At1g08830	Copper/zinc superoxidase dismutase	2.09	0.18	8.11E-05
At5g42180	Peroxidase, putative	-2.22	0.54	1.45E-02
At4g18780	CESA8 (Cellulase synthase 8)	-2.34	0.1	1.52E-04
At3g22231	PCC1 (Pathogen & circadian contr 1)	-2.5	0.42	1.95E-03
At2g11810	MGDG synthase (MGD3), putative	-2.66	0.1	1.12E-04
At1g23130	Bet v I allergen family	-3.48	0.16	4.19E-06
At4g14400	ACD6 (Accelerated cell death 6)	-4.33	0.97	2.12E-02

Table 3-3 (*Continued*)

AGI[a]	Operon annotation	GM	SE [b]	p Value
Carbohydrate and cell wall metabolism				
At4g25810	Xyloglucan endotransglycosylase 6	4.68	0.19	1.53E-04
At3g60140	Glycosyl hydrolase family 1 protein	4.19	0.1	3.46E-05
At2g36780	UDP-glycosyltransferase family	2.87	0.12	1.75E-04
At2g43620	Glycosyl hydrolase family 19 (chitinase)	2.81	0.4	9.08E-04
At4g16260	Glycosyl hydrolase family 17	2.49	0.11	2.06E-04
At4g26530	Fructose-bisphosphate aldolase, putative	-2	0.14	3.06E-05
At4g02290	Glycosyl hydrolase family 9	-2.22	0.25	3.11E-04
Electron transport & Oxidoreductase				
At2g37770	Aldo/keto reductase family	3.05	0.11	1.02E-05
At1g30700	FAD-linked oxidoreductase family	2.58	0.21	1.14E-03
At5g05600	Oxidoreductase	2.42	0.33	5.06E-03
At1g17020	SRG1 (Senescence-related gene 1)	2.26	0.08	1.35E-06
At2g45570	Cytochrome p450 family	2.11	0.34	8.70E-03
At5g20230	*Arabidopsis* blue-copper-binding protein	2.1	0.11	6.36E-06
Lipid metabolism				
At5g14180	Lipase family protein	2.78	0.45	1.59E-03
At1g54010	Myrosinase-associated protein, putative	2.24	0.69	4.77E-02
At3g02040	SRG3 (Senescense related gene 3)	-2.66	0.16	1.24E-05
Protein and amino acid metabolism				
At3g25250	Protein kinase family	2.54	0.38	6.62E-03
At4g04490	Protein kinase family protein	2.51	0.79	4.96E-02
At4g08870	Arginase related	2.45	0.1	2.31E-06
At1g26970	Protein kinase, putative	2.39	0.09	1.50E-06
At1g76600	Similar to unknown protein (*A thaliana*)	2	0.58	2.59E-02
At1g21270	Protein serine/threonine kinase	-2.06	0.24	3.37E-04
At1g65800	ARK2 (*Arabidopsis* receptor kinase 2)	-2.33	0.17	1.73E-04
At4g10540	Subtilase family protein	-2.36	0.07	5.74E-06
At4g21640	Subtilase family protein	-2.45	0.34	2.04E-03
At4g21650	Subtilase family protein	-2.49	0.65	3.13E-02

Table 3-3 (*Continued*)

AGI[a]	Operon annotation	GM	SE [b]	p Value
Transport				
At2g38530	Protease inhibitor/lipid transfer protein	3.91	0.16	2.35E-06
At4g12500	Protease inhibitor/lipid transfer protein	3.34	0.28	7.67E-05
At4g12490	Protease inhibitor/lipid transfer protein	3.32	0.29	9.06E-05
At3g50930	AAA-type ATPase family	3	0.19	1.86E-05
At4g12470	Protease inhibitor/lipid transfer protein	2.8	0.29	1.88E-04
At2g04070	MATE efflux protein family	2.67	0.31	3.24E-03
At5g43610	ATSUC6 (Sucrose-proton symporter 6)	2.5	0.33	6.37E-04
At3g51860	Cation exchanger, putative (CAX3)	2.2	0.24	2.64E-03
At2g04080	MATE efflux protein – related	2.1	0.32	1.17E-03
At4g12480	Protease inhibitor/lipid transfer protein	2.09	0.23	2.45E-04
At4g21680	Peptide transporter – like protein	2.03	0.76	4.41E-02
At5g19530	Spermine synthase (ACL5)	-2.02	0.14	2.72E-05

Foot note: All expression ratios are significant (α=0.05) and are in a log2 scale where fold change is salt treated *ABR17*/ control *ABR17*.

GM- Gene mean

AGI [a] – *Arabidopsis* Genome Initiative

SE [b] - Standard error

Table 3-4 Unknown/unclassified genes exhibiting > 4-fold changes in transcript abundance in NaCl-treated *ABR17*- transgenic seedlings

AGI [a]	Operon annotation	Gene mean	SE [b]	p value
At3g02480	ABA-responsive protein-related	4.55	0.5	7.93E-04
At2g34600	Unknown protein	4.25	0.66	2.30E-02
At5g24640	Unknown protein	4.15	0.28	2.41E-05
At5g43580	Serine-type endopeptidase inhibitor	3.73	0.64	2.14E-03
At4g13220	Similar to OS12G0276100	3.7	0.29	2.26E-04
At4g33720	Pathogenesis-related protein, putative	3.54	0.36	1.96E-04
At3g13600	Calmodulin-binding family protein	3.32	0.69	1.73E-02
At4g39670	Similar to Accelerated cell death 11	3.2	0.12	1.02E-05
A023734_01	Putative ubiquitin-conjugating enzyme	2.63	0.52	1.47E-02
At5g38940	Germin-like protein, putative	2.59	0.34	4.76E-03
At1g66400	Calmodulin-related protein, putative	2.58	0.15	5.88E-05
At1g73260	Trypsin inhibitor –related	2.57	0.37	2.19E-03
At2g36770	Glycosyltransferase family	2.54	0.17	6.14E-04
At5g01920	STN8 (State transition 8)	2.52	0.07	4.94E-05
At4g01430	Nodulin MtN21 family protein	2.5	0.19	9.70E-04
At3g28210	Zinc finger protein (PMZ) –related	2.42	0.27	8.23E-04
At2g32200	Similar to unknown protein (*A. thaliana*)	2.34	0.12	6.21E-06
At1g35140	Phosphate-induced protein –related	2.33	0.44	3.32E-03
At1g23710	Similar to unknown protein (*A. thaliana*)	2.31	0.15	1.19E-04
At5g42830	Hydroxycinnamoyl benzoyltransferase-related	2.3	0.12	3.05E-04
At1g53470	Mechanosensitive ion channel domain-containing protein	2.19	0.15	1.38E-04
At2g36800	Glucosyl transferase –related	2.18	0.16	4.37E-05
At4g24380	Hydrolase, acting on ester bonds	2.16	0.35	1.68E-03
At2g41640	Similar to unknown protein (*A. thaliana*)	2.15	0.15	7.48E-04
At2g30840	2-oxoglutarate-dependent dioxygenase, putative	2.14	0.16	3.57E-05
At5g35510	Unknown protein	2.09	0.16	2.00E-04
At1g17380	Similar to unknown protein (*A. thaliana*)	2.06	0.14	2.27E-05
At5g03210	Unknown protein	2.06	0.55	1.37E-02
At2g36790	Glucosyl transferase –related	2.04	0.49	1.44E-02
At3g03820	Auxin-induced protein, putative	-2	0.24	1.08E-03
At1g12080	Similar to unknown protein (*A. thaliana*)	-2.04	0.33	1.60E-03
At1g78020	Senescence-associated protein –related	-2.07	0.11	9.14E-06
At2g32870	MEPRIN and TRAF homology domain-containing protein	-2.12	0.31	2.40E-03

Table 3-4 (*Continued*)

AGI [a]	Operon annotation	Gene mean	SE [b]	p value
At5g64770	Similar to 80C09_10 (*Brassica rapa*)	-2.19	0.19	8.40E-05
At2g14560	Similar to unknown protein (*A. thaliana*)	-2.22	0.3	6.53E-04
At4g00755	F-box protein family	-2.27	0.14	1.40E-05
At3g32130	Similar to unknown protein (*A. thaliana*)	-2.3	0.17	1.70E-04
At3g45160	Unknown protein	-2.33	0.17	3.44E-05
A003747_01	Histone H2B, putative	-2.36	0.18	1.89E-04
At4g39800	Myo-inositol-1-phosphate synthase	-2.48	0.12	5.20E-06
At2g41090	Calmodulin-like calcium binding protein	-2.48	0.14	1.03E-05
At3g04210	Disease resistance protein, putative	-2.55	0.09	1.04E-06
At5g18030	Auxin-induced protein, putative	-2.58	0.24	1.15E-04
At5g42530	Similar to ECS1 (*A. thaliana*)	-2.59	0.11	2.47E-06
At2g40610	ATEXPA8 (*A. thaliana* expansin 8)	-2.61	0.1	1.50E-05
At5g18080	Auxin-induced protein, putative	-2.61	0.26	1.68E-04
At1g67870	Glycine-rich protein	-2.64	0.1	1.56E-06
At1g29460	Auxin-induced protein, putative	-2.73	0.3	2.72E-04
At1g14880	Similar to unknown protein (*A. thaliana*)	-2.79	0.63	1.14E-02
At1g29430	Auxin-induced protein family	-2.8	0.83	4.29E-02
At1g29510	Auxin-induced protein, putative	-2.88	0.19	2.18E-05
At2g25510	Unknown protein	-2.91	0.14	2.95E-05
At5g61980	ARF GTPase-activating domain-containing protein	-3.03	0.54	5.01E-03
At2g04460	Retroelement pol polyprotein –related	-3.15	0.28	1.54E-03
At1g67860	Similar to unknown protein (*A. thaliana*)	-3.16	0.25	5.21E-05
At5g18010	Auxin-induced protein, putative	-3.21	0.12	1.39E-06
At5g18020	Auxin-induced protein, putative	-3.26	0.2	1.46E-05
At5g35480	Unknown protein	-3.76	0.37	5.27E-04
At4g14400	ACD6 (Accelerated cell death 6)	-4.32	0.97	2.12E-02

All expression ratios are significant (α=0.05) and are in a log2 scale where fold change is salt treated *ABR17*/ control *ABR17*.

AGI [a] – *Arabidopsis* Genome Initiative SE [b] - Standard error

involved in transport across membranes (12) and 60 unknown or unclassified genes. Transcriptional factors are necessary for the proper transcriptional regulation in response to environmental cues (Riechmann et al., 2000) and those exhibiting significant increases in transcript abundance included bHLH, 4 members of APETALA2 (AP2) related, 2 members of NAM, zinc finger (C3HC4-type RING finger) protein family, ATMYB74 (MYB domain protein 74), ATHB-7 (*A. thaliana* HOMEOBOX 7), and WRKY families. *bHLH092* has been indicated among the highly induced transcripts in response to NaCl treatment in the previous transcriptomic studies and are suggested to be important regulators of the NaCl-stress response in *Arabidopsis* (Riechmann et al., 2000). The AP2 domain defines a large family of transcription factors which play important roles in plant growth and development as well as stress tolerance (Ohmetakagi and Shinshi, 1995; Gilmour et al., 1998; Kizis et al., 2001; Guo et al., 2004; Ma and Bohnert, 2007; Shukla et al., 2006).

Similarly, as previously stated, NAM genes have been found to be induced by abiotic stresses implying roles in stress responses in addition to those in plant growth and development (Xie et al., 2000; Olsen et al., 2005). NAM/ NAC proteins contain highly conserved NAC (for NAM, ATAF1, 2, and CUC2) domains in their N-terminal regions, that specifically bind target DNA (Aida et al., 1997). It has also been demonstrated that NAC transcription factors are ABA-responsive (Greve et al., 2003; Fujita et al., 2004) and are also induced by other plant hormones like NAA (α-naphthalene-acetic acid) and ethylene

(Hegedus et al., 2003; Fujita et al., 2004; Tran et al., 2004; He et al., 2005). Overexpression of NAC genes has been shown to result in an increase in lateral roots, and tolerance to abiotic stresses like drought and salt stress. NAC genes are believed to exert their stress ameliorating activity through the regulation of stress-inducible genes (Tran et al., 2004; He et al., 2005; Hu et al., 2006). Similarly, the WRKY family TF genes and myb family genes are known to be biotic and/or abiotic stress responsive (Seki et al., 2002; Jiang and Deyholos, 2006). Thus, it is possible that the increased tolerance of *ABR17*-transgenic seedlings to NaCl is the combined effect of the modulation of the levels of abundance of transcripts for these transcription factors with demonstrated roles in stress tolerance.

The highest transcript abundance of any gene observed in salt treated *ABR17* plants was *XTR-6* (xyloglucan endotransglycosylase-6), which showed a 4.7-fold increase, compared to the untreated *ABR17*-transgenic line. Xyloglucan endotransglycosylase (XET) has been suggested to be a key enzyme involved in the modification of the xyloglucan cross-links that controls the strength and extensibility of the plant cell wall (Silva et al., 1994). Three members of GH family were also seen among genes which were up regulated more than 4-fold (Table 3-3). The importance of GHs genes in plant stress (Vissenberg et al., 2005; Jiang and Deyholos, 2006) has already been discussed in the previous section.

Other salt responsive genes in the *ABR17*-transgenic line included osmotin, mannitol dehydrogenase, steroid sulfotransferases and RD20 (Table 3-3), which are known to be regulated by ABA and are expressed in salt-stressed

plants and have been used to engineer salinity tolerance (King et al., 1986; Singh et al., 1987; Takahashi et al., 2000; Abede et al., 2003; Klein and Papenbrock, 2008). In addition, we also observed an increase in transcript abundance for ribonuclease- RNS1, peroxidases, copper/zinc superoxidase dismutase (*CSD1*), cytochrome p450 family, MATE efflux protein and protein kinases which have been previously demonstrated to accumulate in salt treated tissues by others (Jiang and Deyholos, 2006; Ma et al., 2006). From our microarray results, it appears that many genes involved in mediating responses to salinity stress are increased in transcript abundance as would be expected.

Comparison of salt responses in WT and ABR17 transgenic seedlings

Although transcriptional changes were almost similar both in salt treated *ABR17* and WT seedlings, the transcript abundance of some genes exhibited significant differences in both the trend as well as the degree of modulation of transcript abundance (Table 3-5). For instance, as mentioned previously, transcript abundance of xyloglucan endotransglycosylase (*XTR-6*) (*At4g25810*) increased 4.7-fold in salt treated *ABR17* seedlings, whereas it showed only a 2.4-fold increase in salt-treated WT seedlings (Table 3-5). Similarly, AP2 domain related transcription factor *RAP2.6* (*At1g43160*) increased 4.5-fold in salt treated *ABR17*-plants compared to 1.67-fold in treated WT plants. The expressed proteins- ABA-responsive protein-related (*At3g02480*) and unknown protein (*At5g24640*) also showed increase in transcript abundance of at least 4-fold in salt-treated *ABR17*-transgenic line compared to the 2-fold increase observed for

the WT in response to salt. Other genes which exhibited an increase in transcript abundance of more than 2 fold in salt-treated *ABR17* transgenic but showed less abundance in treated WT included pathogenesis-related protein (*At4g33720*) and glutamine-dependent asparagine synthetase (*At3g47340*). On the other hand, the retroelement pol polyprotein (*At2g04460*) with unknown function showed a down regulation of more than 2-fold in salt-treated transgenic *ABR17* line, but was up regulated in salt treated WT *Arabidopsis* plants.

Interestingly, many members of heat shock protein (Hsp) family and PDF family showed the opposite response in salt-treated *ABR17*-transgenic seedlings compared to the salt-treated WT counterparts, with an increase of transcript abundance in salt-treated *ABR17*-transgenic (Table 3-5). This difference in the direction of the response in gene expression (i.e. induction in the transgenic seedlings versus repression in the WT) may have important consequences with respect to the ability to tolerate salinity (and perhaps other) stress. For example, the Hsp family contains chaperones, which have important roles in protein folding, assembly and in the disposal of unwanted nonfunctional proteins. Hsps are usually induced by environmental stress, and the accumulation of Hsps coincides with enhanced stress tolerance (Kuznetsov et al., 1993; Coca et al., 1996; Campalans et al., 2001; Sun et al., 2001; Koike et al., 2002; Jiang and Deyholos, 2006). In addition, transgenic *Arabidopsis* plants overexpressing *AtHSP17.7* accumulate high levels of AtHSP17.7 protein and show enhanced tolerance to drought and salinity (Kuznetsov et al., 1993; Sun et al., 2001). The

Table 3-5 Comparison of changes in gene expression between NaCl-treated WT and *ABR17*-transgenic *Arabidopsis* seedlings

AGI [a]	Operon annotation	*ABR17*	SE [b]	WT	SE [b]	*ABR17*-WT
		log2 ratio		log2 ratio		
At4g25810	Xyloglucan endotransglycosylase (XTR-6)	4.68	0.39	2.35	1.31	2.33
At3g02480	ABA-responsive protein-related	4.55	1.11	2.4	0.6	2.15
At1g43160	AP2 domain transcription factor RAP2.6	4.52	0.87	1.67	0.6	2.85
At5g24640	Unknown protein	4.15	0.28	2.58	0.36	1.57
At4g33720	Pathogenesis-related protein, putative	3.54	0.89	1.19	0.77	2.35
At3g47340	Glutamine-dependent asparagine synthetase	2.04	0.12	-0.02	0.45	2.06
At2g29500	Small heat shock protein-related	1.69	0.93	-0.21	0.61	1.91
At1g59860	Heat shock protein, putative	1.59	0.8	-1.59	1.03	3.17
At5g12030	*A. thaliana* mRNA for 17.6kDa HSP protein	1.41	0.39	-0.7	0.81	2.11
At5g51440	Heat shock protein, putative	1.32	0.6	0.08	0.3	1.24
At3g09440	Heat shock protein hsc70-3 (hsc70.3)	1.22	0.24	-3.03	0.53	4.25
At5g56010	Heat shock protein, putative	1.19	0.35	-1.06	0.36	2.26
At2g26150	Heat shock transcription factor family	1.06	0.59	-0.55	0.69	1.62
At1g74310	Heat shock protein 101 (HSP101)	1.06	0.27	-2.42	0.75	3.48
At5g48570	Peptidylprolyl isomerase	1.02	0.3	-2.18	0.91	3.19
At5g44420	Plant defensin protein, putative (PDF1.2a)	0.9	0.23	-2.51	0.51	3.41

Table 3-5 (*Continued*)

AGI [a]	Operon annotation	*ABR17* log2 ratio	SE [b]	WT log2 ratio	SE [b]	*ABR17*-WT
At5g44430	Plant defensin protein, putative (PDF1.2c)	0.8	0.25	-2.62	0.42	3.42
At5g56030	Heat shock protein 81-2 (HSP81-2)	0.77	0.17	-1.52	0.34	2.29
At2g26010	Plant defensin protein, putative (PDF1.3)	0.76	0.2	-2.95	0.38	3.7
At3g12580	Heat shock protein hsp70	0.73	0.44	-1.93	1.1	2.66
At5g56000	Heat shock protein 81.4 (hsp81.4)	0.64	0.2	-1.91	0.57	2.55
At5g12020	Class II heat shock protein	0.61	0.29	-0.41	0.49	1.01
At1g75830	Plant defensin protein, putative (PDF1.1)	0.58	0.16	-1.72	0.51	2.3
At4g11660	Heat shock factor protein 7 (HSF7)	0.53	0.35	-0.77	0.75	1.3
At5g02500	Heat shock protein hsc70-1 (hsp70-1)	0.52	0.16	-0.75	0.24	1.27
At5g02490	Heat shock protein hsc70-2 (hsc70.2)	0.43	0.13	-1	0.27	1.43
At2g19310	Small heat shock protein – related	0.39	0.22	-1.98	0.44	2.37
At1g16030	Heat shock protein hsp70b	0.34	0.26	-0.77	0.22	1.11
At2g04460	Retroelement pol polyprotein –related	-3.15	0.56	1.55	0.63	-4.7

Footnote: All expression ratios are significant (α=0.05) and in a log2 scale where fold change is salt treated *ABR17*/control *ABR17* and salt treated WT/control WT. *ABR17*- WT= Difference in log2 ratio of salt treated *ABR17*/control *ABR17* and salt treated WT/control WT.

AGI [a] – *Arabidopsis* Genome Initiative SE [b] - Standard error

abundance of Hsps in plants and their functional characteristics suggest that Hsps play an important role in plant stress tolerance. Thus, the increased abundance of HSP transcripts in the *ABR17*-transgenic seedlings may be important for the increased tolerance of this line to the imposed stress. The up regulation of PDFs in *ABR17*-transgenic *A. thaliana* grown under normal conditions (Table 3-2) and their importance in growth and development has already been discussed earlier. The literature also supports a role for PDFs in stress tolerance (Koike et al., 2002; Huffaker et al., 2006). Most of the previously characterized PDFs exhibit anti-fungal, antibacterial, anti- insect and protease inhibitor activity (Spelbrink et al., 2004). However, the halophyte salt cress (*Thellungiella halophila*), a relative of *Arabidopsis*, over expresses PDFs under normal conditions and hence defensins are believed to play a role in salt tolerance (Taji et al., 2004). It is therefore possible that the observed relatively tolerant phenotype of *ABR17*-transgenic seedlings could be due, at least in part, to the elevated expression of *XTR6*, *RAP2.6* transcription factors, unknown proteins (*At3g02480, At5g24640*), Hsp and PDF gene(s).

qRT-PCR validation of microarray observations

In order to confirm the fact that CK-responsive genes were indeed up-regulated in the *ABR17*-transgenic lines, we performed qRT-PCR experiments with the following genes: plant defensin protein (*PDF1.2a*, *At5g44420*), expansin (*EXPL1*, *At3g45970*), *GRP* (*At1g07135*) and putative *MAPK 11* (*Atg01560*) using qRT-PCR. Among the CK- inducible genes identified from our first set of

microarray experiments, we chose the above 4 genes for qRT-PCR as their transcripts were observed to be at least ~2-fold (1 in log2 ratio) or higher in the transgenic line compared to WT. Our microarray analysis revealed increase in transcripts for defensin, expansin, *GRP* and MAPK in pea *ABR17* seedlings by 2.6, 2.5, 1.9 and 1.9-fold, respectively (Figure 3-3). Our qRT-PCR results were consistent with the microarray data and showed up-regulation of defensin, expansin, *GRP* and *MAPK* by 3.6, 2.5, 2.5 and 2.7-fold, respectively (Figure 3-3). From these results it is apparent that all the four genes that were up-regulated in our microarray analysis also demonstrated up- regulation in the qRT-PCR relative expression experiments (Figure 3-3).

Because of the fact that the specific members of gene families whose transcripts were found to be modulated by *ABR17* cDNA expression in *A. thaliana* were not exactly identical to those specific members of these families identified by other studies, we wanted to investigate whether those specific members detected in our studies were indeed CK-inducible/repressible. In these experiments, we used WT *A. thaliana* tissue germinated and grown for 14 days on medium supplemented with 5 µM zeatin for additional qRT-PCR experiments. This concentration of zeatin was chosen based on our earlier observations that it induced the largest phenotypic responses in *A. thaliana* when exogenously applied (Srivastava et al., 2007). It must also be noted that even though 5 µM zeatin was used in our experiments, it is difficult to estimate how much of this exogenously supplied CK actually gets into the seed in order to exert a

physiological effect. From the results shown in Figure 3-3, it is apparent that the expression of *EXPL1* (*At3g45970*), putative *MAPK* (*Atg01560*) and *GRP* (*At1g07135*) was up-regulated in response to exogenous zeatin by 1.6, 3.8, and 2-fold, respectively (Figure 3-3). In contrast, the expression of defensin gene was observed to be down-regulated in response to the exogenous application of CK (Figure 3-3). The results for expansin, *MAPK* and *GRP* are consistent with our microarray and qRT-PCR results with respect to increased transcript abundance in *ABR17*-transgenic *A. thaliana* previously shown to possess higher endogenous CK concentrations (Srivastava et al., 2007). However, in the case of defensin, even though our microarray and qRT-PCR experiments revealed that this gene was up-regulated in the *ABR17*-transgenic line (Figure 3-3), its expression was not induced by exogenous CKs (Figure 3-3). The reason for this discrepancy is not immediately clear; however, this may be due to the concentration as well as type of CK used in our exogenous experiments. Furthermore, as indicated previously, the amount of the exogenously supplied CK entering the seed to exert physiological effects may also be different from the concentrations required to elicit induction of this gene.

In order to confirm the results from our second and third set of microarray analysis, we performed qRT-PCR experiments with the following 12 genes: unknown proteins (*At3g02480*; *At5g24640*; *At1g14880*), *XTR6* (*At4g25810*), *bHLH* (*At5g43650*), *RAP 2.6* (*At1g43160*), *ATNAC3* (*At3g15500*), *ACD6* (*At4g14400*), *PDF1.2a* (*At5g44420*), *EXPL1* (*At3g45970*), *GRP* (*At1g07135*) and

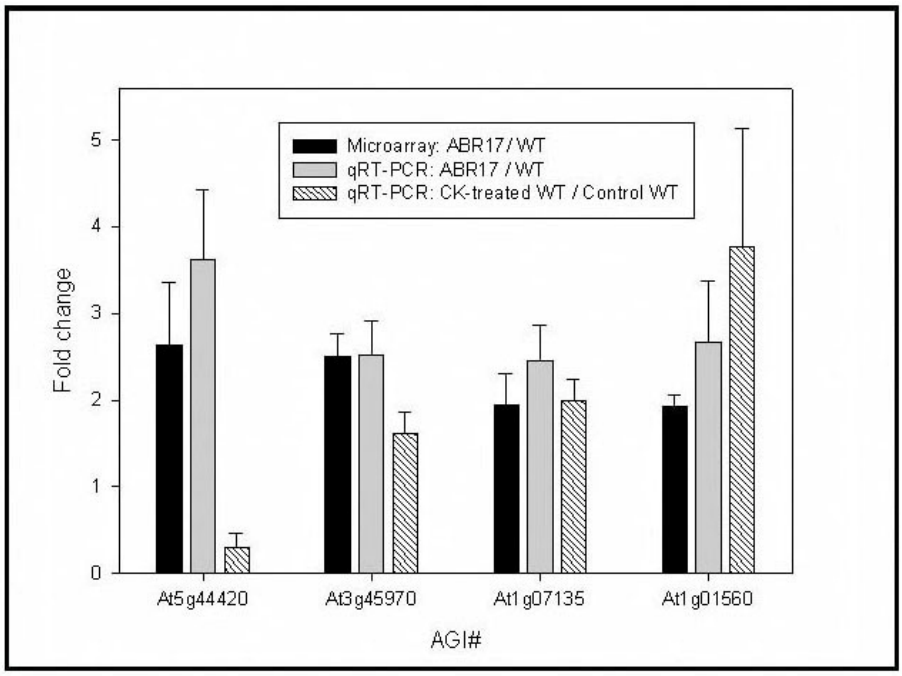

Figure 3-3 Pea *ABR17*-modulated transcriptional changes of selected genes

Transcriptional changes of a selected number of transcripts as identified by microarrays, and their validation using qRT- PCR and effects of CK on these genes in WT *A. thaliana* is given here. The values represented in the graph are fold changes of transcript abundance between transgenic *ABR17-A. thaliana* seedlings versus WT seedlings grown under normal conditions. Error bars are standard error of fold changes driven from (n=3) three biological replicates. The AGI annotations are as follows**: At5g44420**-Plant defensin protein family member PDF1.2, Low-molecular-weight cysteine-rich (LCR77); **At3g45970**-ATEXLA1 (*A. thaliana* expansin-like A1); **At1g07135**-Glycine rich protein; and **At1g01560**-ATMPK11.

MAPK11 (*Atg01560*). The unknown proteins were chosen because expression of two of them (*At3g02480* and *At5g24640*) were among highly induced transcripts in salt treated *ABR17*-transgenic line and also showed comparatively less but high level of transcript abundance in salt treated WT *A. thaliana* lines (Table 3-5, Figure 3-4). Two of these (*At1g14880* and *At4g14400*) were among the highly down regulated genes in both salt treated *ABR17*-transgenic line and WT *A. thaliana* lines (Table 3-3 & 3-4, Figure 3-4). Our qRT-PCR data showed similar trends as observed by microarrays for all the above-mentioned genes in both salt-treated *ABR17*-transgenic and salt-treated WT microarrays (Figure 3-4). The genes *At3g02480, At5g24640, At1g14880* and *At4g14400* showed transcript abundance with fold changes of 5.26, 5.98, 0.27 and 0.06, respectively, in our microarray analysis of salt treated WT *A. thaliana* (Figure 3-4). Our qRT-PCR analysis of salt treated WT *A. thaliana* showed transcript abundance of 18.60, 32.42, 0.03 and 0.06-fold for genes *At3g02480, At5g24640, At1g14880* and *At4g14400* (Figure 3-4) compared to qRT-PCR indicated transcript abundance of 23.36, 17.80, 0.14 and 0.04-fold for genes *At3g02480, At5g24640, At1g14880* and *At4g14400*, respectively (Figure 3-4). Our qRT-PCR analysis of salt treated *ABR17-Arabidopsis* showed transcript abundance of 272.37, 67.49, 0.03 and 0.02-fold for genes *At3g02480, At5g24640, At1g14880* and *At4g14400*, respectively (Figure 3-4). From these results, it is apparent that all of the four genes showed the same trend both in our microarray analysis and qRT-PCR studies (Figure 3-4) although the absolute values were different with these two experimental methods.

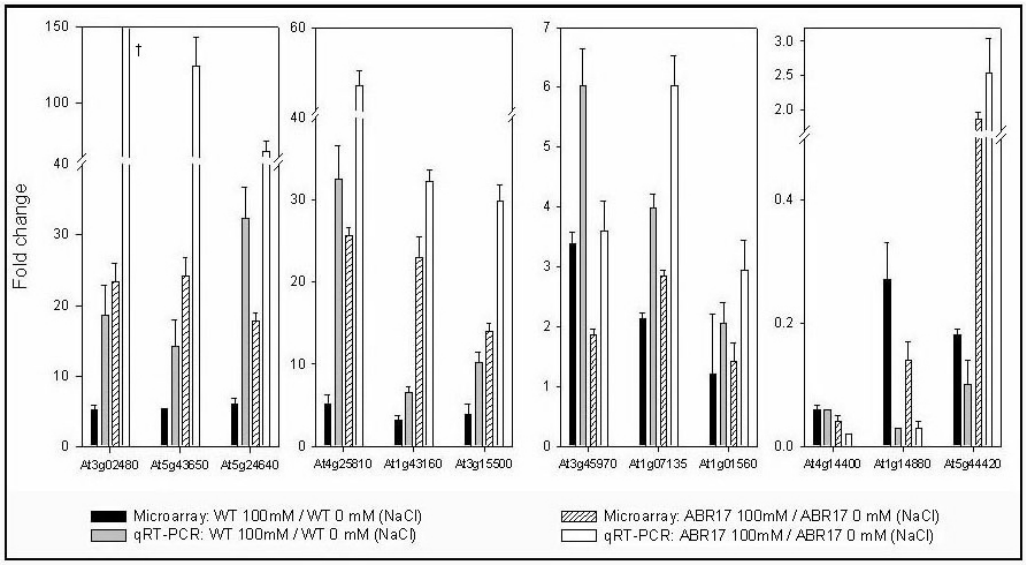

Figure 3-4 Transcript abundance of selected genes in salt treated-WT and *ABR17*- transgenic *A. thaliana* seedlings

The values represented in the graph are fold changes of transcript abundance as identified by microarrays and their validation using qRT-PCR, between salt treated (100 mM NaCl) seedlings versus untreated seedlings either in wild type or in *ABR17* transgenic *A. thaliana*. Error bars are standard error of fold changes driven from (n=3) three biological replicates. The AGI annotations are as follows**:** **At3g02480**-ABA-responsive protein-related; **At5g43650**-basic helix-loop-helix (bHLH) family protein; **At5g24640**-unknown protein; **At4g25810**-XTR6: Xyloglucan endotransglycosylase 6; **At1g43160**-ethylene-responsive transcription factor Related to Apetala 2.6 (Protein RAP2.6); **At3g15500**-ATNAC3 (*A.thaliana* NAC domain containing protein 55); **At4g14400**-ACD6 (Accelerated cell death 6); **At1g14880**-PLAC8 domain containing protein; **At5g44420**-Plant defensin protein family member PDF1.2; **At3g45970**- ATEXLA1 (*A. thaliana* expansin-like A1); **At1g07135**-Glycine rich protein; and **At1g01560**: ATMPK11 (*A. thaliana* MAP kinase 11). †: the fold change in here is 272.27±58.5.

The gene *XTR6* (*At4g25810*) was selected because it was among one of the most highly induced transcripts of any gene on our salt treated *ABR17*-transgenic *A. thalina* microarray (Table 3-3, Figure 3-4). The genes *bHLH* (*At5g43650*), *RAP2.6* (*At1g43160*) and *ATNAC3* (*At3g15500*) were chosen because their expression was the highest among any other transcription factors identified in response to salt in *ABR17*-transgenic line (Table 3-3, Figure 3-4). The genes *At4g25810*, *At5g43650*, *At1g43160* and *At3g15500* showed transcript abundance of 5.10, 5.29, 3.19 and 3.88-fold, respectively in microarray analysis of salt treated WT *A. thaliana*, while our qRT-PCR analysis of salt treated WT *A. thaliana* showed transcript abundance of 32.51, 14.17, 6.58 and 10.23- fold (Figure 3-4). Similarly, microarray analysis of salt treated *ABR17 A. thaliana* showed transcript abundance of 25.62, 24.17, 23.00 and 13.96 (Figure 3-4) and our qRT-PCR analysis values of 54.40, 124.30, 32.27 and 29.88- fold for genes *At4g25810*, *At5g43650*, *At1g43160* and *At3g15500*, respectively (Figure 3-4). Our microarray analysis and qRT-PCR results showed the similar trend in both salt treated-*ABR17* and WT samples (Figure 3-4). The genes *PDF1.2a*, *EXPL1*, *GRP*, and *MAPK11* were chosen as these were validated in our first set of microarrays (ABR17/WT under normal conditions). Once again, a similar trend was observed between microarrays and qRT-PCR analysis thus validating our microarray results.

Relative expression of CK-biosynthetic genes (IPT and CKX) in ABR17-transgenic A. thaliana

As discussed earlier, our observations indicated that many of the genes identified in the transgenic plants as being up-regulated are from families that contain CK-responsive members. We have also previously reported higher endogenous concentrations of CK in this line (Srivastava et al., 2007), which suggested the possibility that this may be due to either enhanced *de novo* CK biosynthesis or decreased degradation. Specifically, the endogenous concentration of total CK in the transgenic line used in this study was ~1-3-fold higher, with the concentration of zeatin (*cis* and *trans* combined) being ~1.4-fold and iP (isopentenyladenine) being ~2-fold higher in these transgenic lines. However, we did not detect any *IPT* (isopentenyltransferases; involved in CK biosynthesis) or *CKX* (cytokinin oxidase; involved in CK degradation) genes as being significantly up- or down-regulated genes in our microarray experiments, suggesting that the elevated endogenous CK concentrations previously reported may not be the result of increased or decreased activities of *IPT* and *CKX* genes, respectively. In order to confirm our microarray results and to lend additional support to our above-mentioned hypothesis with respect to the roles (or lack thereof) of *IPT* and *CKX* expression in *ABR17*-transgenic *Arabidopsis*, we also performed qRT-PCR analysis of the expression of *IPT* and *CKX* genes using qRT-PCR. There are 9 known *IPT* genes and 7 known *CKX* genes but the sequences of *CKX5* and *CKX7* are very similar therefore we performed qRT-PCR analysis on the 9 *IPT* and 6

CKX genes. The results from these experiments are summarized in Table 3-6 and it is apparent that most of the *IPT* genes exhibit similar expression patterns in both transgenic and WT seedlings. The only exception appears to be *IPT8,* where only 0.5-fold expression of this gene was observed in the transgenic line (Table 3-6). Similarly, *CKX* expression in the transgenic line was also quite similar to its expression in the WT (Table 3-6). Our results suggest that the differences in endogenous CK concentrations previously observed in the *ABR17*-transgenic line may not be the result of increased IPT or decreased CKX levels. However, frequently, there is no correlation between transcript abundance and protein levels and therefore it is possible that IPT and/or CKX protein concentrations may have been affected in the transgenic line resulting in increased endogenous CKs as a result of post-translational processes. However, our previously reported proteome studies on this transgenic line did not reveal any differences between transgenic and WT seedlings with respect to the levels of these proteins (Srivastava et al., 2006b). It is possible that the activity of neither IPT nor CKX is responsible for the increased endogenous concentrations of CKs in the *ABR17*-transgenic lines and the increased endogenous CKs previously reported in the *ABR17*-transgenic lines may be the result of tRNA degradation by the previously demonstrated RNase activity of pea ABR17 protein (Srivastava et al., 2007). Thus, an increase in free cellular CK would not necessarily involve enhanced IPT or reduced CKX activity; rather it may reflect an increased access to existing, yet tRNA-bound, CK.

Table 3-6 *IPT* and *CKX* gene expression in *ABR17*-transgenic *A. thaliana*

Gene	Fold change *
IPT1	1.20 ± 0.28
IPT2	1.24 ± 0.17
IPT3	1.29 ± 0.17
IPT4	1.18 ± 0.47
IPT5	1.17 ± 0.26
IPT6	0.99 ± 0.32
IPT7	1.37 ± 0.37
IPT8	0.49 ± 0.09
IPT9	1.10 ± 0.19
CKX1	1.39 ± 0.30
CKX2	1.16 ± 0.31
CKX3	1.50 ± 0.44
CKX4	0.72 ± 0.18
CKX5	0.91 ± 0.22
CKX6	0.79 ± 0.11

Foot note: *The expression of each gene in WT was normalized to 1 and fold change in transgenic line was calculated as described in Methods

3.4 Conclusions

We have demonstrated that pea *ABR17* cDNA expression modulates the level of a number of transcripts related to plant defense, growth and development, which may explain the observed phenotypic differences between WT and *ABR17*-transgenic *A. thaliana.* The gene expression of many transcription factors and defense responsive genes like Hsps and PDFs showed a different degree and kind of response between salt treated-*ABR17* transgenic and WT *A. thaliana*, which explains the observed enhanced germination and early seedling vigor in *ABR17* transgenic lines, compared to its WT counterpart. Many of the genes exhibiting a 2-fold or higher increase in transcript abundance are known CK-responsive genes providing additional evidence of a role for CKs in ABR17 function. Furthermore, a detailed expression analysis of *IPT*s and *CKX*s revealed that the levels of these transcripts were similar in both WT and transgenic seedlings, suggesting the possibility that ABR17 modulates endogenous CKs through an, as of yet, uncharacterized mechanism including the possible degradation of tRNAs which contain CK moieties (Prinsen et al., 1997).

3.5 References

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CHAPTER 4 Functional characterization of four Apetala2-family genes (*RAP2.6, RAP2.6L, DREB19* and *DREB26*) in *Arabidopsis*

4.1 Introduction

Drought and salinity are major abiotic stress factors that affect plant productivity and can reduce average yield for most crops by 50 percent or more (Bray et al., 2000; Bartels and Sunkar, 2005). Areas under drought and salinity are increasing worldwide (Burke et al., 2006) and, therefore, it is important to develop crops that can perform better when subjected to such environmental stresses. To date, many genes have been evaluated for stress tolerance and it has been shown that transcription factors (TFs) are highly effective in engineering stress tolerant plants (Sakuma et al., 2006a & 2006b; Bhatnagar-Mathur et al., 2007; Khong et al., 2008). TFs are DNA-binding proteins and more than 1500 TF genes are present in *Arabidopsis thaliana*, which constitute over 5% of its genome (Riechmann et al., 2000). TFs regulate expression of many genes and, therefore, manipulation of the expression of even a few of these regulatory genes can lead to remarkable changes in plant traits (Martin, 1996; Liu et al., 1999; Udvardi et al., 2007).

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The APETALA2 (AP2) gene family is one of the largest TF gene families of *Arabidopsis* containing 145 loci (Sakuma et al., 2002). These DNA binding proteins have a characteristic AP2 domain, which contains 68 amino acids and is also referred to as AP2/ethylene responsive element binding factor domain (AP2/ERF) (Hao et al., 1998; Riechmann and Meyerowitz, 1998). AP2/ERF genes can be grouped into two classes based on the number of AP2-DNA binding domains. The first class is AP2-like TFs, which encode proteins with two AP2 domains (Riechmann and Meyerowitz, 1998). Examples of proteins belonging to this class are AP2, AINTEGUMENTA (ANT), baby boom (BBM) and Glossy15 (GL15). The second class is ERF-like TFs which encode proteins with only one AP2 domain and includes C-repeat/dehydration responsive element binding factors (CBFs/DREBs), LePtis, ERFs, TINY, abscisic acid insensitive (ABI4), and RAV (related to ABI3/VP1) proteins (Riechmann et al., 2000; Sakuma et al., 2002). Based on their DNA-binding regions, AP2/ERF genes have been classified into five subfamilies: AP2, RAV, DREB, ERF and others (Sakuma et al., 2002). The ERF and DREB subfamily proteins regulate many stress responsive genes by binding to defined *cis*-regulatory sequence (Guo et al., 2005). The ERF subfamily proteins bind to ethylene response elements (ERE) or GCC box found in the promoters of ethylene inducible pathogenesis related genes (Ohme-Takagi and Shinshi, 1995), while the DREB subfamily proteins bind to C-repeat or dehydration response element (DRE) in the promoters of low temperature and/or water deficit responsive genes (Stockinger et al., 1997; Gilmour et al., 1998).

The AP2/ERF family proteins have been implicated in various growth events like plant growth, flower development, meristem determinancy and organ identity, as well as abiotic/biotic stress tolerance (Saleh and Pages, 2003). For instance, *AP2*, *AINTEGUMENTA*, *TINY*, *DRN*, *BD1* genes are involved in floral morphogenesis, organ identity and growth regulation (Kunst et al., 1989; Klucher et al., 1996; Wilson et al., 1996; Chuck et al., 1998; Kirch et al., 2003). The genes *DREB1A*, *DREB2A*, *WXP1*, *CaPF1*, *Pti*, *CaERFLP1* and *NtERF5* have been reported to be involved in biotic and abiotic stress tolerance (Liu et al., 1998; Gu et al., 2002; Fischer and Dröge, 2004; Yi et al., 2004; Lee et al., 2005; Zhang et al., 2007). Some of the AP2 TFs like ABI4, AtERF4, ABR1 and DDF1 are also involved in abscisic acid (ABA), ethylene (ET), gibberellic acid (GA) and brassinosteroid response signaling (Finkelstein et al., 1998; Hu et al., 2004; Magome et al., 2004; Pandey et al., 2005; Yang et al., 2005). Despite the important roles played by AP2 TFs in many aspects of plant physiology, the precise functions of many members of this family are still unknown (Nakano et al., 2006). Nevertheless, there are reports of improving the plant responses to stress through modification of the expression of AP2 TFs (Nakano et al., 2006; Sakuma et al., 2006a & 2006b).

The pea (*Pisum sativum*) abscisic acid-responsive protein ABR17 is a member of the pathogenesis related protein 10 (PR-10) family and is also referred to as PR-10.4 (Iturriaga et al., 1994; Srivastava et al., 2006). ABR17 is significantly homologous to intracellular pathogenesis related (IPR) proteins and has been demonstrated to possess ribonuclease activity (Iturriaga et al., 1994;

Srivastava et al., 2007). In addition to ribonuclease activity, members of this family have demonstrated binding properties with phytohormones like cytokinins and brassinosteroids, and therefore have been implicated in hormone signaling and suggested to function as general hormone carriers (Carpin et al., 1998; Mogensen et al., 2002; Markovic-Housley et al., 2003; Pasternak et al., 2006). An abundance of ABR17 protein has been observed in salt treated pea plants (Kav et al., 2004) and constitutive overexpression of pea *ABR17* in *Arabidopsis* and *Brassica* has resulted in phenotypes with early flowering, increased number of lateral branches and siliques and elevated levels of CKs compared to the WT (Srivastava et al., 2006 & 2007; Dunfield et al., 2007). Furthermore, plants overexpressing *ABR17* have exhibited enhanced seed germination and seedling vigor under multiple abiotic stresses including salinity stress (Srivastava et al., 2006). In our microarray analyses of ABR17-mediated modulation of gene expression (Krishnaswamy et al., 2008), we observed that transcript abundance of four putative AP2 TF genes (*RAP2.6-At1g43160*, *RAP2.6L-At5g13330*, *DREB26-At1g21910* and *DREB19-At2g38340*) was up-regulated significantly in salt treated *ABR17*-transgenic plants compared to unstressed transgenic plants, while only *RAP2.6* and *RAP2.6L* transcripts were observed to increase in abundance significantly in salt treated wild type (WT) plants compared to unstressed WT plants (Krishnaswamy et al., 2008). In addition, the transcript abundance of *RAP2.6* was significantly higher in salt treated *ABR17*-transgenic plants compared to salt treated WT plants (Krishnaswamy et al., 2008). However, there were no significant differences in expression of these AP2 genes between WT and *ABR17*-

transgenic plants under normal/unstressed conditions (Krishnaswamy et al., 2008). It was speculated that the observed enhanced stress tolerant phenotype of *ABR17*-transgenic *Arabidopsis* compared to WT (Srivastava et al., 2006) could be, at least in part, due to increased expression of AP2 TF genes together with the other important genes modulated (Krishnaswamy et al., 2008). AP2 family genes are known to play important roles in the abiotic stress response and based on transcript abundance of *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* in salt treated WT and/or *ABR17*-transgenic *Arabidopsis* plants, we hypothesize that these AP2 genes might participate in plant defense response against salt stress and therefore overexpression of these genes in *Arabidopsis* might enhance tolerance to salt and related stress conditions like drought.

RAP2.6 and *RAP2.6L* belong to the ERF subfamily, while *DREB19* and *DREB26* belong to DREB subfamily (Guo et al., 2005), and all four of them code for proteins with a single AP2 domain (Figure 4-1). *RAP2.6* is activated by the CBF (C repeat binding factor) expression (Fowler and Thomashow, 2002) and has been shown to code for protein that possess transcription activator function (He et al., 2004; Zhu et al., 2010). RAP2.6L has been demonstrated to be involved in gene regulation during shoot regeneration from root explants (Che et al., 2006) as well as in disease resistance (Sun et al., 2010). However, there is no information available on *DREB19* and *DREB26* genes. Here we report and discuss the results from functional characterization of *RAP2.6*, *RAP 2.6L*, *DREB19* and *DREB26* with respect to overexpression, localization/transactivation, spatial/temporal expression and stress/hormonal

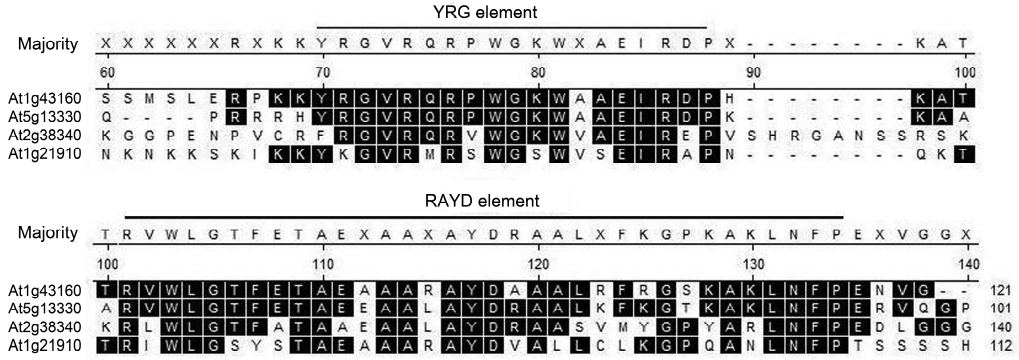

Figure 4-1 Sequence alignment of AP2 domains from AP2 TF proteins

AP2 domains from AP2 TF proteins (RAP2.6-At1g43160; RAP2.6L-At5g13330; DREB19-At2g38340; DREB26-At1g21910) showing YRG and RYD elements. Residues in black box represent conserved amino acid residues between the AP2 TF proteins. Sequence alignment was done using MegAlign (DNASTAR Lasergene8) software.

response experiments. In addition, overexpressed transgenic lines are evaluated under salt and drought stress conditions and the utility of these *AP2* genes in engineering plants for abiotic stress tolerance is discussed.

4.2 Materials and methods

Subcellular localization

RNA was extracted from *A. thaliana* (ecotype WS) using the RNeasy® Plant Mini Kit (Qiagen Sciences), reverse transcribed (iScript® cDNA synthesis kit, Bio-Rad laboratories) and the cDNA was used as a template to amplify AP2 TF genes (*DREB19* and *DREB26*) using the polymerase chain reaction (PCR; High Fidelity PCR system, Roche Diagnostics Corp.). Gene specific primers are given in Table 4-1. The AP2 genes were amplified using the following thermocycling parameters: *DREB19* (94^0C for 2 min; 35 cycles at 94^0C for 1 min, 62^0C for 1 min, 72^0C for 2 min; and a final extension of 72^0C for 7 min) and *DREB26* (94^0C for 2 min; 10 cycles at 94^0C for 30 sec, 40^0C for 30 sec, 72^0C for 45 sec; 30 cycles at 94^0C for 30 sec, 48^0C for 1 min, 72^0C for 50 sec; and a final extension of 72^0C for 5 min). Amplified PCR products were gel purified (QIAquick® gel extraction kit, Qiagen Sciences), restriction digested using *Nco*I or *Bsp*HI (New England Biolabs) and cloned into pCsGFPBT (GenBank: DQ370426). A Gly-Ala-rich peptide linker was used between coding sequence and synthetic green

Table 4-1 List of primers

Subcellular localization	
DREB26	**F:**CATGCCATGGTGAAACAAGAACGCAAGATCCAAACCAGC, **R:**TGCCATGGCAGCTCCACCTCCACCTCCATTGAAACTCCAAAGCGGAATG
DREB19	**F:**CATGCCATGGAAAAGGAAGATAACGGATCGAAACAGAGCTCC, **R:**ATGCCATGGCAGCTCCACCTCCACCTCCGAATCTGAAATACTCAAAATATGAATAGAATC
Trans-activation assay	
RAP2.6	**F:**GCGGCCGGAATTCATGGTGTCTATGCTGACTAATGTTGT, **R:**GCGGTCGGTCGACTTAACCAAAAGAGGAGTAATTGTAT
RAP2.6L	**F:**GATCTCGGAATTCATGGTCTCCGCTCTCAGCCGTGTCAT, **R:**GCGGCCGCTGCAGTTATTCTCTTGGGTAGTTATAATAA
DREB26	**F:**GCGGCCGGAATTCATGGTGAAACAAGAACGCAAGATCC, **R:**GCGGTCGGTCGACTTAATTGAAACTCCAAAGCGGAAT
DREB19	**F:**GCGGCCGGAATTCATGGAAAAGGAAGATAACGGATCG, **R:**GCGGCCGGTCGACCTAGAATCTGAAATACTCAAAATATG
Overexpression	
RAP2.6	**F:**GAGGCGCTCGAGATGGTGTCTATGCTGACTAATGTTGTCTC, **R:**GCCGGCGTCTAGATTAACCAAAAGAGGAGTAATTGTATTGATCATATTC
RAP2.6L	**F:**TAATTAGAAGCTTATGGTCTCCGCTCTCAGCCGTGTCATAG, **R:**GGCCGCGTCTAGATTATTCTCTTGGGTAGTTATAATAATTGTAAC
DREB26	**F:**GCGCCGAAGCTTATGGTGAAACAAGAACGCAAGATCC, **R:**GCGCGCGTCTAGATTAATTGAAACTCCAAAGCGGAATGTC
DREB19	**F:**GCGGCGTCTAGACTAGAATCTGAAATACTCAAAATATGAATCGAATC, **R:**GTGTCGAAGCTTATGGGACGATCACCGTGTTGTGAGAAGAAG
Actin	**F:** TGTTGCCATTCAGGCCGTTCTTTC, **R:** TGGAACCACCACTGAGAACGATGT
18srRNA	**F:** CCAGGTCCAGACATAGTAAG, **R:** GTACAAAGGGCAGGGACGTA
Promoter- GUS fusion	
RAP2.6	**F:**GCGGCCGAAGCTTGTTGTTGTCTTTTCTTCCAAGGAAG, **R:**GCGGTCGTCTAGAGTTTGAAATTGCGGTGGTAGACAAG
RAP2.6L	**F:**GTGGTCGATCGATGCAGTTTAGTACCTGACTAATCTTGCAGCTTTTA, **R:**ATATCAGGGATCCGGCGGTGACATCAGTCTCGTTCCAAGACGAATT
DREB26	**F:** GCGGCCGAAGCTTAAGAAAATTGATATCTCACAACC, **R:**GTGGTCGGGATCCGGTAATGTTGTTGTGTACGTACAGGCT
DREB19	**F:**GCGGCCGAAGCTTAGTAAATTACAAAAAAGTACAAAGTC, **R:**GCGGCCGGGATTCTGGAAAAACACAACACGTACAAACTGTAG

Table 4-1(*continued*)

qRT-PCR	
RAP2.6	**F:** GAGAGGCCAAAAAAATATAGAGGAGTAA
	R: GCCTTGTGTGGGTCTCGAA
RAP2.6L	**F:** CAAGGCCCTACTACCACCACAA
	R: GGTCGAGGAGGAGGTGAGTTC
DREB26	**F:** GGGCACCAAATCAAAAGACAA
	R: GTGCAACATCGTAAGCTCTAGCA
DREB19	**F:** GCTTGGCACGTTTGCTACTG
	R: TGGCATAGGGTCCGTACATGA
Actin	**F:** CCACCATGTTCCCAGGAATT
	R: TTTCTCTCTGGCGGTGCAA

fluorescent protein (sGFP) while generating the fusion protein (Jiang and Deyholos, 2009). Sequences of the constructs were verified by DNA sequence analysis and, along with empty vector controls (VC) transformed into *Agrobacterium tumefaciens* GV3101 using the freeze-thaw method (Weigel and Glazebrook, 2002). *Agrobacterium* strains carrying recombinant pCsGFPBT (with *DREB19* and *DREB26*) and VC were transformed into *A. thaliana* (ecotype WS) using the floral dip method (Clough and Bent, 1998). The T_0 seeds were screened on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 50mg/L hygromycin B (Sigma-Aldrich). Roots from seven-day-old T_1 plants transformed with genes of interest along with VC seedlings were stained with DAPI (4', 6-diamidino-2'-phenylindole, dihydrochloride; 0.5µg/ml) for ten minutes, washed twice with distilled water and mounted on slides. The slides were visualized under the florescence microscope (Zeiss fluorescence microscope) or confocal microscope (Leica DM IRBE, Leica Microsystems Inc.) for the sGFP and DAPI signals. At least five independent T_1 plants from each construct were used in these studies.

Trans-activation assay

The coding sequences of AP2 TF genes (*RAP2.6, RAP2.6L, DREB19* and *DREB26*) were PCR amplified (High Fidelity PCR system, Roche Diagnostics Corp.) using cDNA of *A. thaliana* (ecotype WS) as template. Gene specific primers used for the amplification are given Table 4-1. PCR thermocycling

parameters for *DREB19* and *DREB26* were as described previously. *RAP2.6* and *RAP2.6L* genes were amplified using the following thermocycling parameters: *RAP2.6* (94^0C for 2 min; 35 cycles at 94^0C for 1 min, 62^0C for 1 min, 72^0C for 2 min; and a final extension of 72^0C for 7 min) and *RAP2.6L* (94^0C for 2 min; 35 cycles at 94^0C for 30 sec, 63^0C for 1 min, 72^0C for 50 sec; and a final extension of 72^0C for 7 min). The amplified fragments were gel purified (QIAquick® gel extraction kit, Qiagen Sciences), and double digested using restriction enzymes *Eco*RI-*Sal*I (for *RAP2.6, RAP2.6L* and *DREB19*) and *Eco*RI -*Pst*I (for *DREB26*) (New England Biolabs). The digested fragments were cloned into pBD-GAL4 Cam vector (Stratagene) and their sequences were confirmed by DNA sequence analysis. The sequenced recombinant plasmids (carrying *RAP2.6, RAP2.6L, DREB19* or *DREB26* gene) and empty vector controls (VC) were transformed into yeast strain YRG-2 (Stratagene), according to the manufacturer's instructions. Positive yeast colonies were selected on synthetic drop out (SD) medium for tryptophan (Sigma-Aldrich®). The YRG-2 strain has the auxotrophic marker histidine (his3) as a reporter for detection of trans-activation activity. The positive yeast colonies, confirmed by PCR, were streaked on synthetic drop-out medium for histidine (Sigma-Aldrich®) for determining the trans-activation activity, along with the controls (yeast without vector and with empty pBD-GAL4 Cam plasmid).

Overexpression constructs

The coding sequences of AP2 TF genes (*RAP2.6, RAP2.6L, DREB19* and *DREB26*) were amplified as described previously using cDNA of *A. thaliana* (ecotype WS) as template. Gene specific primers used in the experiment are given in Table 4-1. The PCR conditions used to amplify AP2 genes were as described previously. The amplified products were gel purified (QIAquick® gel extraction kit, Qiagen Sciences) and inserted between cauliflower mosaic virus 35S (CaMV35S) promoter and rbcS32 terminator in the binary vector pKYLX-71 (Schardl et al., 1987), using restriction enzymes, *Xho*I-*Xba*I (for *RAP2.6*) and *Hind*III-*Xba*I (for *RAP2.6L*, *DREB19* and *DREB26*) (New England Biolabs).

The sequenced recombinant plasmids and empty vectors were transformed into *A. tumefaciens* GV3101 using the freeze-thaw method (Weigel and Glazebrook, 2002) and subsequently transformed into *A. thaliana* (WS) as described previously. T_0 seeds were screened for transformants on half strength MS medium (Murashige and Skoog, 1962) containing kanamycin (50mg/L), 1.5% sucrose and 0.7% agar. T_1 seeds were screened for 3:1 ratio and bulked homozygous T_2 seeds were used for further studies. To confirm the presence of transgenes, the homozgygous T_2 plants were grown for a month and the leaf tissue was used to extract RNA (RNeasy® Plant Mini Kit, Qiagen Sciences). cDNA was synthesized (iScript® cDNA synthesis kit, Bio-Rad laboratories) and used as a template for RT-PCRs. The PCRs were carried out using gene specific forward primer and vector specific reverse primer. Plant *Actin* or *18srRNA* primers were

used as internal controls. The genes *18srRNA* and *Actin* were amplified using the following thermal conditions: 94^0C for 2 min; 15 cycles at 94^0C for 1 min, 55^0C for 1 min, 72^0C for 1 min; and a final extension of 72^0C for 10 min.

Promoter activity by GUS fusion

Sequences upstream of the ATG codon from *RAP2.6* (930bp), *RAP2.6L (*999bp), *DREB19* (781bp) and *DREB26* (992bp) were amplified by PCR (High fidelity Pfu polymerase kit, Fermentas Life Sciences), using genomic DNA of wild type (WS) WT *A. thaliana* (ecotype WS) as template. Primers used to amplify upstream sequences of the AP2 genes are given in Table 4-1. The promoter sequences were amplified using the following thermocycling parameters: *RAP2.6* (94^0C for 2 min; 10 cycles at 94^0C for 30 sec, 45^0C for 30 sec, 72^0C for 45 sec; 30 cycles at 94^0C for 30 sec, 55^0C for 1 min, 72^0C for 50 sec; and a final extension of 72^0C for 5 min), *DREB26* (94^0C for 2 min; 10 cycles at 94^0C for 30 sec, 40^0C for 30 sec, 72^0C for 45 sec; 30 cycles at 94^0C for 30 sec, 60^0C for 1 min, 72^0C for 50 sec; and a final extension of 72^0C for 5 min), *RAP2.6L* and *DREB19* (94^0C for 2 min; 10 cycles at 94^0C for 30 sec, 40^0C for 30 sec, 72^0C for 45 sec; 30 cycles at 94^0C for 30 sec, 55^0C for 1 min, 72^0C for 50 sec; and a final extension of 72^0C for 5 min). The amplified fragments were gel purified (QIAquick® gel extraction kit, Qiagen Sciences), and double digested using restriction enzymes *Hind*III-*Xba*I (for *RAP2.6*), *Cla*I-*Bam*HI (for *RAP2.6L)*

and *Hind*III-*Bam*HI (for *DREB19* and *DREB26*) (New England Biolabs). The CaMV 35S promoter of the binary vector pBI121 (GenBank: AF48578) was replaced with the AP2 TF gene promoter to express β-glucuronidase (GUS) gene and the sequence of the recombinant plasmids verified. The sequenced recombinant or empty plasmids (pBI121) were transformed into *Arabidopsis* as described previously. The T_0 transgenic plants were selected on half strength MS medium containing kanamycin (50µg/ml), 1.5% sucrose and 0.7% agar. The presence of transformed promoter was confirmed in T_1 plants by PCR using genomic DNA as a template. Forward promoter specific and reverse *GUS* gene specific primers were used in PCRs.

T_1 plants were used for analyzing promoter activity, and at least 5 independent transgenic lines in each promoter construct were used in the study. Promoter activity was considered in terms of GUS activity that leads to blue color formation by reacting with the substrate X-Gluc (5-bromo-4-chloro-3-indolyl β-D-lucuronide; Sigma-Aldrich ®) (Jefferson et al., 1987). For GUS activity assays, the following samples were used: germinated seeds, 7 day old seedlings, 14 day old seedlings, rosette leaves, inflorescence, immature and mature pods. The samples were permeabilised in cold 90% acetone for 1 hr at -20^0C and washed twice for 5 minutes with 100 mM phosphate buffer (pH 7.6). The samples were incubated overnight at 37^0C in GUS staining buffer (2 mM X-Gluc, 2 mM K_4 [Fe (CN) $_6$].$3H_20$ and 2 mM K_3 [Fe (CN) $_6$]). The samples were washed with 70% ethanol and scored for dark blue staining. Small samples like seed, flower and

immature pods were photographed using dissecting microscope (Wild model M8, Wild Leitz Canada Ltd.) equipped with a digital camera (Nikon DXM1200).

Plant growth conditions

For studying spatial and temporal expression patterns of AP2 TF in *A. thaliana,* WT plants were grown in 15.24 cm pots containing Metro Mix 290 (Grace Horticultural products) in the green house (22^0C day, 18^0C night, 16h photoperiod). The plants were fertilized once every two weeks (Peters 20-20-20, Plant Products containing micronutrients). Tissue was collected at different growth stages of *Arabidopsis* (according to Boyes et al., 2001) from: seedling above ground (growth stage 1.1, 10 rosette leaves > 1 mm in length), rosette leaves and stem (growth stage 3.7, rosette is 70% of final size), early floral buds (growth stage 5.1 when plants start to bolt), inflorescence (growth stage 6.1, 10% of flowers to be produced have opened) and mature siliques (growth stage 7, filled siliques). Tissues were flash frozen in liquid nitrogen and stored at -80^0C, which was later used for quantitative real time-PCR (qRT-PCR) for studying the expression profile of AP2 TF genes.

For observing the differences in the phenotype among AP2 TF overexpression lines and controls (WT and VC), the lines were grown in 15.24 cm pots as mentioned earlier. The flowering time was recorded and the plants were photographed at different stages of growth. The experiment was repeated three times, in each biological replication with 10 plants per line. For recording

the time of flowering, at least 30 plants/line/biological replication were used. The data were analyzed using statistical analysis software (SAS) version 9.1 (SAS Institute Inc.). Significant differences ($p<0.05$) between WT and transgenic genotypes were identified by Student's *t*-test.

Hormone treatments of WT Arabidopsis

WT *Arabidopsis* plants were grown in plastic trays, in the greenhouse for three weeks as described earlier. Jasmonic acid (JA; 50μM), salicylic acid (SA; 1 mM) and abscisic acid (ABA; 50 μM) (Sigma-Aldrich®) were made in 0.1% (v/v) ethanol and applied on *Arabidopsis* plants with a hand-held spray bottle. The ethylene (ET) treatment was performed in an air-tight acrylic chamber (1.5 m × 0.6 m × 0.6 m) placed in the greenhouse, into which 100 ppm ethylene gas in air (Praxair) was passed at the rate of 2 L/min. The control treatment was performed on plants in another chamber into which air (Praxair) was passed at the same rate. Leaves and shoots were collected and pooled after 6 and 24 h post-treatment and flash frozen in liquid nitrogen, and stored at -80 °C. qRT-PCR was performed with these samples to study the response of AP2 TF genes to different stress related hormones. The entire experiment was repeated three times and there were at least 25 plants per treatment in every biological replication.

Imposition of stresses on WT Arabidopsis

The response of AP2 TF genes to different stresses was studied by imparting stress to WT *Arabidopsis* plants. For drought stress, the plants were grown for two weeks in the greenhouse as mentioned earlier. After two weeks, watering was withheld and plants were allowed to wilt (which took another 9-10 days). Control plants were well-watered till the tissues were collected. Leaf samples from wilted and well watered-plants were flash frozen in liquid nitrogen and stored at -80^{0}C. The experiment was repeated three times and there were 30 plants per treatment in each biological replication. For heat and freezing stresses, WT *Arabidopsis* seeds were seeded in Petri dishes containing half strength MS (Murashige and Skoog, 1962) medium (1.5% sucrose and 0.7% agar) and grown for two weeks (at RT and light intensity 40 µmol m^{-2} s^{-1}). Heat stress was imposed by placing the Petri dishes containing seedlings at 48^{0}C for 2 hrs. The plates were subsequently incubated at 22±1^{0}C for another 6 hrs. For inducing freezing stress, the plates were placed at -5^{0}C for 4 hrs and returned to 22±1^{0}C for 6hrs. For salt stress, WT *Arabidopsis* seeds were seeded on Petri dishes containing MS medium (1.5% sucrose, 0.7% agar and 100 mM NaCl) and grown for two weeks (RT and light intensity 40 µmol m^{-2} s^{-1}). The seedlings from salt stress, heat stress (6hrs post treatment), freezing stress (6hrs post treatment) and control treatments were flash frozen in liquid nitrogen and stored at -80^{0}C. qRT-PCR was performed to study the response of AP2 TF genes to different stresses.

Evaluation of transgenic Arabidopsis lines against salt and drought stresses

To evaluate the performance of AP2 transgenic *Arabidopsis* under different stresses, the following lines were used: wild type (WT), vector control (VC), *RAP2.6* (lines A2, A6 and A39), *RAP2.6L* (lines C23, C28 and C31) and *DREB19* (lines D1, D5 and D12). For salt stress, seeds from different lines were seeded on half strength MS (Murashige and Skoog, 1962) medium supplemented with 1.5% sucrose, 0.7% agar and NaCl (0 mM, 125 mM or 150 mM) and incubated for three weeks (at 22 ± 1^0C, light intensity of 18 µmol m^{-2} s^{-1} and 12 hr photoperiod). Germination counts were recorded every week and the plates were photographed after three weeks. The experiment was repeated three times and there were 6 plates (14 seeds per plate) per line in each biological replicate.

For inducing salt stress in the green house, 8 DAS (days after sowing) control and transgenic seedlings were watered with salt water (200 mM NaCl) on alternate days till 25 DAS. Data on a number of plants flowered and number of plants with pods were recorded. For inducing drought stress, on 8 DAS, the trays were watered to saturation and excess water was allowed to drain. After this, the plants were not watered till they wilted (which took another 15-17days) and, subsequently, re-watered. Data on number of plants wilted, number of plants recovered one day after re-watering, number of plants with flowers and number of plants with pods, was recorded. Salt and drought stressed plants were photographed along with the unstressed plants. These experiments were repeated

three times, and there were 16 plants / line / treatment in each biological replicate. The data was statistically analyzed using SAS - version 9.1 (SAS Institute Inc.). During data analysis, the percentages in each observation class (e.g. percent germination) were calculated based on the number of seedlings at the start of the experiment.

qRT-PCR

qRT-PCR was performed to study the response of AP2 TF genes to different stresses and hormones, and to determine their expression at different stages of plant growth, and also to measure their expression levels in overexpressed transgenic plants . RNA was extracted from the pooled tissue as described earlier and was treated with RNase-free-DNase (Qiagen Sciences). RNA was quantified using a NanoDrop ND-1000 (NanoDrop Technologies, Inc.), and was electrophoresed on 1.2% agarose gel in order to evaluate the integrity and reverse transcribed to synthesize cDNA, which was used as template in qRT-PCR (iScript® cDNA synthesis kit, Bio-Rad laboratories). Primers for qRT-PCR were designed using PrimerExpress3.0 (Applied Biosystems) targeting an amplicon size of 80-150 bp. Primer specificity was tested by performing BLAST analysis (http://www.ncbi.nlm.nih.gov/). Primers used in the qRT-PCR analysis are given in Table 4-1. qRT-PCR analysis was performed using the SYBR Green System (Yang et al., 2007) on ABI StepOne thermocycler (Applied Biosystems Inc.). The delta-delta method (Livak and Schmittgen, 2001) was used to calculate the

relative gene expression using either *actin*, *GAPDH* or *UBC21* as an endogenous control. Reactions were performed in triplicate using samples from each biological replicate.

4.3 Results

Gene isolation

We isolated *RAP2.6* (At1g43160), *RAP2.6L* (At5g13330), *DREB19* (At2g38340) and *DREB26* (At1g21910) genes from wild type *A. thaliana* (ecotype WS). Sequence analysis showed differences in coding sequence of *RAP2.6* and *DREB26* compared to available sequence from Columbia genotype (Accession numbers AY062847 and BT024616, respectively). The coding sequence of *RAP2.6* had three substitutions (at positions 61, 405 and 420), however, when translated changed only tryptophan 20 to arginine 20 (W20R). The coding sequence of *DREB26* had three extra bases at nucleotide position 114 and the resulting translated product had one extra amino acid (serine) at the 38th position.

Subcellular localization

Based on consensus sequence analysis, RAP2.6, RAP2.6L, DREB19 and DREB26 proteins were deduced to contain a single DNA binding AP2 domain (Figure 4-1) and were therefore expected to act as TFs and therefore should localize in the nucleus. Nuclear localization of RAP2.6 and RAP2.6L has been

reported (Che et al., 2006; Zhu et al., 2010). In order to confirm the presumed nuclear localization of *DREB19* and *DREB26*, the coding regions were translationally fused to the *N*-terminus of synthetic green fluorescent protein (sGFP) under the control of cauliflower mosaic virus (CaMV) *35S* promoter, and expressed in *Arabidopsis*. Localization was determined by visualizing root samples employing fluorescence confocal microscopy. As shown in Figure 4-2, sGFP was uniformly distributed throughout the cell in control, whereas AP2: sGFP fusion proteins (sGFP: DREB19 and sGFP: DREB26) were detected exclusively in the nucleus, suggesting that these proteins are indeed constitutively nuclear localized. In addition, DAPI and GFP were co-localized in sGFP: DREB19 and sGFP: DREB26 *Arabiopsis* roots (Figure 4-3) confirming their nuclear localization.

Transactivation assay

AP2 TF proteins can function either as transcriptional activators or as repressors based on the presence of a conserved EAR (ethylene-responsive element-binding factors-associated amphillic repression) motif (Stockinger et al., 1997; Fujimoto et al., 2000; Ohta et al., 2001). The AP2 genes *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* lack an EAR motif and therefore were expected to act as transcriptional activators. To verify this, a transactivation assay was performed using the yeast one hybrid system. The full-length coding region of AP2 genes

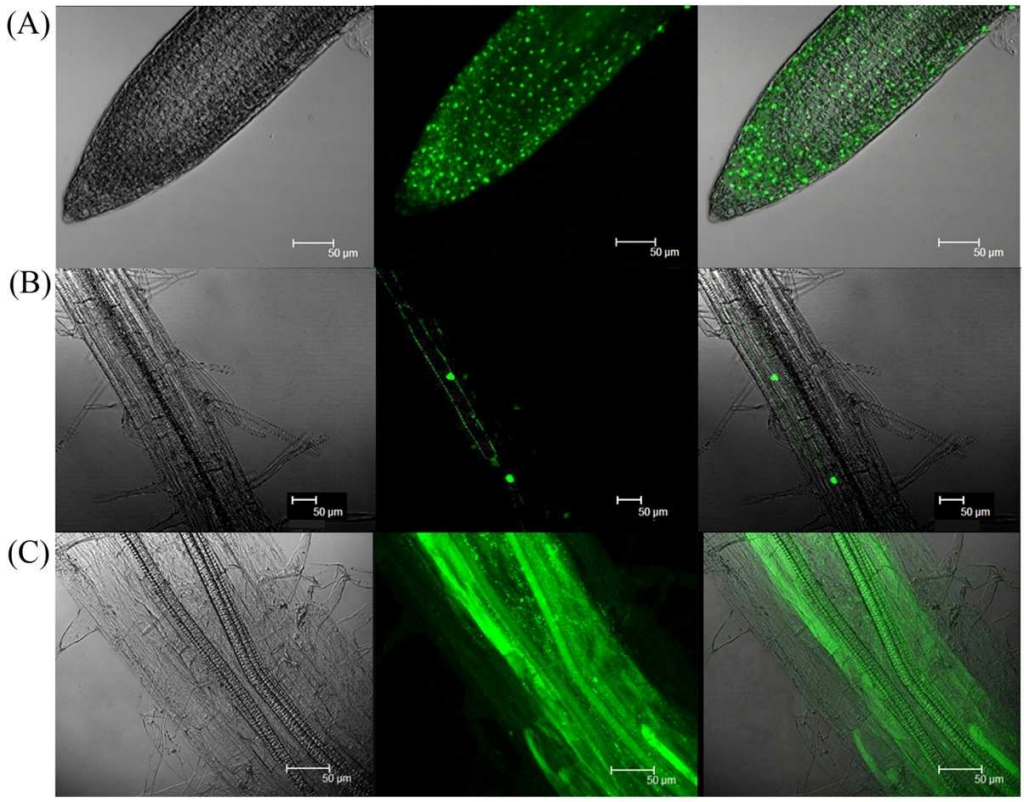

Figure 4-2 Roots from one week old (T_2) transgenic *Arabidopsis* plants showing nuclear localization of AP2 TFs

a DREB19, **b** DREB26 and **c** control pCsGFPBT under confocal microscope. Left panel is bright field, the middle panel is GFP florescence, and the right one is overlay of the two images.

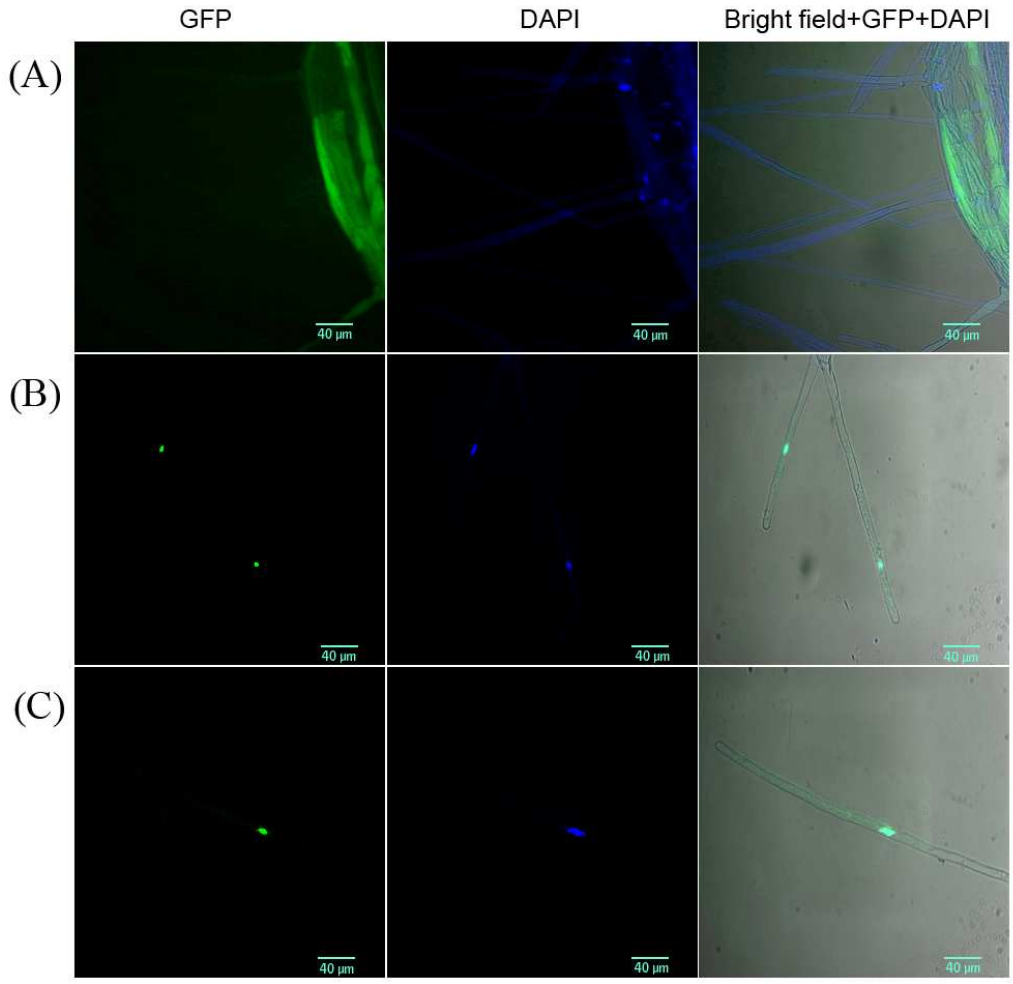

Figure 4-3 Nuclear localization of AP2 TFs (with DAPI stain)

Roots from one week old (T_2) transgenic *Arabidopsis* plants showing nuclear localization of **a** control pCsGFPBT, **b** DREB19 and **c** DREB26 under confocal microscope. Left panel is GFP florescence, the middle panel is DAPI florescence, and the right one is overlay of bright field, GFP and DAPI.

(*RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26*) were fused with GAL4 binding domain using pBD-Gal4 Cam plasmid and tested for the expression of *HIS3* reporter gene in yeast (Figure 4-4). Yeast cells carrying pBD-Gal4 Cam -AP2 TF genes activated the expression of the downstream *HIS3* reporter gene, enabling them to grow on synthetic drop-out/-histidine medium (Figure 4-4A). Yeast cells with or without empty pBD-Gal4 Cam plasmid did not grow on synthetic drop-out/-histidine medium (Figure 4-4A). These results suggest that *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* genes indeed code for transcriptional activators.

Spatial and temporal expression pattern

AP2/ERF family proteins have been reported/shown to play a key role in plant growth and development (Saleh and Pages, 2003). In order to explore the possibility of the involvement of *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* in growth processes, their expression patterns were investigated employing qRT-PCR and GUS reporter fusion system. For qRT-PCR, tissue was collected from the following growth stages (Boyes et al., 2001) of *Arabidopsis*: seedling above ground (stage 1.1, 10 rosette leaves > 1 mm in length), rosette leaves and stem (stage 3.7, rosette is 70% of final size), early floral buds (stage 5.1 when plants start to bolt), inflorescence (stage 6.1, 10% of flowers to be produced have opened) and mature siliques (stage 7, filled siliques). qRT-PCR was used to examine the transcript abundance of AP2 genes in different tissues compared to their levels in rosette leaves and stems (stage 3.7). *RAP2.6* mRNA was more

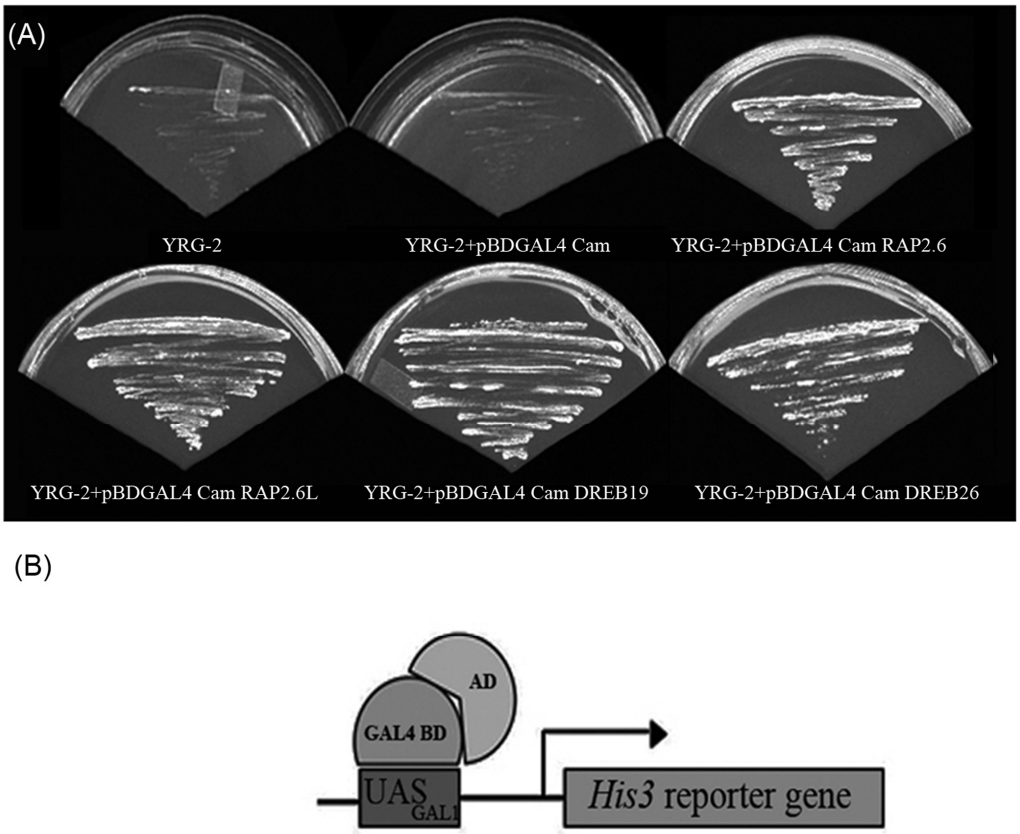

Figure 4-4 Transactivation assay of AP2 TFs

a Transactivation assay with AP2 genes and **b** a schematic representation of yeast-one- hybrid system with *HIS3* reporter. Controls: YRG-2 and YRG-2 with pBD-GAL4-Cam. Growth of the transformants on SD/-his medium indicates that the corresponding gene encodes protein with transactivation activity. The genes *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* code for transactivators.

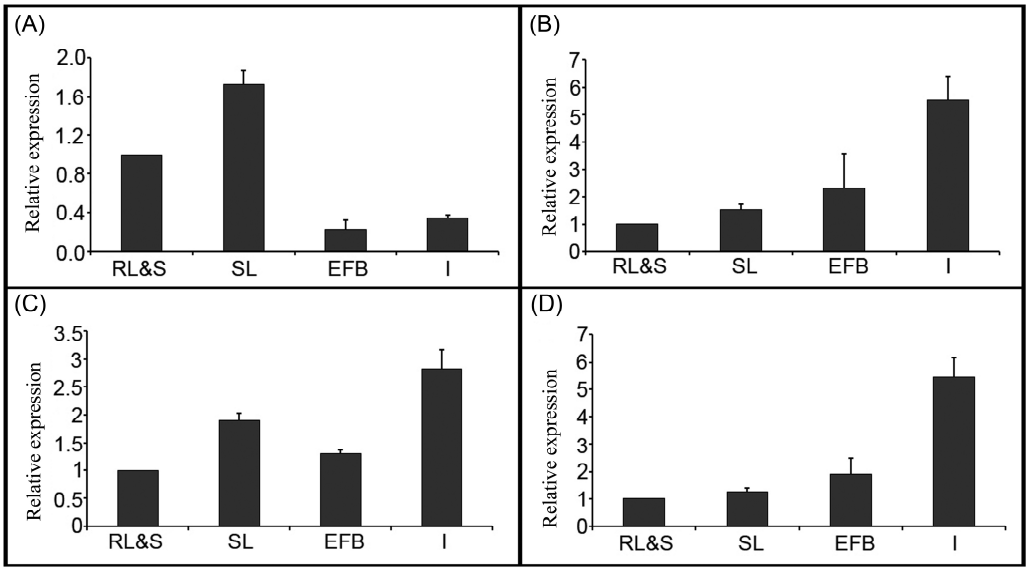

Figure 4-5 Spatial and temporal expression pattern of AP2 TF genes

a *RAP2.6*, **b** *RAP2.6L*, **c** *DREB19*, and **d** *DREB26* at different stages of growth: RL & S (rosette leaves and stem from growth stage 3.7), SL (seedling, growth stage 1.1), EFB (early floral buds) and I (inflorescence). qRT-PCR analysis was performed to compare the transcript abundance of AP2 TF genes in different tissues relative to RL & S.

abundant in seedling as compared to rosette leaves and stem, early floral buds and inflorescence (Figure 4-5A), while transcripts of *RAP2.6L* were more abundant in inflorescence and early floral buds as compared to rosette leaves and seedlings (Figure 4-5B). *DREB19* mRNA was more abundant in seedlings (Figure 4-5C), and *DREB26* mRNA was more abundant in inflorescence as compared to other tissues (Figure 4-5D). Transcript abundance of all the studied AP2 genes decreased from the seedling stage to rosette leaf stage, but subsequently increased during flowering, except for *RAP2.6*, whose transcripts were more abundant in seedlings than in any other tissues sampled (Figure 4-5). None of the examined AP2 transcripts were detected in mature siliques (data not shown).

In order to investigate the tissue specific expression pattern of AP2 genes, germinated seeds, 7 day old seedlings, 14 day old seedlings, flowers and siliques of T_1 *Arabidopsis* containing AP2 TF promoters and β-glucuronidase (GUS) reporter gene fusion constructs (pRAP2.6-GUS, pRAP2.6L-GUS, pDREB19-GUS, pDREB26-GUS and control pCaMV35S-GUS) were tested for GUS activity. As shown in Figure 4-6, strong levels of GUS expression was detected in germinated seeds, seedlings, flowers and siliques of control *Arabidopsis* plants (pCaMV35S-GUS). In plants bearing pRAP2.6-GUS fusions, the GUS expression were detected in roots of 7 day old seedlings, in petals and carpels and in the valves of immature silique (Figure 4-6). The GUS gene expression was detected in anthers, specifically in pollens of plants with pRAP2.6L-GUS fusion construct (Figure 4-6). GUS expression was detected in the tip of the cotyledonary leaves in germinated seeds and in a region where leaves emerge

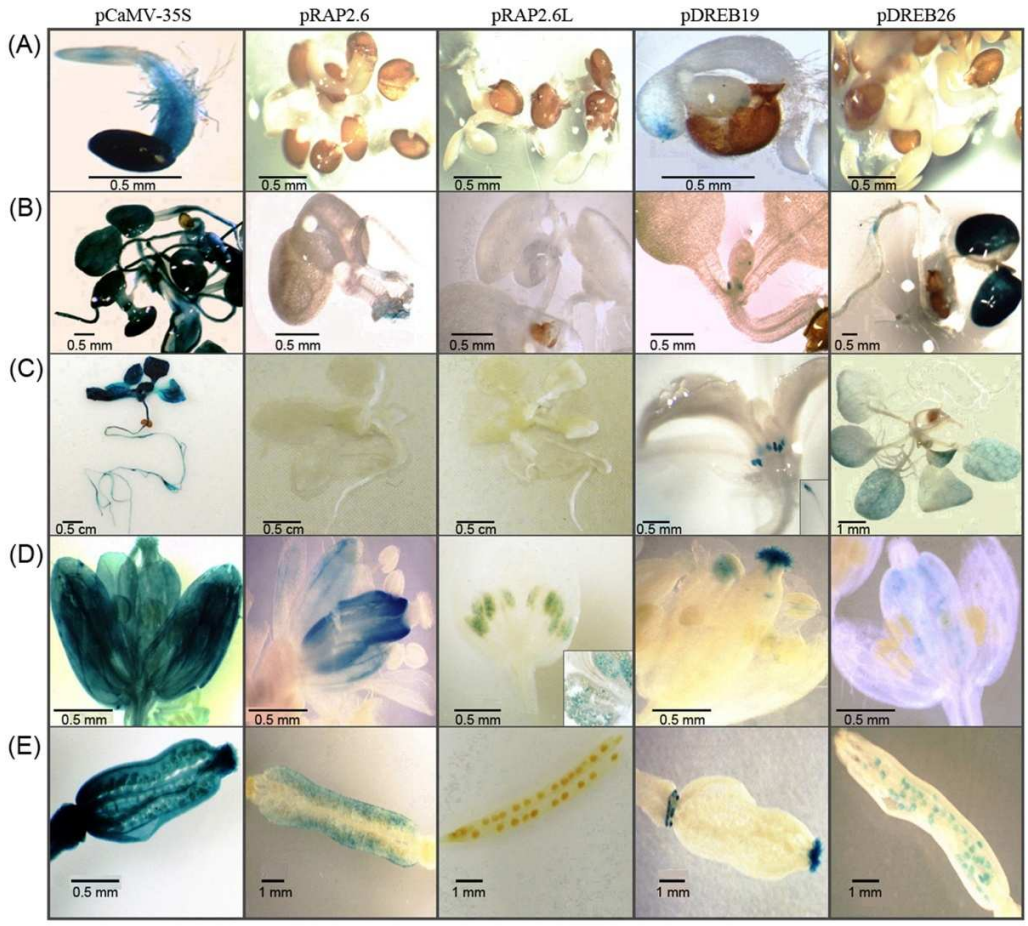

Figure 4-6 Promoter activity of AP2 TF genes as determined by GUS reporter expression

Panels **a-e** represent **a** germinated seeds, **b** 7 days old seedlings, **c** 14 days old seedling, **d** flower, and **e** immature silique of plants containing GUS transgene with different AP2 promoters (CaMV-35S, RAP2.6, RAP2.6L, DREB19 and DREB26). These obersvations were made in atleast 5 independent transgenic lines in each construct.

from shoots in 7 day old seedlings and 14 days old seedlings of plants carrying pDREB19-GUS reporter gene fusion (Figure 4-6). In addition, the GUS expression was detected in xylem tissues and also in stigma, anther and in the region where sepals and petals attach the peduncle in pDREB19-GUS *Arabidopsis* plants (Figure 4-6). A strong expression of GUS was detected in cotyledonary leaves of 7 days old seedlings, ovules and seeds in immature siliques (Figure 4-6) of plants carrying pDREB26-GUS. In addition, a weak level of GUS expression was also detected in 14 days old seedlings containing pDREB26-GUS (Figure 4-6). All the studied AP2 genes (*RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26*) were found to be expressed in *Arabidopsis* flowers, with very specific expression patterns as detected by promoter-GUS fusions (Figure 4-6).

Response to different stresses and stress hormones

In addition to their involvement in plant growth and development, AP2 TF genes have been implicated in biotic and abiotic stress response (Saleh and Pages, 2003; Nakano et al., 2006). We used qRT-PCR to investigate the responses of AP2 genes (*RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26*) in *Arabidopsis* seedlings to different abiotic stresses like salt (100 mM NaCl), heat (48^0C), freezing (-5^0C) and drought (Figure 4-7, left panel). The transcript abundance of AP2 genes in stressed seedlings was compared to their controls. We observed that the tested

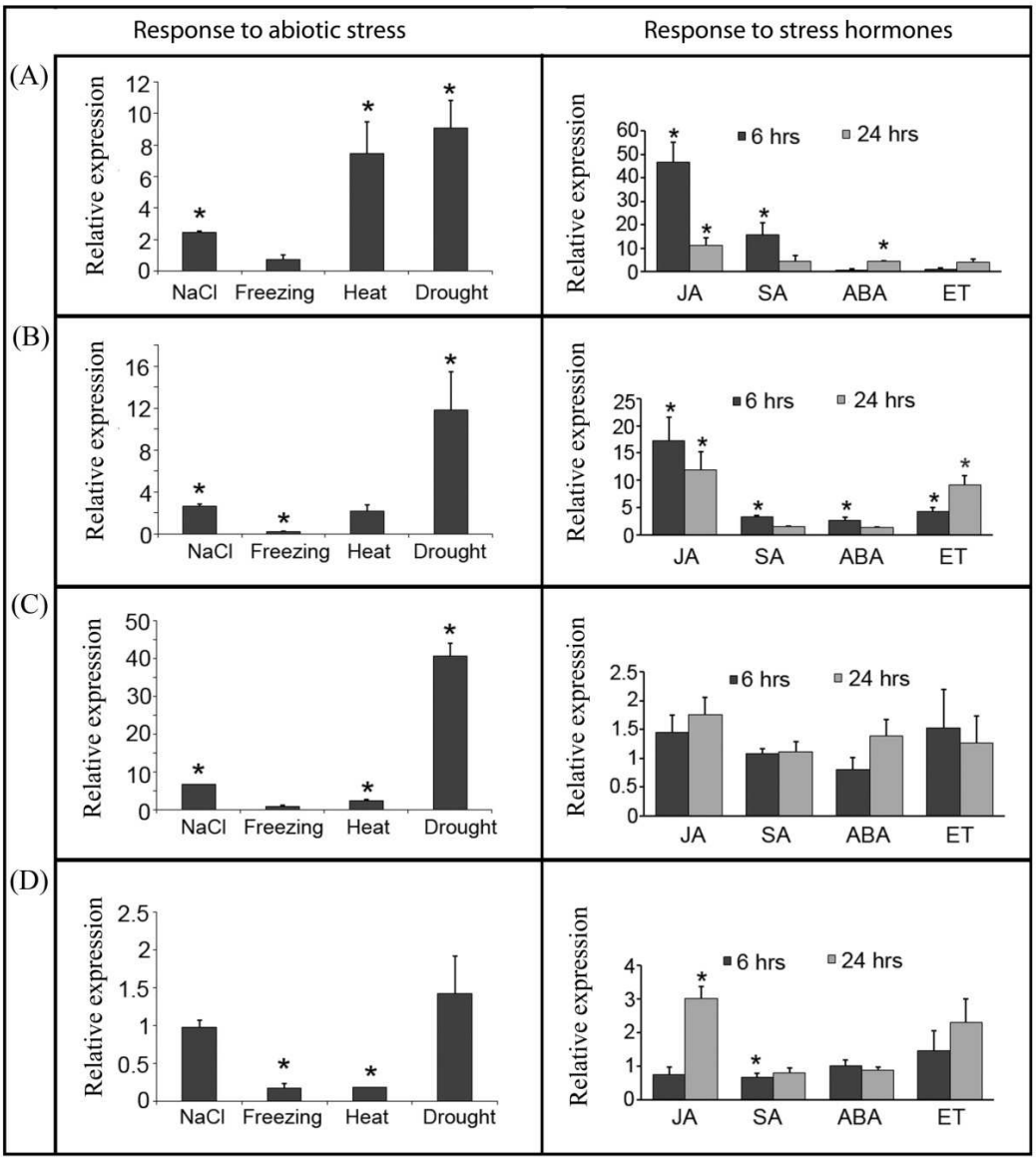

Figure 4-7 Response of AP2 genes to stress and stress hormones

Figure 4-7 details…

qRT-PCR analysis of **a** *RAP2.6*, **b** *RAP2.6L*, **c** *DREB19*, and **d** *DREB26* transcript abundance in 14 day-old *Arabidopsis* seedlings exposed to various (left panel) stresses and (right panel) stress hormones showing transcript abundance in treated plants relative to control. The gene expression levels in control samples have been normalized to 1. Asterisks indicate a statistically significant difference ($P<0.05$) in transcript abundance compared to control. Mean values are from 3 biological replicates, error bars represent the standard error of mean (SEM).

AP2 transcripts exhibited expression modulation following exposure to NaCl, heat, freezing and drought, although the level of the response differed between stresses (Figure 4-7). *RAP2.6* transcripts significantly increased following exposure to NaCl, heat and drought, but were unaffected by freezing (Figure 4-7A). Transcript abundance of *RAP2.6L* was unaffected by heat stress but significantly increased during NaCl and drought stress, and significantly decreased following freezing stress (Figure 4-7B). *DREB19* transcripts were significantly increased in abundance on exposure to NaCl, heat and drought, but did not change in abundance after freezing stress (Figure 4-7C). The transcript abundance of *DREB26* was not altered as a result of exposure to NaCl or drought, although their abundance was significantly decreased following exposure to heat and freezing stress (Figure 4-7D). All the tested AP2 genes except *DREB26* exhibited an increase in transcript abundance on exposure to NaCl and drought (Figure 4-7). Our results suggest an important role for these TFs in mediating plant responses to abiotic stresses.

In addition to different stresses, we also investigated the response of AP2 genes (*RAP2.6, RAP2.6L*, *DREB19* and *DREB26*) in *Arabidopsis* upon exposure to stress hormones SA, JA, ABA and ET (Figure 4-7, right panel). qRT-PCR was used to compare the transcript abundance of AP2 genes in plants exposed to a variety of hormones to that of mock treated control plants at 6 hrs and 24 hrs after exposure. *RAP2.6* transcripts were significantly increased in abundance at 6 hrs after exposure to both JA and SA, and although decreased at 24 hrs of exposure, they were still significantly high in JA treated tissue (10 times higher than

control) (Figure 4-7A). Transcript abundance of *RAP2.6* was not altered at 6 hrs after exposure to ABA, however it significantly increased at 24hrs after exposure to ABA (Figure 4-7A). *RAP2.6* did exhibit alterations in transcript abundance in response to ET (Figure 4-7A). The transcript abundance of *RAP2.6 L* was significantly high at 6 hrs after exposure to SA, JA, ABA and ET but decreased to normal level by 24hrs post-exposure in SA, ABA, but in case of JA treatment, the transcript levels, although decreased were still 10 times higher than control (Figure 4-7B). In the case of ET treated tissue, the transcript abundance of *RAP2.6L* increased from 6hrs to 24 hrs after exposure (Figure 4-7B). *DREB19* did not exhibit statistically significant ($P<0.05$) alteration of transcript abundance in response to any of the tested hormones (Figure 4-7C), while *DREB26* showed a moderate increase in transcript abundance at 24 hrs after exposure to JA, and 6hrs after exposure to SA (Figure 4-7D). Thus, *RAP2.6* and *RAP2.6L* were most responsive to different stress hormones compared to *DREB19* and *DREB26* (Figure 4-7).

Overexpression of RAP2.6, RAP2.6L, DREB19 and DREB26

The AP2 TF genes (*RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26)* were characterized by separately overexpressing each gene under the control of CaMV35S promoter in *A. thaliana* (WS). In the case of *DREB26*, we obtained 12 independent T_0 transgenic lines, of which only 4 lines set seeds (T_1) and very few at that. The presence of the transgene was confirmed using RT-PCR in all transgenic lines

(Figure 4-8), and the *DREB26* expression levels were measured in eight T_0 *DREB26* overexpressed *Arabidopsis* plants using qRT-PCR. The transcript abundance of *DREB26* was significantly higher (ranged from 20 to 120 fold higher) in transgenic plants compared to WT and VC (Table 4-2). *DREB26* transgenic plants (T_0) exhibited abnormal morphology with tiny leaves, few or no secondary branches and deformed flowers (Figure 4-9A). In addition, the T_1 *DREB26* plants died early in the vegetative stage, and therefore, we were unable to characterize them any further.

In the case of *RAP2.6*, *RAP2.6L* and *DREB19*, we selected three independent transgenic lines in each gene based on initial screening for phenotype and confirmed the presence of transgene using RT-PCR in two week old homozygous T_2 plants (Figure 4-8). In addition, the expression levels of *RAP2.6*, *RAP2.6L* and *DREB19* genes were quantified using qRT-PCR in transgenic *RAP2.6*, *RAP2.6L* and *DREB19 Arabidopsis* lines, respectively (Table 4-3). The expression of *RAP2.6* was higher (ranged from 31019.10 ± 14694.86 to 78003.17 ± 26651.89 in three lines) in *RAP2.6* overexpressed lines (A2, A6 and A39) compared to the controls (Table 4-3). Similarly, the transcript abundance of *RAP2.6L* was higher (ranged from 8907.49 ± 512.38 to 16631.41 ± 896.90 in three lines) in *RAP2.6L* overexpressed lines (C23, C28 and C31) compared to the controls (Table 4-3). The expression levels of *DREB19* were higher (ranged from 134.83 ± 24.28 to 967.47 ± 235.16 in three lines) in *DREB19* overexpressed *Arabidopsis* lines (D1, D5 and D12) compared to the controls (Table 4-3). These results (Table 4-3) demonstrate that AP2 genes are indeed getting overexpressed

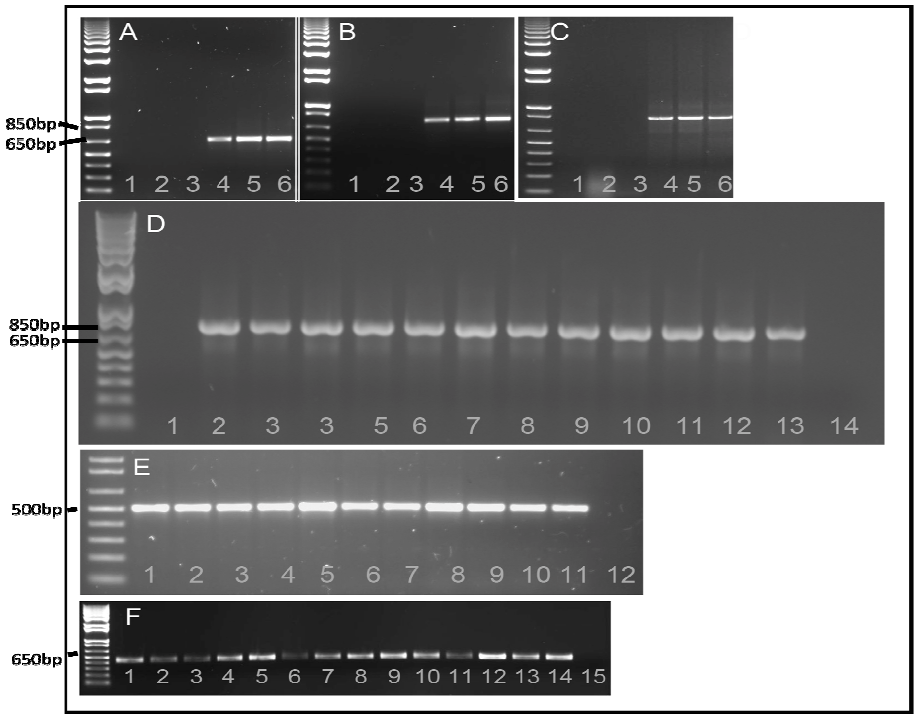

Figure 4-8 RT-PCR showing the amplification of AP2 genes in transgenic plants

a (RAP2.6): 1 to 6 refers to -ve C, WT, VC, A2, A6 and A39

b (RAP2.6L): 1 to 6 refers to -ve C, WT, VC, C23, C28 and C31

c (DREB19): 1 to 6 refers to -ve C, WT, VC, D1, D5 and D12

d (DREB26): 1 WT, 2-13 DREB26 lines and 14 –ve C

e (Actin): 1 to 12 refers to WT, VC, A2, A6, A39, C23, C28, C31, D1, D5, D12 and –ve C

f (18sRNA) 1 WT, 2-14 DREB26 lines and 15 –ve C

mRNA: *RAP2.6*-578bp, *RAP2.6L*-639bp, *DREB26*-693bp & *DREB19*-734bp. The amplicon sizes are little above mRNA size as forward gene specific primer and reverse vector specific primer have been used for the amplification. *18sRNA* & *actin* amplication is for testing cDNA quality. WT: wild type, VC: vector control, -ve C: negative control. *RAP2.6* lines are A2, A6 & A39. *RAP2.6L* lines are C23, C28 & C31. *DREB19* lines are D1, D5 & D12.

Table 4-2 Transcript abundance of DREB26 in *DREB26*-overexpressed, wild type (WT), vector control (VC) genotypes as detected by qRT-PCR

Genotypes	**Fold change** [a]
DREB26-1	55.71
DREB26-2	18.15
DREB26-3	110.77
DREB26-4	28.02
DREB26-5	97.03
DREB26-6	52.69
DREB26-7	38.61
DREB26-8	74.55
VC	2.55
WT	1

Foot notes: [a] Expression levels of *DREB26* in different T_0 *DREB26*-transgenic lines were calculated relative to WT, and expression levels in WT was normalized to 1.

in the respective transgenic plants and their transcript abundance is significantly higher compared to the WT and VC.

The differences in phenotype between WT and homozygous T_2 transgenic (*RAP2.6*, *RAP2.6L* and *DREB19*) *Arabidopsis* plants was studied in the greenhouse. Representative pictures of adult plants and siliques of control and *RAP2.6*, *RAP2.6L* and *DREB19* transgenic *Arabidopsis* plants are shown in Figure 4-9. *RAP2.6L* and *DREB19* did not show any phenotypic differences compared to controls except for flowering time, while *RAP2.6* showed observable phenotypic difference compared to controls (WT and VC) (Figure 4-9, Table 4-3). *RAP2.6* transgenic plants were dwarf with many secondary branches and shorter siliques, compared to their controls (Fig. 7). However, no differences were found between *RAP2.6* transgenic plants and the controls in terms of germination, growth and morphology up to bolting stage. Apart from this, significant differences were observed in flowering time between *RAP2.6* transgenic lines and controls (Table 4-2). *RAP2.6* transgenic lines (A2, A6 and A39) flowered 2-3 days earlier than the controls (Table 4-4). A significant difference in flowering time was also observed in *RAP2.6L* and *DREB19* transgenic *Arabidopsis* lines compared to controls (WT and VC) (Table 4-4). *RAP2.6L* transgenic lines (C23, C28 and C31) flowered 3-4 days earlier and *DREB19* transgenic lines (D1, D5 and D12) flowered nearly 3 days earlier than controls (Table 4-4). In summary, overexpression of *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* altered the

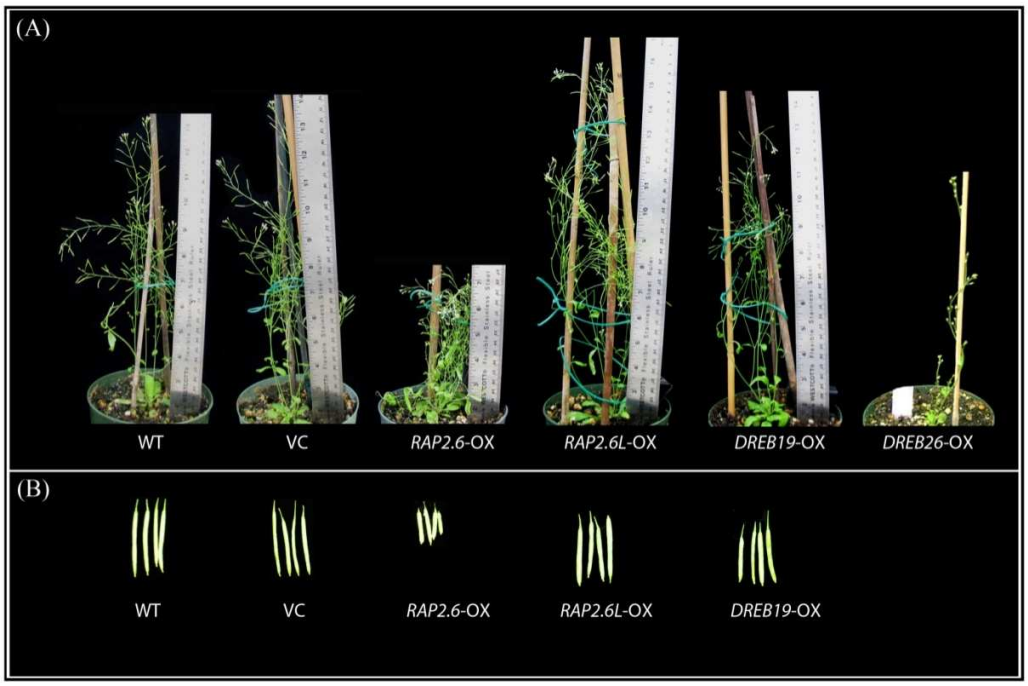

Figure 4-9 Phenotype of representative of AP2 genes overexpressed *Arabidopsis*

a adult plants, and **b** siliques of control (WT and VC) and AP2 TFs overexpressed genotypes

Table 4-3 Transcript abundance of AP2 genes in AP2 TFs overexpressed, wild type (WT), vector control (VC) genotypes as detected by qRT-PCR

Gene	Genotypes	Line	Fold change [a]	Standard error
RAP2.6	*RAP2.6*-OX	A2	78003.17	26651.89
	RAP2.6-OX	A6	31019.10	14694.86
	RAP2.6-OX	A39	44975.16	18392.29
	VC	-	1.14	0.30
	WT		1	-
RAP2.6L	*RAP2.6L*-OX	C23	8907.49	512.38
	RAP2.6L-OX	C28	16631.41	896.90
	RAP2.6L-OX	C31	15977.00	1512.19
	VC	-	1.55	0.79
	WT	-	1	-
DREB19	*DREB19*-OX	D1	967.47	235.16
	DREB19-OX	D5	383.61	94.71
	DREB19-OX	D12	134.83	24.28
	VC	-	1.19	0.57
	WT	-	1	-

Foot notes: [a] Expression levels of AP2 genes in different genotypes were calculated relative to WT, and expression levels in WT were normalized to 1, and mean values are from three biological replicates.

Table 4-4 Days required for floral initiation in wild type (WT), vectors control (VC) and AP2 TFs overexpressed *Arabidopsis* genotypes under normal conditions

Genotypes	Line	Number of observations	Average number of days required for floral initiation [a]	SEM [b]
RAP2.6-OX	A2	65	23.74*	0.32
	A6	65	22.52*	0.23
	A39	63	22.25*	0.17
RAP2.6L-OX	C23	63	22.57*	0.29
	C28	65	21.94*	0.21
	C31	65	22.69*	0.31
DREB19-OX	D1	65	23.38*	0.25
	D5	65	23.12*	0.21
	D12	65	22.92*	0.27
WT	-	85	26.00	0.20
VC	-	78	26.41	0.35

Foot notes:

* indicates a significant difference ($P<0.05$) when compared to WT

[a] Mean values from three biological replicates

[b] SEM standard error of mean

phenotypes in terms of growth and appearance and/or flowering time in *Arabidopsis*.

Evaluation of RAP2.6, RAP2.6L and DREB19 transgenic plants for abiotic stress tolerance

Transgenic *RAP2.6* lines (A2, A6 and A39), *RAP2.6L* lines (C23, C28 and C31) and *DREB19* lines (D1, D5 and D12) were evaluated for abiotic stress tolerance (Figure 4-10 to 4-12). Salt tolerance in the early vegetative stage was studied by plating transgenic and control (WT and VC) seeds on MS medium containing 0 mM NaCl, 125 mM or 150 mM NaCl (Figure 4-10). Without stress, three week old *RAP2.6*, *RAP2.6L* and *DREB19 Arabidopsis* plants appeared developmentally advanced in terms of growth and floral bud initiation compared to WT and VC seedlings (Fig. 8a). All *RAP2.6*, *RAP2.6L* and *DREB19* transgenic lines appeared developmentally more advanced than the control (WT and VC) seedlings even at 125 mM NaCl stress (Figure 4-10A). The AP2 transgenic plants had greater shoot and root mass and also the true leaves appeared earlier in transgenic plants compared to the control (Figure 4-10A). In addition, at 150 mM NaCl stress, *RAP2.6L* transgenic lines were developmentally advanced compared to WT (Figure 4-10A). Although there were no differences in germination rate between any of the transgenic lines and controls without stress, significant differences were observed in percent germination of *RAP2.6L* (lines C23, C28 and C31) and *RAP2.6* (lines A6 and A39) transgenic plants compared to WT,

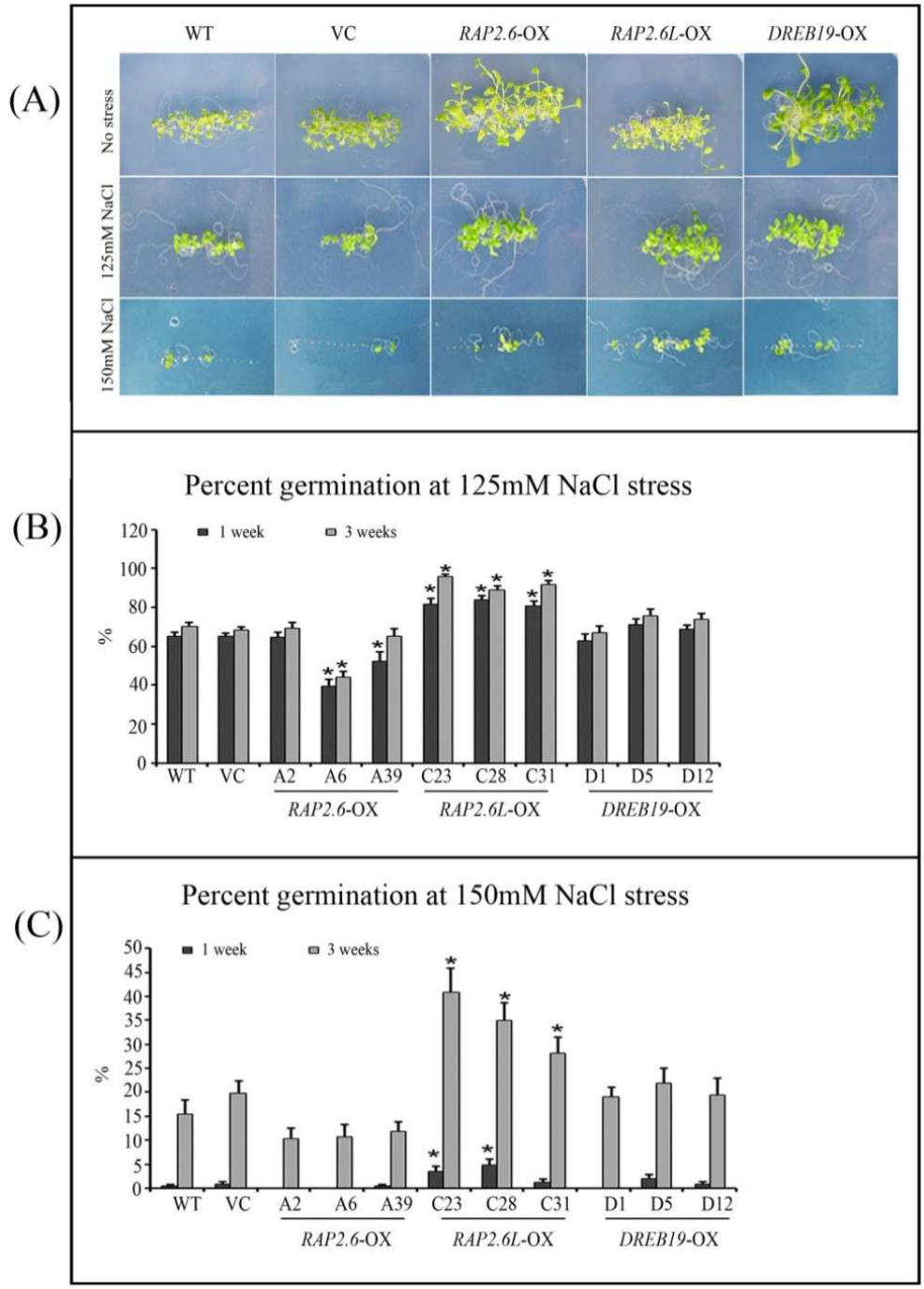

Figure 4-10 Salinity stress screening of AP2 genes overexpressed *Arabidopsis*

Figure 4-10 details…

a Photographic representation of representative AP2 TF overexpressing *Arabidopsis* plants along with wild type (WT) and vector control (VC) on MS medium containing NaCl (0 mM, 125 mM or 150 mM), and percent germination of AP2 TF overexpressing *Arabidopsis* lines and controls, on MS medium with **b** 125 mM NaCl, and **c** 150 mM NaCl. Asterisks indicate a significant difference ($P<0.05$) when compared to WT. Mean values from three biological replicates are shown. Error bars = standard error of mean (SEM) and N=252.

after 1 week on 125 mM NaCl medium (Figure 4-10B). Percent germination was higher in *RAP2.6L* transgenic lines (ranged from 80% to 84% in three lines) and lower in *RAP2.6* transgenic lines (ranged from 40% to 50% in three lines) compared to WT (65%; Figure 4-8B). The germination percent was significantly higher in all three *RAP2.6L* transgenic lines (ranged from 88% to 95% in three lines) compared to WT (70%) even after 3 weeks on medium containing 125 mM NaCl (Figure 4-10B). Percent germination was significantly lower (44%) in one of the *RAP2.6* transgenic lines (A6) compared to WT after 3 weeks on 125 mM NaCl medium (Figure 4-10B). Other than this, no significant differences were observed between WT and VC or other tested AP2 transgenic lines for percent germination in 125 mM NaCl stress (Figure 4-10B). Although the percent germination was less in at least two *RAP2.6* transgenic lines (A6 and A39) at 125 mM NaCl stress, the seedlings appeared to be developmentally advanced in terms of shoot and root growth compared to controls (Figure 4-10A). The percent germination was much less after 1 week of seeding on medium containing 150 mM NaCl in all of the tested *Arabidopsis* lines (Figure 4-10C). However, nearly 5% germination was observed in at least two *RAP2.6L* transgenic lines (C23 and C28) and the difference was significant when compared with WT (Figure 4-10C). At 150 mM NaCl stress, three weeks after plating (Figure 4-10C), *RAP2.6L* transgenic lines had significantly higher percent germination (ranged from 30% to 40% in three lines) compared to WT (15%). There were no significant differences in percent germination or differences in appearance between WT and VC or other transgenic lines (*RAP2.6* and *DREB19* lines) at 150 mM NaCl stress.

The AP2 transgenic lines (*RAP2.6*, *RAP2.6L* and *DREB19*) were also evaluated for salt stress tolerance in the greenhouse, by watering on alternate days with salt water (200 mM NaCl) from 8 days after seeding (DAS) to 25 DAS. Many seedlings died and growth was retarded in NaCl treated plants (Figure 4-11A). However, there were no significant differences between any of the transgenic lines and WT in terms of percent death (Figure 4-11A). Nevertheless, significantly higher percent flowering and percent pod set was observed in AP2 transgenic lines compared to WT under salt stress (Figure 4-11A). *RAP2.6L* transgenic lines had the highest percent flowering (ranged from 80% to 90% in three lines) followed by *DREB19* lines (ranged from 40% to 60% in three lines) and *RAP2.6* lines (ranged from 30% to 50% in three lines). *RAP2.6L* transgenic lines had the highest percent pod set (ranged from 50% to 80% in three lines) followed by *DREB19* transgenic lines (ranged from 25% to 37% in three lines) and *RAP2.6* transgenic lines (ranged from 20% to 27% in three lines). In contrast, the control genotypes (WT and VC) set very few or no pods (Figure 4-11A).

The transgenic plants (*RAP2.6*, *RAP2.6L* and *DREB19*) were also evaluated for drought tolerance by imposing drought stress in the greenhouse. As shown in Figure 4-11B, more than 85% of the plants wilted in WT, VC, *RAP2.6* (lines D1, D5 and D12) and *RAP2.6L* (lines C23, C28 and C31) transgenic lines while only 60-65% wilted in the *DREB19* transgenic lines (D1, D5 and D12). However, all of the tested genotypes recovered (> 85%) within a day when re-watered except for two of the *RAP2.6* transgenic lines (A2 and A39), which had significantly less percent recovery (Figure 4-11B). There were significant

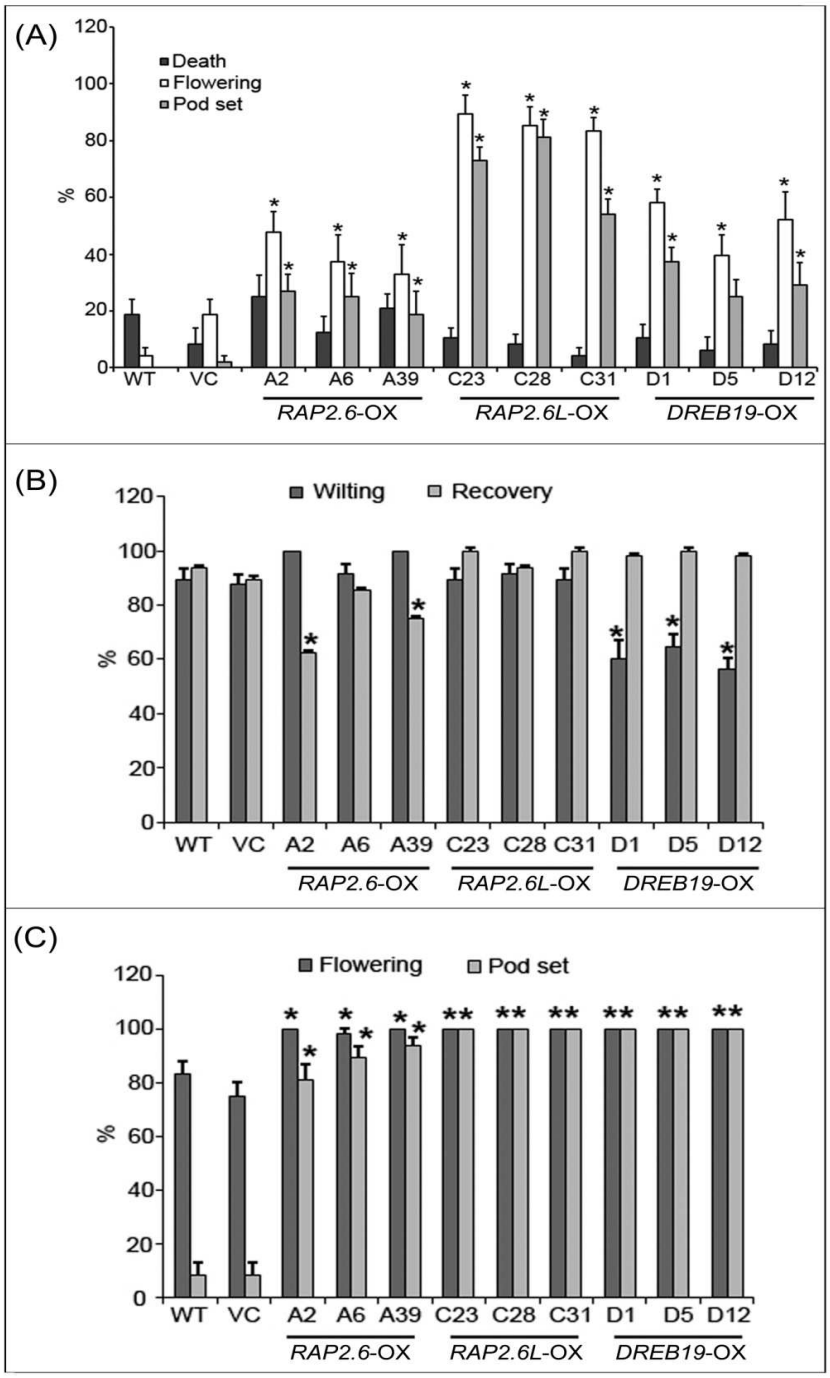

Figure 4-11 Abiotic stress screening of AP2 genes overexpressed *Arabidopsis* (in green house)

Figure 4-11 details…

a Performance of wild type (WT), vector control (VC) and AP2 TF transgenic *Arabidopsis* plants at 200 mM NaCl stress in greenhouse showing percent of plants that not survived, flowered and set pods. All the percentages were calculated based on the total number of initial plants. None of the dead plants had flowers or pods as they died before the onset of flowering. All the survived plants did not flower and some of the flowered plants did not set pods, **b** performance of WT, VC and AP2 TF transgenic plants under drought stress in terms of wilting and recovery, and **c** performance of WT, VC and AP2 TF transgenic plants under drought stress in terms of flowering and pod set. Asterisks indicate a significant difference ($P<0.05$) when compared to WT. Mean values from three biological replicates are shown. Error bars are the standard error of mean (SEM) and N=48.

differences in terms of percent flowering and percent pod set between AP2 transgenic lines and WT (Figure 4-11C). More than 95% of the plants flowered and 70-90% of the plants set pods in AP2 transgenic lines (*RAP2.6*, *RAP2.6L* and *DREB19* transgenic lines), whereas only 75-80% of the plants flowered and only 8% of the plants set pods in controls (WT and VC) following exposure to drought stress (Figure 4-11C). In absence of any stress, transgenic plants flowered earlier as previously observed (Table 4-4) and had a higher number of secondary branches compared to control plants (Figure 4-12A). At 200 mM NaCl stress condition, *RAP2.6L* and *DREB19* transgenic lines were taller and produced more flowers and pods compared to WT, VC and *RAP2.6* plants (Figure 4-12B). Similarly, *RAP2.6L* and *DREB19* plants looked stronger and had higher number of flowers and pods than control plants (WT and VC) and *RAP2.6* transgenic lines under drought stress (Figure 4-12C-D). In summary, greenhouse stress studies demonstrated the enhanced performance of *RAP2.6L* and *DREB19* transgenic plants under salt and drought conditions compared to WT plants.

4.4 Discussion

Salinity and drought are the two major environmental constraints in crop production and more than 10 percent of the World's arable land is affected by salinity and drought (Bray et al., 2000; Jenks et al., 2007). Since the completion of the *Arabidopsis* genome project and subsequent ongoing efforts in genomic research, many genes have been functionally characterized for stress tolerance. TFs represent most important molecular targets in genetic engineering of crop

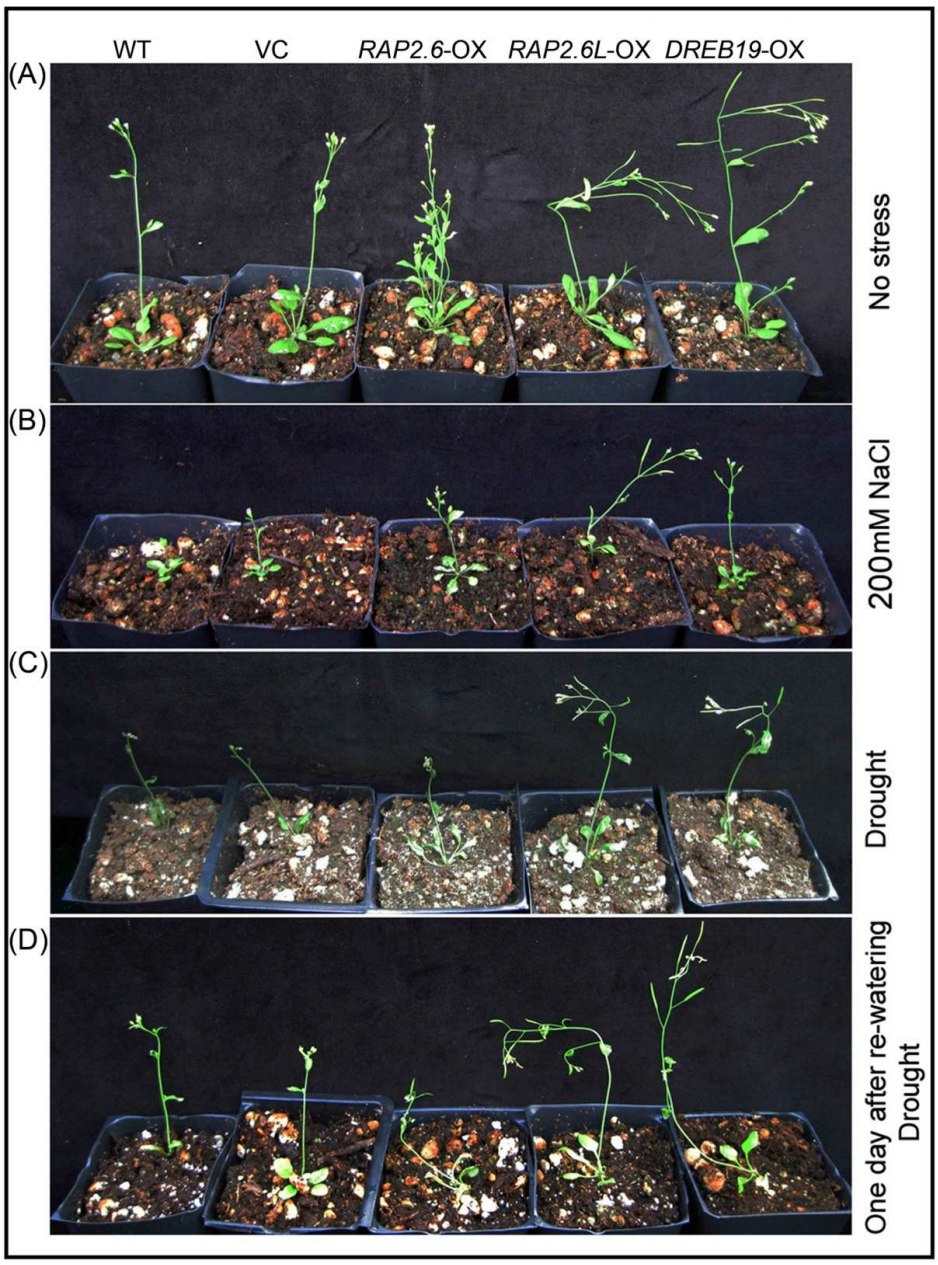

Figure 4-12 Photographic representation of WT, VC and AP2 TF overexpressing *Arabidopsis*

a without stress, **b** at 200 mM NaCl stress , **c** drought stress, and **d** recovery one day after re-watering.

plants for stress tolerance (Nakashima and Shinozaki, 2006; Khong et al., 2008). This is due to the fact that a single TF can regulate the expression of numerous genes including its own gene and activates the adaptation process of an organism to a changed environment (Khong et al., 2008). Some examples of the application of TF in stress tolerance include, *AtMYB44* and *GhDREB,* which conferred enhanced abiotic stress tolerance in *Arabidopsis* and wheat when overexpressed (Jung et al., 2008; Gao et al., 2009). Similarly, a stress responsive TF gene *SNAC1*, when overexpressed, enhanced drought tolerance in rice (Hu et al., 2006). Furthermore, overexpression of AP2 TF genes *OsDREB1F* and *HARDY* enhanced multiple abiotic stress tolerance in both *Arabidopsis* and Rice (Karaba et al., 2007; Wang et al., 2008). These examples also illustrate that knowledge obtained from research on the model plant *Arabidopsis* can be applied in improving crop plants. Nevertheless, the biological role of many *Arabidopsis* TF genes is yet to be explored and many of them may be very useful in engineering crop plants for stress tolerance. In this study, we have made an attempt to investigate the biological role of two ERF (*RAP2.6* and *RAP2.6L*) and two DREB (*DREB19* and *DREB26*) subfamily AP2 TF genes. We chose to study these genes because of their increased transcript abundance in *ABR17*-overexpressed *Arabidopsis* compared to the WT, under NaCl stress (Krishnaswamy et al., 2008). *ABR17* transgenic *Arabidopsis* plants have demonstrated enhanced tolerance to salt and other abiotic stresses (Srivastava et al., 2006). It was speculated that the higher expression of *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* genes in *ABR17*-transgenic plants under NaCl stress could be partially responsible for the observed

salt tolerant phenotype (Srivastava et al., 2006; Krishnaswamy et al., 2008). In this study we have tested if higher expression of these AP2 genes (*RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26*) enhances salt and drought tolerance, by overexpressing them in *Arabidopsis.* Results from a functional assay, expression analysis as well as overexpression studies of these AP2 genes are discussed below.

RAP2.6, RAP2.6L, DREB19 and DREB26 are transcription factors

The genes *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* code for proteins with one AP2 DNA binding domain (Figure 4-1) and sequence analysis suggests that the proteins do not contain EAR motif seen in AP2 transcriptional repressors (Stockinger et al., 1997; Fujimoto et al., 2000; Ohta et al., 2001; Dong and Liu, 2010). They are therefore expected to localize in the nucleus and act as transcriptional activators. Recently, nuclear localization and transcriptional activity has been demonstrated for RAP2.6 and RAP2.6L (Che et al., 2006; Sun et al., 2010; Zhu et al., 2010). However, the subcellular localization and function of DREB19 and DREB26 proteins is not known. In order to study the subcellular localization of DREB19 and DREB26, they were expressed as GFP fusion proteins in *Arabidopsis* (Figure 4-2 & 4-3) and our results demonstrate that DREB19 and DREB26 proteins localize to the nucleus (Figure 4-2 & 4-3) and therefore these proteins might act as transcription factors. However, all nuclear localizing proteins are not transcription factors, therefore, we carried out a

transactivation assay using yeast one hybrid assay with *HIS3* reporter gene (Figure 4-4) to investigate the role of DREB19 and DREB26 in transcriptional regulation. Our results indicate that DREB19 and DREB26 are indeed transactivators (Figure 4-4). We also verified transcriptional activation of RAP2.6 and RAP2.6L using the *HIS3* reporter gene and the results were consistent with the recent reports (Sun et al., 2010; Zhu et al., 2010). Our study (Figure 4-2 & 4-4) and previous studies indicate that the putative AP2 like proteins RAP2.6, RAP2.6L, DREB19 and DREB26 act as TFs.

RAP2.6, RAP2.6L, DREB19 and DREB26 might be important in early vegetative as well as reproductive stages of plant growth

Studying spatial/temporal as well as the tissue specific expression pattern of any gene would give information on the importance of that gene in different growth phases, growth transitions as well as tissue/organ development. We carried out spatial/temporal expression studies of *RAP2.6, RAP2.6L, DREB19* and *DREB26* genes by quantifying their transcript abundance using qRT-PCR in WT *Arabidopsis* plants (Figure 4-5). We observed both similar and divergent expression patterns among the four genes in different stages of plant growth (Figure 4-5). For instance, transcripts of all four genes were enriched in seedlings compared to the rosette leaves stage (Figure 4-5). *RAP2.6* was most abundant in seedlings compared to any other tissue assayed (Figure 4-5). Consistent with this, *RAP2.6* expression has been reported to be high in the stem compared to flowers

(Zhu et al., 2010). Unlike *RAP2.6*, the transcript abundance of *RAP2.6L*, *DREB19* and *DREB26* transcripts was most abundant in the inflorescence compared to any other tissue assayed (Figure 4-5). The transcript abundance of all the four genes increased from floral bud initiation stage to the inflorescence stage indicating their importance in flower development (Figure 4-5) and again, there was no expression of these four AP2 genes in fully matured siliques. These results indicate that all the four genes, especially *RAP2.6*, might be very important in the early vegetative stage. In addition, all of them might be more important in the transition from the vegetative stage to the reproductive stage and in flower development than in silique maturation. In fact, *RAP2.6L* has been implicated in shoot regeneration, since *RAP2.6L* knockdown mutants reduced the efficiency of shoot formation in tissue culture of roots (Che et al., 2006).

The tissue specific expression pattern of *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* was studied indirectly by detecting GUS gene expression in *Arabidopsis* plants containing a promoter-GUS fusion system for these genes (Figure 4-6). In case of germinated seeds, GUS expression was observed only for *DREB19* in cotyledonary leaves. Consistent with qRT-PCR expression analysis, GUS expression was also observed in 7 day old seedlings with *RAP2.6*, *DREB19* and *DREB26* promoters and 14 day old seedlings with *DREB19* and *DREB26* promoters, once again supporting the importance of these genes in the early vegetative stage (Figure 4-6). *DREB19* expression was confined to only the region where leaves emerge from the stem and also in xylem tissues in roots, while *DREB26* expression was detected in cotyledonary leaves, and true leaves

and *RAP2.6* expression was seen only in roots (Figure 4-6). *DREB19* might be involved in leaf emergence as well as in the regulation of genes involved in nutrient/water uptake by xylem tissue and *DREB26* might be involved in leaf and plant development. Although qRT-PCR showed enriched expression of all the four genes in the seedlings, GUS expression was not detected in 7 and 14 day old plants with *RAP2.6L* promoter as well as in 14 day old plants with the *RAP2.6* promoter (Figure 4-6). This could be because the elements needed for the expression of the gene in such stages might be absent within the cloned promoter region. However, abundant GUS expression has been observed in seedlings when more than 1kb of the *RAP2.6L* promoter was cloned (Che et al., 2006). Promoter-GUS fusion studies together with qRT-PCR studies suggest the importance of these four AP2 genes in early vegetative stages. Furthermore, consistent with our qRT-PCR results, GUS expression was observed in flowers with promoters of all the four genes, although each of the AP2 genes tested demonstrated a unique expression pattern within the flower (Figure 4-6). For instance, *RAP2.6* was detected in petals and carpels, while *RAP2.6L* was detected in pollen grains, whereas *DREB26* was detected in ovules, and *DREB19* was detected on the stigmatic surface (Figure 4-6). Although, there was no expression of these genes in mature siliques as detected by qRT-PCR, GUS expression was detected in developing young siliques with promoters of *RAP2.6* and *DREB26* (Figure 4-6). GUS expression was detected in valves of the siliques with *RAP2.6* promoter, while it was detected in early seeds with *DREB26* promoter. These results suggest that they may have very specific roles in flower and silique development.

RAP2.6 may be important in sepal, carpel and overall silique development, while *RAP2.6L* may be important for pollen grain development and function. Similarly, *DREB26* might have an essential role during seed development. In fact, genes from the AP2 TF family are known for their key role in floral morphogenesis and seed development (Kunst et al., 1989; Jofuku et al., 1994; Klucher et al., 1996).

RAP2.6, RAP2.6L, DREB19 and DREB26 are involved in plant defense response

Expression of *RAP2.6, RAP2.6L*, *DREB19* and *DREB26* in response to different abiotic stresses and stress hormones were measured in order to evaluate the involvement of these genes in plant stress signaling. Results indicate that *RAP2.6* and *RAP2.6L* are responsive to both abiotic stresses and the hormones JA, SA, ABA and ET (Figure 4-7). The phytohormone ABA is involved in abiotic stress signaling whereas hormones JA, SA and ET are part of biotic stress response (Fujita et al., 2006). This suggests the participation of the ERF subfamily genes, *RAP2.6* and *RAP2.6L,* in both biotic and abiotic stress signaling. Indeed, *RAP2.6* has been associated with signal transduction during infection of *Arabidopsis* with *Pseudomonas syringae* (He et al., 2004) and role of *RAP2.6L* in bacterial resistance has been demonstrated by mutating *RAP2.6L* in *Arabidopsis* (Sun et al., 2010). It has been shown that among different TF families, ERF family is most responsive to JA and *Alternaria brassicola* (McGrath et al., 2005). The gene *ERF1,* a member from the ERF subfamily, has been suggested to

integrate the JA and ET signaling pathways in *Arabidopsis* and has also been demonstrated to confer resistance to fungal pathogens when overexpressed (Berrocal-Lobo and Molina, 2002; Lorenzo et al., 2003). In the case of the DREB genes, *DREB19* was not responsive to stress hormones, while it was found to be most responsive to salt, heat and drought (Figure 4-7). Salt and drought responsive genes *DREB2A* and *DREB2B* are members of group A-2 of DREB subfamily to which the gene *DREB19* belongs (Sakuma et al., 2006b). *DREB2A* and *DREB2B* are also reported to be highly responsive to salt, heat and drought, and less responsive to phytohormones like ABA, JA and SA (Liu et al., 1998; Sakuma et al., 2006b). Transcript abundance of *DREB26* moderately changed on exposure to JA and SA, but did not altered in response to abotic stresses (Figure 4-7). It appears that *DREB19* is more involved in abiotic stress compared to *DREB26*. The different responses of AP2 genes to different stress and stress hormones suggest that they have very specific physiological roles.

Ovexpression of RAP2.6, RAP2.6L, DREB19 and DREB26 alters phenotype in terms of plant development and/or flowering time

RAP2.6, *RAP2.6L*, *DREB19* and *DREB26* were overexpressed in *Arabidopsis* using CaMV35S promoter in order to investigate their roles in plant growth and development as well as in abiotic stress tolerance. Our overexpression studies indicate that all the four genes tested might have very essential roles in plant growth and development, as overexpression lead to the

altered phenotype with respect to growth or/time of flowering (Figure 4-9 and Table 4-4). Transgenic T_0 *DREB26* lines had altered/deformed phenotype in *Arabidopsis*; over expressed lines being abnormal, dwarf with thin stem, very few leaves and less/no secondary branches (Figure 4-9). Only a few lines set seeds, and the seedlings from those seeds died during germination. Our expression studies (Figure 4-5 & 4-6) have demonstrated that *DREB26* is expressed in cotyledonary leaves, true leaves at the seedling stage as well as in flowers and developing seeds (Figure 4-5 & 4-6). Although the expression of *DREB26* appears to be important in these stages, a balanced expression might be very essential for appropriate plant development, as overexpression leads to deformed plants with no/leaves, deformed flowers and poor pod set. In addition, *DREB26* was less responsive to stress and stress hormones (Figure 4-7), which suggests that *DREB26* might have major role in growth and development, rather than in defense response. Indeed, our qRT-PCR expression studies also indicated less/no response to abiotic stresses and stress related hormones (Figure 4-7). Dwarf phenotype has also been previously reported in AP2 TF overexpressing transgenic *Arabidopsis* plants (Magome et al., 2004; Tong et al., 2009). For instance, molecular analyses of gibberellin deficient mutant *dwarf and delayed flowering 1* (*ddf1*) revealed increased expression of the putative AP2 TF (Magome et al., 2004). Furthermore, overexpression of the AP2 TF gene *DDF2* that is closely related to *DDF1* resulted in the dwarf phenotype in *Arabidopsis* (Magome et al., 2004). Overexpression of chrysanthemum *DREB1B* in *Arabidopsis* resulted in expression of a GA deactivation enzyme (GA2ox7) and dwarfism (Tong et al.,

2009). Whether dwarfism and poor growth of *DREB26* transgenic plants is because of altered GA biosynthesis is not clear since overexpressed *DREB26* plants did not survive and thus we were unable to perform further studies. Expression of *DREB26* under the control of a stress inducible promoter and also loss-of-function analysis might shed more light on the importance of *DREB26* in plant development and the stress response.

In the case of other AP2 TF (*RAP2.6*, *RAP2.6L* and *DREB19*) overexpressed plants, we characterized three independent transgenic lines in each of *RAP2.6*, *RAP2.6L* and *DREB19* overexpressed plants. We observed a comparatively altered phenotype in *RAP2.6* transgenic lines, being dwarf and having many secondary branches compared to controls with small siliques (Figure 4-9). In addition, pod size was comparatively smaller than the WT (Figure 4-9). There were no obvious differences between WT and *RAP2.6* transgenic *Arabidopsis* plants untill the secondary branches started to emerge. Once the secondary branching starts, *RAP2.6* transgenic plants developed more secondary branches and became dwarf (Figure 4-9 & 4-12A). Our expression analysis (Figure 4-5 & 4-6) studies with WT *Arabidopsis* had suggested the importance of *RAP2.6* in vegetative stage and silique development. However, higher expression of *RAP2.6* appears to inhibit apical dominance and promote lateral branching and inhibit silique development. Our qRT-PCR expression analysis showed more than a 1000 fold higher expression of *RAP2.6* in *RAP2.6* transgenic lines (A2, A6 and A39) compared to WT (Table 4-3). In addition to the altered morphology, *RAP2.6* overexpressing lines flowered earlier than the WT (Table 4-4). Indeed,

the early flowering phenotype was also observed in *RAP2.6L* and *DREB26* transgenic *Arabidopsis* lines (Table 4-4). Although a few members of the AP2 family genes have been reported to be involved in the regulation of flowering time, they are known to regulate negatively. For instance, it has been demonstrated that AP2 genes are targets of *miR172,* and overexpression of *miR172* down regulates AP2 genes (*AP2, TOE1* and *TOE2*) and promotes early flowering (Aukerman and Sakai, 2003). In addition, delayed flowering has been observed in overexpressed *TOE1 Arabidopsis* and early flowering has been observed in *ap2* mutants suggesting the function of *TOE1* and *AP2* as floral repressors (Aukerman and Sakai, 2003; Ohto et al., 2005). Furthermore, overexpression of another AP2 family gene *CBF2* results in delayed bolting and flowering in *Arabidopsis* (Schwager et al., 2010). Enriched transcript abundance of *RAP2.6, RAP2.6L* and *DREB19* in early floral buds and inflorescence as well as the GUS expression pattern driven by their promoters (Figure 4-5 & 4-6), suggesting a role for these AP2 genes in flower development. However, the early flowering phenotype of *RAP2.6, RAP2.6L* and *DREB19* overexpressed plants could be an indirect effect of upregulation of stress related genes which are likely to promote flowering similar to the one observed in stressed plants. The AP2 TF genes *RAP2.6, RAP2.6L* and *DREB19* belong to the ERF and DREB subfamilies, whose members are known to bind defined *cis*-elements present in the promoters of pathogenesis related proteins, low temperature and water deficit responsive genes in order to regulate their expression (Stockinger et al., 1997; Gilmour et al., 1998; Guo et al., 2005). Furthermore, an early flowering phenotype has been

observed in plants overexpressing stress related genes. For example, overexpression of the stress related gene phosphatidylinositol-phospholipase C2 in canola promotes early flowering (Fawzy et al., 2009). Indeed, early flowering phenotype has been observed in both *ABR17*- transgenic *Arabidopsis* and *Brassica* compared to the WT under normal conditions, in addition to enhanced stress tolerance (Srivastava et al., 2006; Dunfield et al., 2007). However, studying RNAi or T-DNA insertion lines would confirm the role of *RAP2.6*, *RAP2.6L* and *DREB19* in flowering time.

Overexpression of RAP2.6L and DREB19 enhances salt and drought tolerance

RAP2.6, *RAP2.6L* and *DREB19* transgenic *Arabidopsis* plants were evaluated under abiotic stresses to investigate the importance and utility of these genes in abiotic stress tolerance. The *RAP2.6L* transgenic lines performed better than any transgenic and WT genotypes under NaCl stress in Petri plate experiments. They germinated earlier and had high seedling vigor with enhanced rooting compared to the WT (Figure 4-10). In addition, the *RAP2.6L* transgenic lines performed better than the WT by exhibiting increased percent flowering and percent pod set under NaCl stress in green house conditions (Figure 4-11 & 4-12). Although there were no differences with respect to wilting and recovery, *RAP2.6L* transgenic lines had higher percent flowering and pod set compared to the WT, even under drought stress (Figure 4-11 & 4-12). Therefore, these results suggest that *RAP2.6L* might have a major role in salt tolerance, although it appears to

participate also in drought tolerance. In addition, significantly higher expression of *RAP2.6L* in response to salt and drought stress (Figure 4-7) in the present study, as well as its upregulation in our previous salt microarray studies (Krishnaswamy et al., 2008), further supports the role of *RAP2.6L* in salt and drought stress.

Similar to *RAP2.6L* transgenic plants, *DREB19* overexpressing lines exhibited high seedling vigor compared to the WT at 125 mM NaCl stress (although there was no difference in germination rate, Figure 4-10), and also had high percent flowering and pod set compared to WT under NaCl stress in greenhouse conditions (Figure 4-11). Furthermore, under drought stress, *DREB19* transgenic lines performed better than the WT or any other transgenic lines tested, with less percent wilting in addition to high percent flowering and pod set (Figure 4-11 & 4-12). These results suggest that although *DREB19* is involved in salt tolerance, it appears to be more important in drought tolerance. Detection of *DREB19* promoter driven GUS expression in xylem tissues of roots (Figure 4-6) and also the significantly increased expression of *DREB19* in response to drought and salt stress (Figure 4-7) suggest a role for it in the abiotic stress response (Figure 4-7). In fact, among four AP2 studied, *DREB19* was the most responsive to drought stress (Figure 4-7). Furthermore, other genes (*DREB2A* and *DREB2B*) from the same A-2 group of the DREB subfamily have been demonstrated to impart drought and salt tolerance (Sakuma et al., 2006a & 2006b) suggesting that *DREB19* might be one of the important genes involved in drought signaling.

Unlike *RAP2.6L* and *DREB19* transgenic plants, the performance of *RAP2.6* transgenic plants was comparable to that of the WT under salt stress, although *RAP2.6* and *RAP2.6L* belong to the same group (B-4) of the ERF subfamily. In fact, two of the *RAP2.6* lines (A6 and A39) exhibited reduced germination compared to the WT when grown on medium containing 125 mM NaCl, although the seedling vigor was higher than the WT (Figure 4-10). However, they had significantly higher percent flowering and pod set compared to WT under salt stress in green house conditions, where stress was induced after germination (Figure 4-11 & 4-12). Our observations suggest that overexpression of *RAP2.6* affects germination under salt stress but not seedling growth once germinated. *RAP2.6* transgenic plants did not perform better either under drought stress as at least two of the *RAP2.6* transgenic lines (A2 and A39) had less recovery than the WT following drought stress, although they had higher percent flowering and pod set (Figure 4-11 & 4-12). The differences in performance between three independent transgenic *RAP2.6* lines under salt and drought stress might be due to the position effect, as it could not be correlated with the expression levels of *RAP2.6* (Table 4-3). Although, expression analysis of *RAP2.6* in response to stress and stress hormones (Figure 4-7) as well as previous studies (Fowler and Thomashow, 2002; Krishnaswamy et al., 2008) suggest a role of *RAP2.6* in plant stress signaling, overexpression of *RAP2.6* was not helpful in getting a stress tolerant phenotype. However, very high expression levels of *RAP2.6* were observed in CaMV35S-*RAP2.6* overexpressed lines *(*Table 4-3) which may not be ideal for the plants as they also showed a negative effect on

plant growth under normal conditions (Figure 4-9). A combination of a stress inducible promoter and *RAP2.6* would give better stress tolerance with no/less negative effect on phenotype has previously been reported in another AP2 family gene *DREB1A* (Kasuga et al., 1999 & 2004). Expression of *DREB1A* with sress inducible promoter rd29A gave rise to greater tolerance to stress conditions, with a minimal effect on plant growth than with CaMV35S promoter (Kasuga et al., 1999 & 2004).

Zhu et al., (2010) have reported that *RAP2.6* overexpressed lines are hypersensitive to NaCl and ABA compared to WT. However, we did not observe the sensitivity of *RAP2.6* in any of the three independent *RAP2.6* transgenic lines to NaCl, although we did see a low germination percentage in two lines (A6 and A39) at 125 mM NaCl (Figure 4-10). Furthermore, we also did not observe hypersensitivity of *RAP2.6* overexpressing lines to ABA (Figure AI-1). In addition, Zhu et al. (2010) have not reported any phenotype differences between overexpressed *RAP2.6* lines compared to WT under normal conditions, which were very much evident in our study (Figure 4-9). These differences could be due to a positional effect or to differences in ecotype and sequence. In the present study, three independent *RAP2.6* transgenic lines have been used compared to only one or two independent transgenic lines in the aforementioned study (Zhu et al., 2010). We have isolated *RAP2.6* from ecotype WS which has one amino acid difference (W20R) from the reported sequence of ecotype Columbia (Zhu et al., 2010), and we have overexpressed in WS background unlike the Columbia

ecotype used in their study (Zhu et al., 2010). These differences could also be due to posttranscriptional modifications that convert the inactive form to an active form, like was observed in another DREB family gene *DREB2A* (Sakuma et al., 2000a).

In summary, the results from our study suggest that: (i) DREB19 and DREB26 localize in nucleus and act as transcription activators similar to RAP2.6 and RAP2.6L, (ii) these AP2 genes have divergent physiological roles as they have different expression patterns and invoke varied responses when subjected to abiotic stresses and stress hormones, (iii) they play a very important role both in plant development and stress responses, since overexpression leads to altered phenotypes and altered responses to abiotic stresses, and (iv) increased transcript abundance of *RAP2.6L* and *DREB19* enhances abiotic stress tolerance as speculated based on our previous salt microarray study of *ABR17*-transgenic plants (Srivastava et al., 2006; Krishnaswamy et al., 2008). Early germination, high seedling vigour, early flowering and maturity traits observed in *RAP2.6L* and *DREB19* transgenic plants under salt and/or drought stresses are the characteristics of stress tolerant plants, as they contribute to escape or avoidance of stress conditions (Munns et al., 2000; Price et al., 2002). Similarly, better root growth observed in these transgenic plants may help in sequestration of toxic ions and enhance tolerance to salt. For example, salt tolerance in barley has been linked with early flowering, fast development and better root growth (Munns et al., 2000). In addition, direct positive yield component parameters like higher germination rates, flowering and pod set and better development that were

observed in stressed *RAP2.6L* and *DREB19* overexpressed plants (Figure 4-10 to 4-12) are the traits that are considered while engineering seed plants for abiotic stress tolerance (Basra et al., 2003; Munns et al., 2006; Zadeh and Naeini, 2007; Blum, 2009). Therefore, future studies on overexpressing *RAP2.6* and *DREB19* in crop plants for developing salt and drought tolerant plants could be a worthwhile endeavor.

4.5 References

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CHAPTER 5 General discussion and conclusions

5.1 Discussion

The area under salinity and drought are increasing rapidly, affecting the total available arable land and food production (Wang et al., 2003; UNEP, 2008). It is estimated that worldwide, abiotic stress decreases crop yield by more than fifty percent in many crops (Bray et al., 2000). Therefore, it is very important to understand the stress signaling networks and develop stress tolerant crop plants.

Pathogenesis related (PR) proteins are part of plants multi-component defense signaling network in response to biotic and abiotic stresses (Sels et al., 2008). PR proteins are classified into 17 families and members of the family 10 are called PR-10 proteins. They are low molecular weight (16-19kDa), protease resistant cytosolic proteins that are both constitutively expressed as well as induced in response to stress (Van Loon et al., 1994; Liu et al., 2006). These proteins, which are homologues to Bet v 1 and cytokinin-specific binding proteins (CSBPs), are known for their ligand binding activity with cytokinins (CKs), brassinosteriods and flavonoids and also for their ribonuclease (RNase) activity (Biesiadka et al., 2002; Mogensen et al., 2002; Marković-Housley et al., 2003; Park et al., 2004; Pasternak et al., 2006; Srivastava et al., 2007). Structural studies of PR-10 proteins have revealed the presence of a glycine rich loop (GxGGxGxxK) that is similar to the "P" loop motif observed in nucleotide binding proteins as well as a long forked cavity that assist in ligand binding

(Biesiadka et al., 2002; Mogensen et al., 2002; Marković-Housley et al., 2003; Pasternak et al., 2006; Fernandes et al., 2008). Studies have attributed the ligand binding property of PR-10 proteins to their function as general plant hormone carrier during stress and their RNase activity to antiviral/antifungal activity observed in the case of some PR-10 proteins (Biesiadka et al., 2002; Mogensen et al., 2002; Marković-Housley et al., 2003; Park et al, 2004; Chadha and Das, 2006; Pasternak et al., 2006; Fernandes et al., 2008). Nevertheless, their exact biological role in mediating plant responses to stress and/or during normal growth and development of plants is not clear. In this dissertation, we have made an attempt to understand the biological function of PR-10 proteins *in planta* by studying pea PR-10 member PR-10.4 (also referred as abscisic acid responsive 17; ABR17) (Iturriaga et al., 1994). We have investigated the role of specific amino acids hypothesized to be involved in RNase activity of pea ABR17 and also the expression changes brought by the transgenic expression of pea *ABR17* in *A. thaliana* by employing various genomics strategies. We then focused on and functionally characterized stress related putative AP2 (APETALA 2) transcription factor (TF) genes whose transcripts were high among ABR17-inducible transcripts under salt stress.

The first objective of the study was to test the hypothesis- "highly conserved amino acid residues histidine 69 and glutamic acid 148 are important for catalysis during the RNase activity of pea ABR17". The RNase activity of two pea PR-10 proteins, including ABR17, has been previously demonstrated

(Srivastava et al., 2006a & 2007). In order to get insights into the amino acids responsible for the observed RNase activity of ABR17, variants H69L (histidine 69 leucine) and E148A (glutamic acid 148 alanine) were generated using site-directed mutagenesis. Residues H69 and E148 are conserved among different pea PR-10 proteins and they are also known for their involvement in the catalysis of reactions by many enzymes including RNases. For example, histidine residue can act as both proton donor and acceptor and often reported in RNase mechanisms (Mosimann et al., 1994). Furthermore, the importance of E147 in RNase activity of SPE16 (a PR-10 protein from *Pachyrrhizus erosus*) has been previously demonstrated (Wu et al., 2003). In solution RNase activity assay with recombinant, mutant ABR17 purified proteins from *Escherichia coli* demonstrated that RNase activity was reduced in H69L variant while it was enhanced in E148 variant compared to WT-ABR17, suggesting the importance of both H69 and E148 residues in the RNase activity of pea ABR17 protein.

Our homology modeling results demonstrated the conservation of three dimensional structures between ABR17 and *Lupinus luteus* llpr10.1b proteins (Biesiadka et al., 2002). Previous co-crystallization and molecular docking experiments of *Lupinus* PR-10 protein with zeatin and N, N”-diphenylurea molecules have reported that residue His69 adopts a double conformation that facilitates substrate stabilization, reaction kinetics and also formation of hydrogen bond networks with neighboring residues (Biesiadka et al., 1994; Fernandes et al., 2009). The zeatin binding site should be compatible with the RNA binding

site since zeatin is a nucleoside analog (Biesiadka et al., 1994) and, therefore it is speculated that H69 in pea ABR17 protein might also be involved in polar interactions and substrate stabilization in RNase activity. Substitution of H69 with leucine results in loss of these polar interactions and, therefore, results in decreased RNase activity in H69L-ABR17. On the other hand, E146 in *Lupinus* PR-10 forms polar interactions with Ser11 and the backbone amide group of the β1 main chain. Therefore, in variant E148A of pea ABR17, when E148 is substituted with alanine, these polar interactions might be lost and facilitate the widening of the C-terminal helix (α3) thus increasing substrate accessibility and RNase activity (Krishnaswamy et al., 2011). H69L and E148A variants of pea ABR17 would be helpful in testing the existence of speculated links between observed RNase activity of recombinant pea PR-10 proteins and enhanced CK levels observed in *PR-10* transgenic plants (Srivastava et al., 2004, 2006a, 2006b & 2007).

The second objective of the study was to test the hypothesis- "pea ABR17 enhances stress tolerance in ABR17-transgenic Arabidopsis by modulating the expression of stress responsive genes". The overexpression of pea *ABR17* in *Arabidopsis* exhibited precocious flowering, a higher number of lateral branches, and an increased number of seed pods, as well as elevated concentrations of endogenous CKs compared to the wild type (WT) counterpart (Srivastava et al., 2007). Early flowering increased lateral branching and enhanced CK levels of *ABR17*-transgenic are suggestive of a role for CKs in

ABR17 action (Bonhomme et al., 2000; Tanaka et al., 2006; Srivastava et al., 2007). Furthermore, PR-10 proteins were found abundantly in pea (*Pisum sativum*) under salt stress, and pea PR-10 (*PR-10.1* and *ABR17*) overexpressed canola and *Arabidopsis* plants demonstrated enhanced germination and early seedling growth under abiotic stress conditions (Kav et al., 2004; Srivastava et al., 2004; Srivastava et al., 2006b). In addition, exogenous application of CKs enhanced the germination of wild type *Arabidopsis* (Srivastava et al., 2007), supporting the suggestion of ABR17 action through CKs (Srivastava et al., 2007).

In order to gain additional insights into the possible ways in which ABR17 may mediate plant responses to stress, *ABR17*-mediated global gene expression changes in *A. thaliana* both under normal and stressed conditions were investigated using microarrays. Significantly responsive transcripts due to the expression of pea *ABR17* in *A. thaliana* under normal conditions included plant defensins, mitogen-activated protein kinase (*MAPK*), expansins, glycine-rich proteins, proline-rich protein (*PRP*) , xyloglucon endotransglycosylase (*XTH*), glycosyl hydrolase (*GH*), phytosulfokine precursor 2 (*PSK2*), No Apical Meristem (*NAM*) family protein and glutaredoxins (Krishnaswamy et al., 2008). These genes have been earlier reported to be involved in ancestral non-specific innate immune defense system and/or developmental processes like cell cycle regulation, cell wall synthesis, organ development, cell growth and differentiation (Cassab, 1998; Reinhardt et al., 1998; Sablowski and Meyerowitz, 1998; Bernhardt and Tierney, 2000; Cho and Cosgrove, 2000; Reidy et al., 2001;

Goujon et al., 2003; Igasaki et al., 2003; Vissenberg et al., 2005; Xing et al., 2005; Huffaker et al., 2006). This led us to suggest that significantly higher expression of the above mentioned plant growth and development related genes might be responsible for the *ABR17*-transgenic phenotype which includes early flowering, increased lateral branching and seed pods (Krishnaswamy et al., 2008). Furthermore, members of most of the gene families described above have been previously reported to be regulated by CKs (Rashotte et al., 2003; Brenner et al., 2005).

In addition, qRT-PCR (quantitative real time- polymerase chain reaction) analysis of *EXPL1* (*At3g45970*), putative *MAPK* (*Atg01560*) and *GRP* (*At1g07135*) genes in CK treated *Arabidopsis* tissue showed significantly higher expression, suggesting that they are indeed CK-responsive genes (Krishnaswamy et al., 2008). However, neither our microarray and qRT-PCR analysis nor our previously reported proteome studies of *ABR17*-transgenic plants (Srivastava et al., 2006b; Krishnaswamy et al., 2008) showed significant differences in expression of any *IPT* (isopentenyl transferase; involved in CK biosynthesis) or *CKX* (CK oxidase; involved in CK degradation) genes/proteins. Therefore, it is possible that the previously reported enhanced CKs in *ABR17*-transgenic lines (Srivastava et al., 2007) may be the result of possible degradation of CK containing free tRNAs (Prinsen et al., 1997) rather than modulation of CK biosynthetic genes (Srivastava et al., 2007).

Our microarray analysis of salt treated versus untreated *ABR17*-*Arabidopsis* seedlings also revealed an increase in the abundance of transcripts for transcription factors (TFs) including *AP2* (*APETALA* 2)- related, *NAM*, zinc finger (C3HC4-type RING finger), *bHLH*, *ATMYB74*, *ATHB*-7 and *WRKY* family genes that have been implicated previously in the plant abiotic stress response in *ABR17* plants (Krishnaswamy et al., 2008). Other up-regulated genes in *ABR17* salt stressed tissue included xyloglucan endo-transglycosylase (*XET*), members of GH family, osmotin, mannitol dehydrogenase, steroid sulfotransferases, *RD20*, ribonuclease- *RNS1*, peroxidases, copper/zinc superoxidase dismutase (*CSD1*), cytochrome p450 family, *MATE* efflux protein and protein kinases (Krishnaswamy et al., 2008). These genes have also been reported to have major role in plant abiotic stress signaling (King et al., 1986; Singh et al., 1987; Silva et al., 1994; Takahashi et al., 2000; Abede et al., 2003; Vissenberg et al., 2005; Ma et al., 2006; Klein and Papenbrock, 2008). Furthermore, the transcript abundance of some genes exhibited significant differences in the degree of modulation between salt stressed-WT and salt stressed-*ABR17* tissues. For example, xyloglucan endotransglycosylase (*XTR-6*) (*At4g25810*), RAP2.6 (*At1g43160*), ABA-responsive protein-related (*At3g02480*), unknown protein (*At5g24640*), PR-related protein (*At4g33720*), glutamine-dependent asparagine synthetase (*At3g47340*), heat shock proteins (Hsps) and plant defensins were highly abundant in salt treated *ABR17*-transgenic tissue compared to salt treated WT tissue (Krishnaswamy et al., 2008). These results suggested that higher expression

of the stress related genes in *ABR17*-transgenic seedlings might be responsible for the observed abiotic stress tolerant phenotype.

The third objective of the study was to perform functional validation of our microarray results. Based on these we speculated that some of the putative TFs whose transcript abundance was high in salt treated *ABR17*-transgenic *Arabidopsis* are important for the observed stress tolerance in ABR17 plants. Four AP2 family genes [*RAP2.6* (*At1g43160*), *RAP2.6L* (*At5g13330*), *DREB26* (*At1g21910*) and *DREB19* (*At2g38340*)] were identified among significantly upregulated TFs in salt treated *ABR17*-transgenic *Arabidopsis* (Krishnaswamy et al., 2008), and these genes were selected for functional characterization as their function in abiotic stress tolerance was not known. The AP2 family proteins have been previously implicated in various physiological process including plant growth and development, and abiotic/biotic stress tolerance (Saleh and Pages, 2003). Therefore, it was hypothesized that *RAP2.6, RAP2.6L, DREB19,* and *DREB26* might participate in plant defense response against salt stress and overexpression of these genes in *Arabidopsis* might enhance salt and other abiotic stresses (Krishnaswamy et al., 2010).

The genes *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* code for proteins with one AP2 DNA binding domain, and nuclear localization and transcriptional activity has been demonstrated for RAP2.6 and RAP2.6L (Che et al. 2006; Zhu et al. 2010; Sun et al. 2010). Our study demonstrated nuclear localization and transcriptional activity for DREB19 and DREB26 proteins (Krishnaswamy et al.,

2010). Our spatial/temporal expression studies of *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* genes in *Arabidopsis* indicated enriched transcript abundance of the these four genes in the early seedling and inflorescence stage, suggesting their importance in early vegetative growth and flower development. Promoter activity studies using GUS reported gene also supported these observations. In addition, promoter studies also showed the unique expression pattern of all four AP2 genes within a flower. For instance, *RAP2.6* promoter was active in petals and carpels, while *RAP2.6L* was expressed in pollen grains, whereas *DREB26* was detected in ovules, and *DREB19* was detected on the stigmatic surface suggesting their specific role in floral morphogenesis. Abiotic stress and hormone response studies indicated that ERF (Ethylene responsive factors binding) subfamily genes *RAP2.6* and *RAP2.6L* are responsive to stress hormones [jasmonic acid (JA), salicylic acid (SA), ABA and ethylene (ET)] in addition to abiotic stresses.

The hormones JA, SA and ET are biotic stress signaling molecules, and members from the ERF subfamily have been previously implicated in JA and ET signaling pathways and biotic stress tolerance (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003; McGrath et al., 2005). Furthermore, both *RAP2.6* and *RAP2.6L* have been associated with biotic stress signal transduction (He et al., 2004; Sun et al., 2010). Therefore, it appears that *RAP2*.6 and *RAP2.6L* may be involved in both biotic as well as abiotic stress signaling. In the case of DREB (dehydration responsive element binding) genes, *DREB19* was highly responsive to abiotic stresses (salt, heat and drought) but was not responsive to the stress

hormones tested. On the other hand, a modest increase in *DREB26* transcript abundance in response to stress hormones was observed, while it was not responsive to abotic stresses. The results suggest that *DREB19* might be very important in abiotic stress tolerance while *DREB26* may not be that important.

The AP2 genes were overexpressed in *Arabidopsis* to characterize their phenotype under normal as well as under stressed conditions. *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* overexpressed plants showed an altered phenotype with respect to growth or/time of flowering, suggesting the importance of these four TFs in plant growth and development similar to spatial/temporal expression studies. *DREB26*-transegnic plants had an altered/deformed phenotype with thin stem, few leaves and less/no secondary branches. Furthermore, *RAP2.6* overexpressed lines exhibited a dwarf phenotype with numerous secondary branches and small siliques. In addition, *RAP2.6*, *RAP2.6L* and *DREB19* transgenic plants flowered earlier compared to the controls. Although an altered phenotype and late flowering have been previously reported in AP2 family genes there are no reports of early flowering in overexpressed AP2 plants (Ohto et al., 2005; Tong et al., 2009; Schwager et al., 2010). The early flowering phenotype observed in this study could be due to the fact that these are stress related TFs and therefore studying knock out/knock down lines would confirm the role of these AP2 genes in regulating flowering time (Guo et al. 2005; Fawzy et al. 2009; Dunfield et al. 2007).

The AP2 transgenic plants (*RAP2.6*, *RAP2.6L* and *DREB19*) were also characterized for salinity and drought tolerance in Petri dish tests as well as in greenhouse conditions. *DREB26*-transgenic plants were not included in this experiment as they were deformed and did not set enough seeds needed for the experiment. The results from stress studies demonstrated that the performance of *RAP2.6* transgenic plants was just comparable to the WT and VC controls under salt and drought stress. However, the very high expression of *RAP2.6* that was observed in our CaMV35S-*RAP2.6* overexpressed lines may not be ideal for the plants and therefore, a combination of an inducible promoter and *RAP2.6* would give better stress tolerance with no/less negative effect on phenotype similar to the one previously observed in an AP2 family gene *DREB1A* (Kasuga et al., 1999 & 2004). In the case of *RAP2.6L* and *DREB19* transgenic plants, they were developmentally advanced with a better root system, germinated earlier, flowered earlier and set more pods under under salt and/or drought stresses.

Early germination and early flowering characters might enhance stress tolerance as they could help plants to escape or avoid stress conditions, while better root growth may enhance salt tolerance by sequestering toxic ions (Munns et al., 2000; Price et al., 2002). In addition, higher germination rate, flowering and pod set are direct positive yield component parameters that are essential in enhancing crop productivity under abiotic conditions (Munns et al., 2006; Blum, 2009). Based on the characterization of AP2 (*RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26*) transgenic *Arabidopsis*, we suggest that it is worthy undertaking

overexpression of *RAP2.6* and *DREB19* in crop plants for the purpose of salinity and drought tolerance (Krishnaswamy et al., 2010). Furthermore, we suggest that the multiple abiotic stress tolerance exhibited by *ABR17*-transgenic *Arabidopsis* plants could be in part associated with upregulation of AP2 TF family genes, supporting our microarray studies.

5.2 Conclusions

The possible mechanisms of pea PR-10 action in overexpressed plants are illustrated in Figure 5-1. PR-10 proteins are known for their CK binding and RNase activity (Liu and Ekramoddoullah, 2006). Pea ABR17 (PR-10.4) has also exhibited RNase activity and highly conserved amino acids like histidine 69 and glutamic acid 148 appear to be very important for the RNase activity of pea ABR17 (Srivastava et al., 2007; Krishnaswamy et al., 2011). Pea *ABR17* overexpressed plants exhibited precocious flowering and developmentally advanced phenotype with a greater number of secondary branches under normal conditions (Srivastava et al., 2006b). In addition, they possessed enhanced CK levels compared to WT plants (Srivastava et al., 2007). Taken all together, it appears that under normal conditions, the accumulation of more PR-10 proteins may act as a CK reservoir (through their CK binding activity) or they may hydrolyse CK containing tRNA (transfer RNA) molecules (through their RNase activity) to enhance CK levels in *PR-10* transgenic plants. The enhanced CK could be responsible for higher expression of CK responsive genes, which included genes related to growth and development, which was observed in our

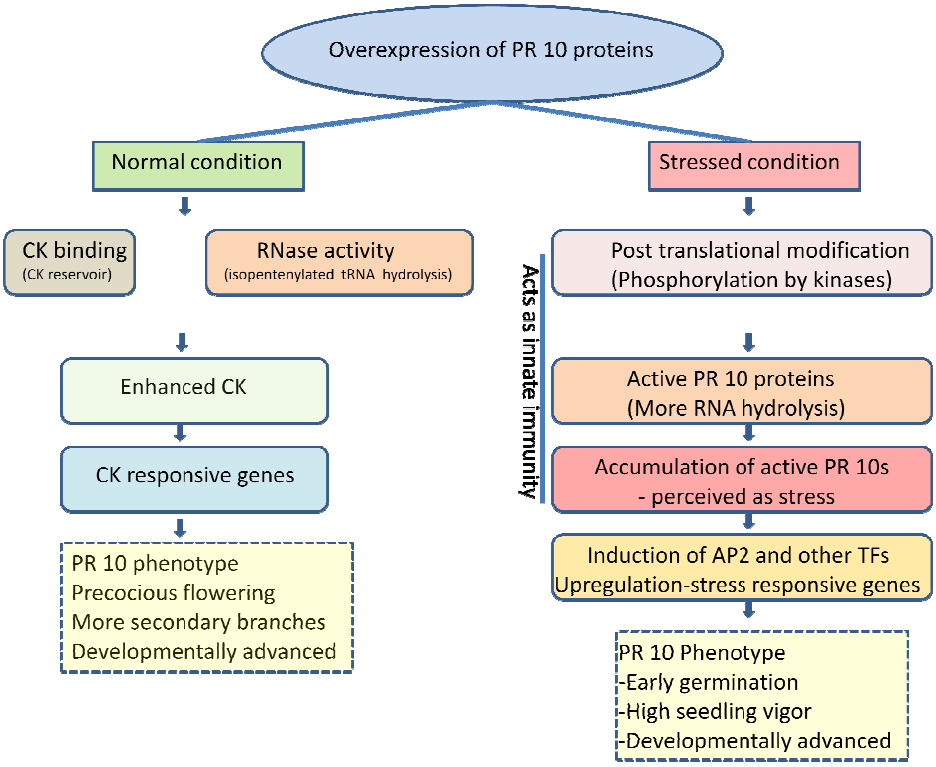

Figure 5-1 Possible ways in which PR-10 may mediate its action in *PR-10* transgenic plants

microarray results (Krishnaswamy et al., 2008). This could attribute to early flowering and the developmentally advanced phenotype observed in *ABR17* plants. Similarly, under stress conditions, PR-10 proteins may undergo posttranslational modifications like phosphorylation by stress induced kinase cascades (Park et al., 2004; Jain et al., 2006). The accumulation of active PR-10 proteins may increase RNA hydrolysis and may be perceived as stress. In a way, accumulation of active PR-10 proteins might act as priming or innate immunity. This results in activation of AP2 and other TFs, which upregulate stress responsive genes whose products are necessary for stress tolerance. Therefore, *PR-10* overexpressed plants may channelize stress tolerance related networks and adapt to stressed conditions earlier than wild type plants. This helps *PR-10* plants to germinate and grow better under abiotic stress conditions. It appears that, in nature, PR-10 proteins may enhance tRNA dependent CK biosynthesis, and accumulation of PR-10 proteins under stress may act as positive feedback for the upregulation of stress related TFs. Such TFs include AP2 TF family genes like *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26*. Furthermore, our studies suggest that overexpression of *RAP2.6L* and *DREB19* could be employed in enhancing the abiotic stress tolerance of crop plants.

The study decribed in this dissertation have some limitations. For example, in the first objective, in addition to RNase assay and homology modeling studies, measuring rate of reactions for WT and its variants (histidine 69 and glutamic acid 148) could be considered. In the second objective, in order to

investigate the differentially expressed genes between WT and *ABR17*-transgenic *Arabidopsis* plants under salinity conditions, statistical comparison of WT-stressed/WT-normal and *ABR17*-stressed/*ABR17*-normal microarray results was carried out. Although results are reliable as evidenced by qRT-PCR with this design, the best design would be to carry out microarray of *ABR17*-stressed/WT-stressed. Furthermore, more than one endogenous control should have been considered for performing qRT-PCR. In the third objective, knockout or knockdown analysis for *RAP2.6*, *RAP2.6L*, *DREB19* and *DREB26* genes would further support results from over-expression studies. Future studies may be planned that could address some of these limitations and further our understanding of the structure and function of plant PR-10 proteins.

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Appendix I

Table AI-1 List of primers used in qRT-PCR

Gene	Primer pairs and probe used in real time PCR
AtExpansin *AT3G45970.1*	Forward; 5'-CTCCTCTGCCTCTGCTCTCTCT-3' Reverse; 5'-TCCGGCGAAGAAACTCGTA-3' Probe; 5'-TGTGCTTATGGCTCTATG-3'
AtGlycine-rich protein *AT1G07135.1*	Forward; 5'-GGGTGGTTCCGATTCATACG-3' Reverse; 5'-TGTGATCCTGCCGATCCA-3' Probe; 5'-TGGTAATGGCAGTCTGG-3'
AtMAPK *AT1G01560.1*	Forward; 5'-GGCTTGCGAGGACTAAATCAGA-3' Reverse; 5'-GCTCGGTACCAACGTGTAACAA-3' Probe; 5'-ACAGACTTCATGACAGAAT-3'
Plant defensin PDF1.2a *AT5G44420.1*	Forward; 5'-CCCTTATCTTCGCTGCTCTTGT-3' Reverse; 5'-TTCTGTGCTTCCACCATTGC-3' Probe; 5'-CTCTTTGCTGCTTTCGA-3'
AtIPT1 *AT1G68460*	Forward; 5'-CGCCGGTGGATCTAACTCTTT-3' Reverse; 5'- AACTTTGGGTCGAATCGTTGA-3' Probe; 5'-TCCACGCACTCTTAG-3'
AtIPT2 *AT2G27760*	Forward; 5'-TGGAATGCGCAAGTGGTTAA-3' Reverse; 5'-TTCGGTTTCTGTCTCCAGGAA-3' Probe; 5'-TGCTTCAGAGATCATCAG-3'
AtIPT3 *AT3G63110*	Forward; 5'-TTTCCGGAGTTTGACAGGTTTT-3' Reverse; 5'-CAGTTCTTCTCTGTCTTCCACATTCA-3' Probe; 5'-CAGGAACGAGCAGTTC-3'
AtIPT4 *AT4G24650*	Forward; 5'-TGGAGTGCCACATCACCTTCT-3' Reverse; 5'-ATTCTGCCGCTGTGACTTCTC-3' Probe; 5'-TGAACTAAACCCGGAGGC-3'
AtIPT5 *AT5G19040*	Forward; 5'-GCCGGTGGTTCCAATTCTT-3' Reverse; 5'-CGGAAGTCAACGCAATCGT-3' Probe; 5'-CATCGAGGCTCTGGTC-3'
AtIPT6 *AT1G25410*	Forward; 5'-GACGCTACGGCGGCAAT-3' Reverse; 5'-CCTTCTCCCTTTGCCGTACTT-3' Probe; 5'-ATGGCTGAGCTGAATC-3'
AtIPT7 *NM_113267*	Forward; 5'-CATTTGGGTCGACGTTTCCT-3' Reverse; 5'-GCGGTCGACACGTTTTGAG-3' Probe; 5'-CCCGTACTTAACTCCTTT-3'
AtIPT8 *AT3G19160*	Forward; 5'-CCGGATCAGGCAAGTCATG-3' Reverse; 5'-CGATCTCGCCAGAGAAACG-3'

	Probe; 5'-CTCTCAATCGATCTAGCAAC-3'
AtIPT9	Forward; 5'-TTGGCAGTGGCGTATTTTTG-3'
AT5G20040	Reverse; 5'-AAGGCTTGGCGGTTGAAGT-3'
	Probe; 5'-CCCGTACCTGTTACTTG-3'
AtCKX1	Forward; 5'-CGGGCTTGGACAGTTTGG-3'
gi|20196946	Reverse; 5'-CGGTGCTGGTTCAAGAGAGAT-3'
	Probe; 5'-ATAATCACCCGGGCACG-3'
AtCKX2	Forward; 5'-CTCCCCATCATCAGCAAGGT-3'
AT2G19500	Reverse; 5'-ATGAACCCGGGCAAGTAACTTA-3'
	Probe; 5'-TGACACATTAACGAAAACA-3'
AtCKX3	Forward; 5'-CCAAGGACATGAACTCGGATCT-3'
AT5G56970	Reverse; 5'- TTATAATGCCGAATTGACCCAAAC-3'
	Probe; 5'-TTCTTCGCGGTGTTAGGA-3'
AtCKX4	Forward; 5'-CCATCTCTGCCGCTTCTCA-3'
AT4G29740	Reverse; 5'-GCGCCGGGATTTTCG-3'
	Probe; 5'-ACTTCGGTAACATAACCG-3'
AtCKX5	Forward; 5'-TGCGGGTCGGTTCTTTATTG-3'
AF303981	Reverse; 5'-TGAGTTGGAATCGGAGTCTCTGT-3'
	Probe; 5'-TCGAACTCGGTCTTCA-3'
AtCKX6	Forward; 5'-GATGTCGACGGCCACTTCA-3'
AT1G75450	Reverse; 5'-GTCTGAGGAGACGGAGGCTAAG-3'
	Probe; 5'-CGTCCACCCTTCC-3'
AtActin2/7	Forward; 5'-GCCATTCAGGCCGTTCTTT-3'
AY102779	Reverse; 5'-ATCGAGCACAATACCGGTTGT-3'
	Probe; 5'-TCTATGCCAGTGGTCG-3'
ABA-responsive protein	Forward; 5'-GCCACTGGCCAGACTAAGGA-3'
At3g02480	Reverse; 5'- CAAGGAGTCTTGAGCTGAAGCA-3'
XTX 6	Forward; 5'- TTTCCTAAGAACCAGCCAATGAG-3'
At4g25810	Reverse; 5'-TTGACGAGACCACCCCTTGT-3'
bHLH	Forward; 5'- CCGACATCTCGGGTGATAGAA-3'
At5g43650	Reverse; 5'- CCTCAGTTCCGTGTCCTTCATAT-3'
RAP2.6	Forward; 5'- TGTCCTTGGAGAGGCCAAAA-3'
At1g43160	Reverse; 5'- CATACACGTGTCGCCTTGTGT-3'
unknown protein	Forward; 5'- CACCACCAGTTTTTGGAGATT-3'
At5g24640	Reverse; 5'- CCGCTCACTTTCTCCGATGA-3'
ATNAC3	Forward; 5'-TCGACGGAGGGAAGAAGAGTT-3'
At3g15500	Reverse; 5'- TTGGTTTTGGTTCCTTTTGGA-3'
ACD6	Forward; 5'- CCCATGTGAAATGGCTTTTAGTC-3'

At4g14400	Reverse;5’- AGGGCCAAGGATAAAGATTGC-3'
PLAC8	Forward; 5’- TTTGCTGTAACCTCTGTGCTTTG-3'
At1g14880	Reverse; 5’- TGCCCATCCAAGGCTCATAT-3'

Table AI-2 Transcriptional profiling: genes exhibiting more than 1.5-fold increase/decrease in transcript abundance in *ABR17* transgenic *Arabidopsis*

AGI [a]	Operon annotations	log2 ratio	SE
At5g20230	plastocyanin-like domain containing protein	1.55	0.14
At4g36060	BHLH family protein	1.49	0.19
At5g44420	plant defensin protein, putative (PDF1.2a)	1.40	0.38
At5g42040	hypothetical protein	1.38	0.41
At4g22450	hypothetical protein	1.37	0.17
At5g44430	plant defensin protein, putative (PDF1.2c)	1.35	0.40
At3g45970	expansin protein family (EXPL1)	1.32	0.16
At5g01920	protein kinase family	1.24	0.22
At2g26010	plant defensin protein, putative (PDF1.3)	1.17	0.35
At5g10040	expressed protein	1.04	0.31
At1g75830	plant defensin protein, putative (PDF1.1)	1.04	0.30
At2g26020	plant defensin protein, putative (PDF1.2b)	0.96	0.26
At1g07135	glycine-rich protein	0.95	0.19
At1g01560	mitogen-activated protein kinase (MAPK 11), putative	0.94	0.10
At3g43850	hypothetical protein	0.94	0.13
At2g26560	patatin, putative	0.90	0.12
At5g39890	expressed protein	0.88	0.18
At1g10070	tat-binding protein –related	0.88	0.06
At2g22860	(AtPSK) phytosulfokine precursor 2	0.85	0.06
At5g52760	heavy-metal-associated domain-containing protein	0.85	0.20
At2g27080	expressed protein	0.82	0.16
At1g19530	expressed protein	0.82	0.19
At3g55980	expressed protein	0.82	0.19
At2g47880	glutaredoxin protein family	0.82	0.22
At3g62680	proline-rich protein family	0.82	0.11
At4g14365	expressed protein	0.82	0.11
At3g15500	No apical meristem (NAM) protein family	0.82	0.06
At2g26560	patatin, putative	0.81	0.15
At1g56240	F-box protein (SKP1 interacting partner 3-related)	0.80	0.12
At1g08630	expressed protein	0.79	0.22
At1g77120	alcohol dehydrogenase (ADH)	0.78	0.20
At2g14610	pathogenesis-related protein 1 (PR-1)	0.77	0.15
At1g75040	pathogenesis-related protein 5 (PR-5)	0.77	0.08
At1g02660	*A. thaliana* chromosome I BAC T14P4	0.76	0.04
At5g45340	cytochrome P450 family	0.76	0.19
At2g18690	expressed protein	0.75	0.19
At3g49160	pyruvate kinase -related protein	0.75	0.10
At1g72940	disease resistance protein (TIR-NBS class), putative	0.75	0.08

At1g18570	myb family transcription factor	0.75	0.11
At1g76410	RING zinc finger protein –related	0.75	0.06
A005153_01	RESISTANCE TO POWDERY MILDEW8 (RPW8)	0.74	0.09
At2g35290	expressed protein	0.73	0.06
At2g38470	WRKY family transcription factor	0.73	0.16
At1g80840	WRKY family transcription factor	0.70	0.18
At4g30280	xyloglucan endotransglycosylase, putative	0.70	0.12
At5g54710	*A. thaliana* chromosome 5, TAC clone:K5F14	0.70	0.19
At2g40000	nematode-resistance protein –related	0.70	0.11
At2g31945	expressed protein	0.68	0.03
At1g72060	expressed protein	0.68	0.10
At5g46710	expressed protein	0.67	0.10
At2g15890	expressed protein	0.67	0.10
At1g69490	No apical meristem (NAM) protein family	0.67	0.08
At4g31800	WRKY family transcription factor	0.66	0.14
At1g21400	branched-chain alpha keto-acid dehydrogenase –related	0.66	0.13
At3g49960	peroxidase, putative	0.66	0.03
At5g47230	ethylene responsive element binding factor 5 (AtERF5)	0.66	0.19
At1g07000	exocyst subunit EXO70 family	0.65	0.06
At1g28330	dormancy-associated protein –related	0.65	0.12
At1g02660	lipase (class 3) family	0.64	0.22
At4g25920	expressed protein	0.64	0.18
At1g05250	peroxidase, putative	0.64	0.08
At1g02640	glycosyl hydrolase family 3	0.64	0.09
At4g10270	probable wound-induced protein	0.64	0.07
At4g11890	protein kinase family	0.64	0.10
At4g02270	expressed protein	0.62	0.17
At2g14900	gibberellin-regulated proteins –related	0.62	0.12
At3g23170	expressed protein	0.62	0.04
At2g19190	light repressible receptor protein kinase, putative	0.62	0.11
At5g58660	oxidoreductase, 2OG-Fe(II) oxygenase family	0.62	0.12
At2g18150	peroxidase, putative	0.61	0.16
At1g02610	*A. thaliana* chromosome I BAC T14P4	0.61	0.10
At5g13080	WRKY family transcription factor	0.60	0.16
At2g17040	No apical meristem (NAM) protein family	0.59	0.16
At4g23160	hypothetical protein	0.59	0.08
At3g08720	ribosomal-protein S6 kinase (ATPK19) –related	0.59	0.07
At2g41640	expressed protein	0.59	0.13
At3g10040	expressed protein	0.59	0.08
At2g35460	harpin-induced protein 1 family (HIN1)	0.58	0.11
At5g57020	N-myristoyl transferase	-0.59	0.09
At2g41650	expressed protein	-0.60	0.07

At1g19150	PSI type II chlorophyll a/b-binding protein (Lhca2*1)	-0.60	0.08
At1g64390	glycosyl hydrolase family 9 (endo-1,4-beta-glucanase)	-0.60	0.15
At5g02230	haloacid dehalogenase-like hydrolase family	-0.60	0.07
At1g62180	phosphoadenylyl-sulfate reductase, putative	-0.61	0.07
At5g06530	ABC transporter family protein	-0.63	0.13
At2g03090	expansin, putative (EXP15)	-0.63	0.14
At5g23060	expressed protein	-0.63	0.06
At4g16370	isp4 like protein	-0.64	0.07
At1g23740	oxidoreductase, zinc-binding dehydrogenase family	-0.65	0.07
At4g36030	armadillo repeat containing protein	-0.65	0.15
At4g37980	mannitol dehydrogenase (ELI3-1), putative	-0.65	0.16
At2g05100	light-harvesting chlorophyll a/b binding protein	-0.66	0.11
At1g44000	expressed protein	-0.68	0.12
At2g31380	salt tolerance-like protein	-0.68	0.08
At1g01060	myb family transcription factor	-0.70	0.04
At5g48490	seed storage/lipid transfer protein family	-0.71	0.18
At1g73870	CONSTANS B-box zinc finger family protein	-0.71	0.20
At3g47420	glycerol 3-phosphate permease, putative	-0.72	0.11
At4g26850	expressed protein	-0.72	0.08
At5g55570	hypothetical protein	-0.72	0.13
At5g67370	expressed protein	-0.73	0.09
At5g05250	expressed protein	-0.75	0.08
At5g05270	chalcone-flavanone isomerase family	-0.75	0.06
At3g02380	Zinc finger protein CONSTANS-LIKE 2 (COL2)	-0.75	0.06
At5g53450	protein kinase family	-0.76	0.11
At3g27690	chlorophyll A/B binding protein, putative	-0.77	0.19
At5g02120	one helix protein (OHP)	-0.90	0.09
At5g48850	male sterility MS5 family	-0.99	0.17
At1g56430	nicotianamine synthase, putative	-1.13	0.08
At3g56980	bHLH protein family	-1.36	0.13

All expression ratios are significant (α=0.05) and are in a log2 scale where fold change is ABR17/WT.

AGI [a] – *Arabidopsis* Genome Initiative SE [b] - Standard error

Table AI-3 Transcriptional profiling: genes exhibiting more than 4-fold increase/decrease in transcript abundance in salt- treated Wild type *Arabidopsis*

AGI [a]	Operon annotation	log2 scale	SE
A023244_01	*A. thaliana* ABA-regulated gene cluster	3.96	0.82
A023734_01	Genomic sequence for *A. thaliana* BAC F15O4	3.94	0.59
At1g54010	ESTs	3.90	0.28
At2g38530	nonspecific lipid transfer protein 2 (LTP 2)	3.62	0.18
At4g13220	expressed protein	3.59	0.35
At2g02990	ribonuclease, RNS1	3.59	0.22
At4g12500	lipid transfer protein (LTP) family	3.45	0.21
At2g03760	steroid sulfotransferase, putative	3.45	0.06
At4g12490	lipid transfer protein (LTP) family	3.40	0.16
At2g43620	glycosyl hydrolase family 19 (chitinase)	3.38	0.24
At4g04220	disease resistance protein family	3.33	0.24
At3g57470	protease-related protein	3.19	0.50
At4g12470	lipid transfer protein (LTP) family	3.17	0.11
At3g43180	zinc finger (C3HC4-type RING finger) family	3.09	0.09
At5g43570	hypothetical protein	3.01	0.16
At1g62420	expressed protein	2.91	0.31
At3g60140	glycosyl hydrolase family 1, beta-glucosidase	2.91	0.30
At1g69930	glutathione transferase, putative	2.81	0.13
At3g44040	hypothetical protein	2.76	0.58
At3g02240	expressed protein	2.71	0.17
At4g02330	expressed protein	2.69	0.12
At2g43510	trypsin inhibitor –related	2.67	0.13
At5g24640	expressed protein	2.58	0.36
At5g43580	hypothetical protein	2.52	0.61
At4g11650	osmotin-like protein (OSM34)	2.52	0.23
At3g29970	germination protein –related	2.50	0.22
At5g42830	hydroxycinnamoyl benzoyltransferase-related	2.49	0.19
At2g34600	expressed protein	2.48	0.55
At2g16060	class 1 non-symbiotic hemoglobin (AHB1)	2.40	0.22
At3g02480	expressed protein	2.40	0.24
At2g13510	hypothetical protein	2.38	0.57
At5g14180	expressed protein	2.36	0.30
At4g25810	xyloglucan endotransglycosylase (XTR-6)	2.35	0.54
At3g21720	isocitrate lyase –related	2.34	0.25
At5g01330	pyruvate decarboxylase-related protein	2.34	0.19
At1g10585	*A. thaliana* BAC T10O24 from Chromosome 1	2.33	0.13
At1g17020	oxidoreductase, 2OG-Fe(II) oxygenase family	2.32	0.11

At5g36925	expressed protein	2.32	0.10
At2g19800	expressed protein	2.31	0.16
At1g21910	transcription factor TINY family	2.29	0.16
At4g12530	lipid transfer protein (LTP) family	2.28	0.57
At2g18490	C2H2-type zinc finger protein –related	2.26	0.22
At2g30840	2-oxoglutarate-dependent dioxygenase, putative	2.26	0.22
At3g08860	alanine--glyoxylate aminotransferase , putative	2.25	0.16
At4g12480	lipid transfer protein (LTP) family	2.25	0.33
At4g08870	arginase –related	2.25	0.15
At4g36700	cupin domain-containing protein	2.22	0.65
At5g36920	hypothetical protein	2.19	0.15
At5g13330	AP2 domain transcription factor family	2.16	0.17
At5g01920	protein kinase family	2.16	0.35
At3g46270	expressed protein	2.15	0.10
At1g30700	FAD-linked oxidoreductase family	2.13	0.22
At5g24030	expressed protein	2.12	0.34
At1g42040	hypothetical protein	2.09	0.28
At5g64120	*A. thaliana* mRNA for peroxidase ATP15a	2.08	0.20
At5g19550	aspartate aminotransferase, cytoplasmic isozyme 1	2.08	0.11
At2g36780	UDP-glycosyltransferase family	2.08	0.14
At4g30290	xyloglucan endotransglycosylase, putative	2.06	0.13
At4g36430	peroxidase, putative	2.06	0.45
At3g51860	cation exchanger, putative (CAX3)	2.05	0.24
At1g35140	phosphate-induced (phi-1) protein –related	2.05	0.29
At2g36770	glycosyltransferase family	2.04	0.11
At4g09600	gibberellin-regulated protein GASA3 precursor	2.04	0.17
At4g15910	drought-induced protein (Di21)	2.03	0.08
At4g05390	ferredoxin--NADP(+) reductase, putative	2.02	0.22
At4g01700	glycosyl hydrolase family 19 (chitinase)	2.02	0.17
At2g44370	CHP-rich zinc finger protein, putative	2.01	0.14
At4g37870	*A. thaliana* BAC F15O4 from chromosome I	2.01	0.13
At4g16260	glycosyl hydrolase family 17	2.01	0.21
At5g06330	harpin-induced protein, putative (HIN1)	2.00	0.24
At5g22580	expressed protein	-2.00	0.20
At4g26850	expressed protein	-2.00	0.11
At3g04210	disease resistance protein, putative	-2.00	0.20
At1g25440	CONSTANS B-box zinc finger family protein	-2.02	0.16
At3g08940	chlorophyll a/b-binding protein -related	-2.03	0.18
At3g19320	leucine rich repeat protein family	-2.03	0.06
At3g54890	chlorophyll a/b binding protein	-2.04	0.17
At3g51750	expressed protein	-2.06	0.34
At5g04550	expressed protein	-2.07	0.38

At1g62360	homeobox protein –related	-2.07	0.45
At3g45160	expressed protein	-2.08	0.10
At1g29420	auxin-induced protein family	-2.08	0.16
At1g14150	PsbQ domain protein family	-2.10	0.10
At3g17930	expressed protein	-2.11	0.21
At5g17670	expressed protein	-2.11	0.37
At5g09660	malate dehydrogenase, glyoxysomal	-2.11	0.15
At3g03830	auxin-induced protein, putative	-2.12	0.26
At4g24700	expressed protein	-2.12	0.10
At3g62960	glutaredoxin protein family	-2.13	0.17
At2g39470	oxygen-evolving complex 25.6 kD protein, putative	-2.14	0.28
At3g04140	expressed protein	-2.14	0.12
At1g78020	senescence-associated protein –related	-2.14	0.13
At5g18030	auxin-induced protein, putative	-2.14	0.31
At2g47880	glutaredoxin protein family	-2.16	0.16
At2g26500	expressed protein	-2.16	0.13
At5g61980	ARF GTPase-activating domain-containing protein	-2.16	0.58
At1g32080	expressed protein	-2.17	0.08
At5g48570	peptidylprolyl isomerase	-2.18	0.37
At1g68010	glycerate dehydrogenase	-2.18	0.17
At1g51300	*A. thaliana* chromosome I BAC F11M15	-2.19	0.42
At5g24580	copper-binding protein family	-2.22	0.30
At3g14200	DnaJ protein family	-2.23	0.35
At1g12080	expressed protein	-2.23	0.39
At2g45660	MADS-box protein (AGL20)	-2.24	0.12
At1g61520	chlorophyll a/b binding protein	-2.24	0.13
At4g17460	homeobox-leucine zipper protein HAT1	-2.24	0.35
At2g26020	plant defensin protein, putative (PDF1.2b)	-2.27	0.47
At5g45820	CBL-interacting protein kinase 20	-2.29	0.31
At3g28830	expressed protein	-2.30	0.20
At5g54270	chlorophyll a/b binding protein, putative	-2.32	0.27
At3g51895	sulfate transporter ATST1	-2.34	0.08
At4g10540	*A. thaliana* BAC F3H7	-2.35	0.10
At5g02160	expressed protein	-2.35	0.09
At3g59370	expressed protein	-2.36	0.31
At3g16120	dynein light chain protein -related	-2.37	0.45
At5g44780	expressed protein	-2.38	0.08
At2g06230	hypothetical protein	-2.38	0.23
At3g15270	squamosa promoter binding protein-related 5	-2.41	0.61
At1g74310	heat shock protein 101 (HSP101)	-2.42	0.31
At3g47070	expressed protein	-2.44	0.22
At4g21650	*A. thaliana* DNA chromosome 4, BAC clone F17L22	-2.45	0.41

At4g11320	cysteine proteinase	-2.47	0.20
At1g77490	thylakoid-bound ascorbate peroxidase, putative	-2.51	0.19
At5g44420	plant defensin protein, putative (PDF1.2a)	-2.51	0.21
At1g29450	auxin-induced protein, putative	-2.53	0.19
At1g09340	RNA-binding protein –related	-2.57	0.12
At5g18080	auxin-induced protein, putative	-2.58	0.16
At4g15460	glycine-rich protein	-2.60	0.14
At4g12830	hydrolase, alpha/beta fold family	-2.60	0.18
At5g42040	hypothetical protein	-2.61	0.19
At2g33810	squamosa-promoter binding protein -related	-2.61	0.66
At5g44430	plant defensin protein, putative (PDF1.2c)	-2.62	0.17
At2g40610	expansin, putative (EXP8)	-2.66	0.08
At3g15540	auxin-responsive protein IAA19	-2.68	0.22
At5g64770	expressed protein	-2.69	0.20
At4g00755	F-box protein family	-2.70	0.25
At4g26530	fructose-bisphosphate aldolase, putative	-2.72	0.25
At5g22430	expressed protein	-2.73	0.50
At1g29490	*A. thaliana* chromosome 1 BAC F15D2	-2.80	0.21
At3g32130	*A. thaliana* chromosome 3, BAC clone: F1M23	-2.81	0.05
At5g18010	auxin-induced protein, putative	-2.83	0.11
At1g58520	ERD4 protein-related	-2.83	0.31
At5g18020	auxin-induced protein, putative	-2.89	0.18
At1g29460	auxin-induced protein, putative	-2.89	0.10
At2g26010	plant defensin protein, putative (PDF1.3)	-2.95	0.16
At5g58770	dehydrodolichyl diphosphate synthase, putative	-2.95	0.42
At1g67870	glycine-rich protein	-2.97	0.18
At5g39860	bHLH protein	-2.98	0.10
At4g39800	myo-inositol-1-phosphate synthase	-2.99	0.17
At3g09440	heat shock protein hsc70-3 (hsc70.3)	-3.03	0.22
At4g21640	subtilisin-related protease	-3.04	0.31
At2g15020	expressed protein	-3.04	0.42
At4g28395	lipid transfer protein, putative	-3.14	0.64
At2g40300	ferritin –related	-3.19	0.17
At1g29510	auxin-induced protein, putative	-3.27	0.13
At3g24500	ethylene-responsive transcriptional coactivator -related	-3.34	0.29
At1g29430	auxin-induced protein family	-3.63	0.29
At5g62080	lipid transfer protein (LTP) family	-3.78	0.68
At1g23130	Bet v I allergen family	-3.89	0.06
At4g14400	*A. thaliana* chromosome 4, contig fragment No. 38	-3.90	0.39
At1g67860	expressed protein	-4.04	0.26
At5g35480	expressed protein	-4.12	0.18

All expression ratios are significant (α=0.05) and are in a log2 scale where fold change is salt-treated wild type/control wild type.

AGI [a] – *Arabidopsis* Genome Initiative SE [b] - Standard error

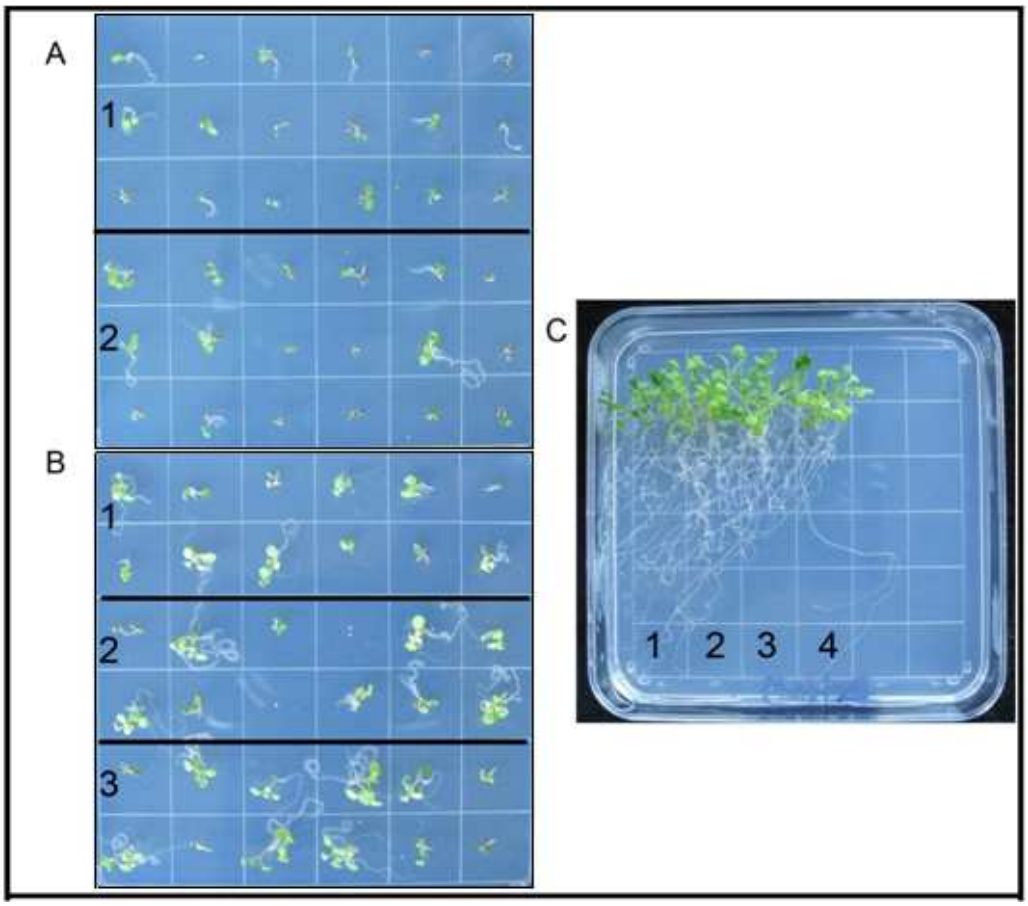

Figure AI-1 Growth of *RAP2.6*-transgenic *Arabidopsis* on MS-ABA medium

a Germination of 1) WT (wild type) and 2) VC (vector control) after 3 weeks, **b** Germination of 1) A2, 2) A6, and 3) A39 after 3 weeks, **c** Root elongation of 1) WT, 2) A2, 3) A6 and 4) A39 after three weeks. MS medium had 1 µM ABA (abscisic acid). For root elongation assay, seeds were germinated on MS medium for 5 days and seedlings were transferred on to MS medium with 1 µM ABA. Plates were placed vertically at RT and light intensity 40 µmol m^{-2} s^{-1}**.**