Functional characterization of *Brassica juncea* Annexin-2 in salt stress and expression analysis of annexin homologs in *Brassica rapa*

Thesis submitted to the University of Hyderabad for the award of

DOCTOR OF PHILOSOPHY

By

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"DECLARATION"

I, **Israr Ahmed** hereby declare that this thesis entitled **"Functional characterization of *Brassica juncea* Annexin-2 in salt stress and expression analysis of annexin homologs in *Brassica rapa***" submitted by me under the supervision of **Prof. P. B. Kirti**, is an original and independent research work. I also declare that it has not been submitted previously in part or in full for any degree or diploma of any other University or Institution.

Date:

Israr Ahmed
(Research Scholar)

CERTIFICATE

This is to certify that the thesis entitled **"Functional characterization of *Brassica juncea* Annexin-2 in salt stress and expression analysis of annexin homologs in *Brassica rapa*"** Submitted by **Israr Ahmed** bearing registration number **09LPPH21** in partial fulfilment of requirements for award of Doctor of Philosophy in the **School of Life Sciences** is a bonafide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Parts of this thesis have been:

A. Published in the following publications:
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Supervisor **Head of Department** **Dean of School**

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Abbreviations

µg	Microgram
µl	Microliter
µM	Micro molar
ABA	Abscisic acid
ATP	Adenosine triphosphate
BAP	Benzyl amino purine
bp	Base pairs
CAMv	Cauliflower mosaic virus
cDNA	Complementary DNA
cm	Centimeter
CTAB	Cetyl trimethylammonium bromide
d	Day
DEPC	Diethyl pyrocarbonate
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy nucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
g	Gram
h	hours
IPTG	Isopropyl-β-D-thiogalactoside
Kb	Kilobases
KDa	Kilodalton
LB	Luria Bertani

M	Molar
min	Minutes
ml	Milliliter
MS	Murashige and Skoog
ng	Nano gram
NS	Null segregant
O.D	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribo nucleic acid
Rpm	Revolutions per minute
TE	Tris.EDTA
Tris	Tris (hydroxymethyl) amino methane
WT	Wild type

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Chapter 1

Introduction

Introduction

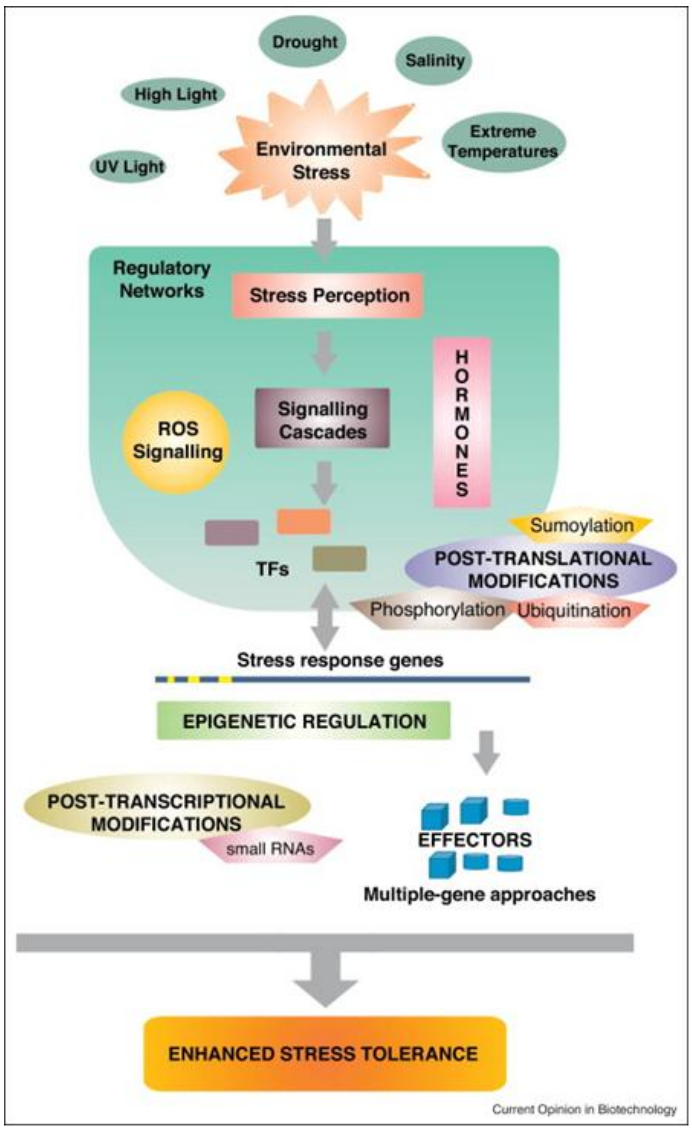
Plants being sessile in nature have to deal with various environmental cues during its growth and development. These environmental cues reduce their growth and yield below the optimum levels. These stresses are broadly classified into two types; biotic and abiotic stress. Abiotic stress includes drought, salinity, temperature, heavy metals, nutrient, light, ozone, etc. whereas biotic stress includes insect pests and pathogens. Together, these constraints significantly reduce crop production and productivity. Among the abiotic stresses, drought and salinity together are the more severe ones affecting more than 10% of arable land, which result in more than 50% decline in the average yields of major crops worldwide (Bray et al. 2000). Salinity affects more than 800 million hectares of land across the world (Munns and Tester 2008). The annual global losses in agricultural production from salt-affected soils are more than US$12 billion (Flowers and Colmer 2008; Qadir et al. 2008) and are as expected rising. Salinization of agricultural lands is increasing due to intensive farming practices and frequent use of ground water for irrigation. Saline soils can be reclaimed either by the use of soil amendments like gypsum, which is rich in calcium; or by leaching down the excess salts from the rooting zone to the deeper layers or by growing salt tolerant crops (Ashraf 1994; Flowers et al. 1997; Grieve and Shannon 1999). In line with the scarcity of arable land across the globe, global food production needs to be increased by 70% by the year 2050 to meet the food demands of the estimated 9.3 billion human population (Shabala 2013; Tester and Langridge 2010). In the present scenario, plant biologists face a challenge to develop new technologies and approaches that will increase the global food production under these environmental conditions.

During the course of evolution, plants have evolved several adaptive mechanisms, which enable them to survive under the ever-changing external environment. Understanding these adaptive responses could play a significant role in engineering or breeding crops that can make better use of limited resources in a sustainable manner. Plant adaptations to most abiotic stresses involve a range of changes at the physiological, biochemical, and molecular levels which contributes towards their survival under the variable environmental conditions. Physiological responses include stomatal closure to reduce the transpiration rate, reduction in leaf area, stunted growth, root growth, leaf abscission, wilting, change in relative water content, electrolyte leakage. On the other hand, biochemical changes include generation of

reactive oxygen species, accumulation of osmoprotectants like proline, soluble sugars, glycine betaine, etc., changes in chlorophyll and lipid peroxidation. Molecular responses include signal perception, activation of signaling cascade to activate transcription factors which further induce a set of genes. These genes synthesize the compatible molecules or regulate the downstream signaling for signal transduction (Agarwal et al., 2006). The general plant signaling pathway in response to the stresses has been shown in Figure 1.1. Plant adaptive strategies to stress are coordinated and fine-tuned by adjusting growth, development, cellular and molecular activities (Ahuja et al. 2010) . Recent research has made efficient use of transcriptomic, proteomic and molecular approaches to identify the complex networks linked to stress perception and response in the model and crop plants (Alvarez et al. 2008; Coolen et al. 2016; Nakabayashi and Saito 2015; Shulaev et al. 2008).

Conventional breeding technologies have been vital in the introgression of QTLs associated with the desired trait to develop tolerant crops. However, in certain situations, it brings about certain undesirable agronomic characteristics from the donor parents. Traditional breeding methods fail to introduce the desired genes due to pollination barrier across the species and distant relatives or from non-plant sources. In such condition, insertion of the specific genes through genetic engineering provides a potential solution for the development of stress tolerant crops. Further, genetic engineering allows control over the expression of a particular gene in specific time and tissue for their optimal function. Constitutive expression of a stress-induced gene or transcription factor often leads to deleterious effect due to the accumulation of the gene product or continuous energy consumption and is desired only under stress. To cope up with such problems, stress inducible promoters have been identified and fused with the desired gene to develop stress tolerance in plants (Chen et al. 2015; Kasuga et al. 2004; Lee et al. 2003; Pino et al. 2007).

With the completion of several genome sequencing projects, it has become possible to study the genes responding to abiotic stress in a comprehensive manner. Comparative transcriptome studies between the tolerant and the susceptible cultivars helped in the identification of the stress related genes and their relationship with the *cis*- regulatory elements (Kilian et al. 2007; Ma and Bohnert 2007; Weston et al. 2008). Stress-induced genes are regulated by complex signaling networks.



Source: Cabello et al. (2014)

Figure1.1: Plant abiotic stress response and intervention points for genetic engineering strategies

Broadly, the translation products of stress-induced genes are classified into two categories (a) protein with known functions that are directly involved in abiotic stress tolerance (eg LEA proteins, HSP, transporters etc) and the other group includes (b) regulatory proteins involved in intracellular signaling and stress-induced gene expression (eg. protein kinases, phosphatases and transcription factors). Single action genes such as those involved in the synthesis of osmoprotectants, ROS detoxification and transport were the initial targets for plant transformation. Several reports are

available where single gene overexpression increased the stress tolerance of the plants (Abebe et al. 2003; Cortina and Culianez-Macia 2005; Gao et al. 2000; Garg et al. 2002; Holmstrom et al. 2000; Pilonsmits et al. 1995; Sakamoto et al. 1998; Shen et al. 1997). ROS are produced as a result of abiotic stresses and need to be detoxified to protect the cells from oxidative injury. Engineering plants with the expression of ROS-detoxifying genes such as such as glutathione peroxidase, superoxide dismutase, ascorbate peroxidases and glutathione reductases (Lee et al. 2007; McKersie et al. 1996; Murgia et al. 2004; Sarowar et al. 2005; Sengupta et al. 1993; Wang et al. 1999; Yoshimura et al. 2004) alleviates oxidative stress in transgenic plants. Another strategy to cope up with the salt stress is the reestablishment of the ionic homeostasis in cells. This can be achieved either by sequestering the excessive ions inside the vacuoles or effluxing out from the roots to the outer environment. Overexpression of NHX1, SOS1 and HKTs resulted in salt tolerance in the transgenic plants (Apse et al. 1999; Hauser and Horie 2010; Horie et al. 2009; Shi et al. 2003; Sunarpi et al. 2005; Yang et al. 2009b).

Regulatory proteins like protein kinases, phosphatases, and transcription factors regulate signal transduction and gene expression in the stress response (Jang et al. 2003; Mizoi et al. 2012; Nakashima et al. 2009; Osakabe et al. 2013; Qin et al. 2011a). In field condition, plants are exposed to a combination of stresses simultaneously, and their responses are governed by multi genes. This has led to the manipulation of the regulatory genes, which regulate the expression of several downstream stress responsive genes. In many signal transduction pathways, the external stimuli are perceived by the membrane localized receptors that initiates a signaling cascade of phosphorylation and dephosphorylation and finally activate or suppresses a transcription factor. Among the kinases, mitogen activated protein kinase (MAPK) and calcium dependent protein kinase (CDPK) are widely studied. MAPK signaling cascade is composed MAPKK kinase (MAP3K), MAPK kinase (MAP2K) and MAP kinase (MAPK). Arabidopsis genome contains of than 80 MAP3Ks, ten MAP2Ks, and 20 MAPKs. The sequential activation of MAPK finally phosphorylates some transcription factors or other signaling components that elicit the response to the external stimuli (Andreasson and Ellis 2010; Danquah et al. 2014; Popescu et al. 2009).

Transcription factors constitute ~ 7% of the Arbidopsis genome and are classified into different families such as WRKY, bZIP, MYB, AP2/EREBP, and NAC (Udvardi et al., 2007; Golldack et al., 2011). A transcription factor binds to the *cis* acting elements present in the regulatory regions of the stress responsive genes and thus, regulates their expression. Manipulation of plants with genes coding for the transcription factors confers multiple stress tolerance (Agarwal and Jha 2010; Gao et al. 2011; Hossain et al. 2010; Mao et al. 2012; Orellana et al. 2010).

Calcium is the most important intracellular secondary signaling molecules. Abiotic stresses cause a rapid rise in the intracellular calcium; mostly cytosolic $[Ca^{2+}]_{cy}t$ but in some cases nuclear $[Ca^{2+}]_{nuc}$ or organellar $[Ca^{2+}]_{org}$. The change in the calcium levels are stimuli specific and differ in terms of the location (cytosolic, nuclear or organellar), magnitude, duration and frequency (Allen et al. 2001; Allen et al. 2000; Johnson et al. 1995; Mazars et al. 2009; McAinsh and Pittman 2009; Tracy et al. 2008). These spatial and temporal patterns of cellular Ca^{2+} changes are termed Ca^{2+} signatures. These Ca^{2+} signatures are specific stimuli and are sensed by Ca^{2+} binding proteins, which program the cell to generate a specific response by activating the downstream signaling cascades (Batistic and Kudla 2004; Kudla et al. 2010; Luan et al. 2002; McCormack et al. 2005). The change in calcium fluxes is mediated by membrane-localized calcium channels and transporters. Plants regulate the calcium fluxes across the membranes through three major classes of calcium transporters: channels, pumps (ATPases) and Ca^{2+}/ H^{+} antiporters. Apart from these, some other proteins are also involved in transporting Ca^{2+}, which include Cyclic nucleotide gated channels (CNGC), Glutamate receptor homologs (GLR) and annexins (Chin et al. 2009; Kudla et al. 2010; Singh et al. 2014; Steinhorst and Kudla 2013; Swarbreck et al. 2013; Vincill et al. 2012).

Annexins belong to a multigene family with wide taxonomic distribution across different kingdoms including higher vertebrates, plants, fungi, and protists (Benz and Hofmann 1997; Clark et al. 2012; Gerke and Moss 2002; Mortimer et al. 2008; Moss and Morgan 2004). Mostly, these are soluble proteins but have the capacity to bind or associate with the plasma or endomembranes in a calcium-dependent and independent manner (Balasubramanian et al. 2001; Blackbourn et al. 1991; Gerke and Moss 2002). Plant annexin expression is developmentally and tissue-specifically regulated (Clark et al. 2001; Jami et al. 2008; Jami et al. 2009; Laohavisit et al. 2010; Mortimer et al.

2008; Yadav et al. 2015). Expression profiling studies of annexins in different plant species revealed that they are regulated by different signaling molecules and abiotic stress inducers (Cantero et al. 2006; Jami et al. 2009; Lu et al. 2012; Xu et al. 2016; Yadav et al. 2015; Zhou et al. 2013). Genetic manipulation of the plants with annexin expression showed alterations in growth, development and stress response of the plants (Jami et al. 2008; Divya et al. 2010; Konopka postupolska et al. 2009; Tang et al. 2014). Recently, several reports suggest their involvement in plant defense mechanisms against biotic and abiotic stresses.

Previously, six annexins (*AnnBj1*, *AnnBj2*, *AnnBj3*, *AnnBj4*, *AnnBj6* and *AnnBj7*) were identified in *Brassica juncea* (Jami et al. 2009; Jami et al. 2010). Among these, role of *AnnBj1* and *AnnBj3* were studied in stresses. Ectopic expression of *AnnBj1* in tobacco and cotton conferred multiple stress tolerance (Jami et al. 2008; Divya et al. 2010). Overexpression of *AnnBj3* in Arabidopsis and *Saccharomyces cerevisiae* alleviated methyl viologen induced oxidative stress (Dalal et al. 2014a; Dalal et al. 2014b). In the present investigation, we focused on the characterization of *AnnBj2* in salt stress by constitutive expressing it in the model plant tobacco (*Nicotiana tobacum*) and in the native system (*B.juncea*). Further, we analyzed the expression *of Brassica rapa* annexins in response to different signaling molecules.

Objectives:

1. Preparation of *AnnBj2* overexpression construct and Generation of *AnnBj2* overexpressing transgenic tobacco plants and its evaluation under salt stress
2. Genetic transformation of *B.juncea* with *AnnBj2* and evaluation of its role in salt stress
3. Expression analysis of annexin homologs in *B.rapa* in response to different signaling molecules.

Chapter 2

Review of Literature

2.1. Economic importance of *Brassica juncea*

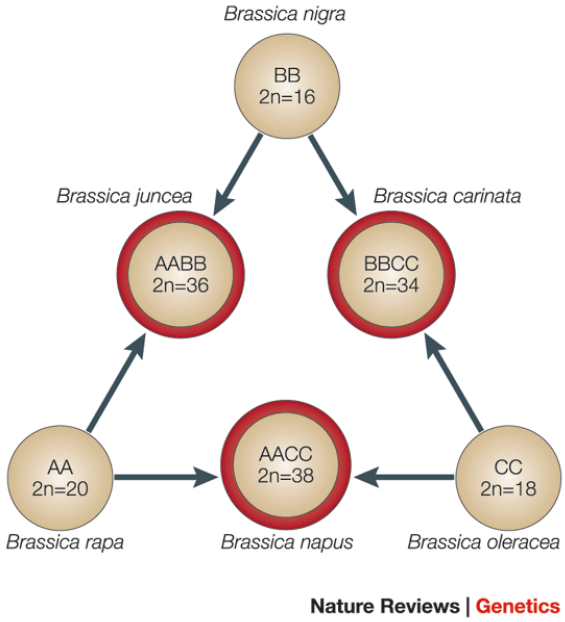
Indian mustard (*Brassica juncea*, AABB; 2*n* = 36) is a natural amphiploid derived from the interspecific hybridization between *B. rapa* (AA; 2*n* = 20) and *B. nigra* (BB; 2*n* = 16). It belongs to family Bracicaceae. It is one of the most important oilseed crops of India. Its oil content varies from 35-45% oil and protein content 20-24%. India is one of the biggest consumers of vegetable oil with more than half of its demand being met mainly through imports. Mustard is the second most important oilseed crop next to groundnut, contributing about 32% of the total oilseed production in the country. In the year 2013-14, it was grown on an area of 6.6 million hectares, producing 7.8 million tonnes with an average yield of 1185 kg/ha in the country. In India, it is grown both as a rainfed and irrigated crop under different agro climatic conditions. Rajasthan, Uttar Pradesh, Madhya Pradesh, Haryana, Gujarat and West Bengal states accounted for nearly 86.5% area and 91.4% production of Rapeseed-Mustard in the country during 2012-13.

Mustard is grown in temperate and subtropical climatic conditions. It needs cool and dry weather conditions and hence in India, it is mostly cultivated as a rabi season crop particularly in northern parts. It requires a temperature of 10-25°C and annual rainfall in the range of 600-1000 mm. Well drained sandy loamy soil with pH 5.5 to 7.5 is good for mustard cultivation.

2.2. Genomic relationship of *Brassica* species

Brassica is one of the most important genera within the family Brassicaeae. It is closely related to the model plant Arabidopsis and comprises 38 species. Many of these species are economically important oilseed crops, condiments and vegetables. The genomic relationship between the important cultivated species is well explained by the 'U' triangle as shown in Figure 2.1. It includes three diploid species, *B. rapa* (AA genome, 2n=20), *B. nigra* (BB genome, 2n=16) and *B. oleracea* (CC genome, 2n=18). These three diploid species have undergone natural process of interspecific hybridization followed by chromosome doubling to give rise to the amphidiploid species *B. juncea* (AABB genome, 2n=4x=36), *B. napus* (AACC genome, 2n=4x=38), and *B. carinata* (BBCC genome 2n=4x=34). The relationship among these species were confirmed by genomic in situ hybridization (Iwabuchi et al. 1991; Snowdon et al. 1997), comparative genome mapping (Lagercrantz and Lydiate 1996)

and artificial synthesis of the amphidiploids by crossing the donor diploid parents (Song and Osborn 1992).



Source: Stewart et al. (2004)

Figure 2.1: Genomic relationships of *Brassica* species as represented by 'U' triangle.

2.3. Abiotic stress

Plants encounter a number of environmental stresses, which adversely affect their growth and development and results in significant yield reduction. The most common abiotic stresses affecting plant growth are drought, salinity, temperature, light and heavy metals. Among the various abiotic stresses, drought and salinity together affect more than 10% of total arable land resulting in 50% yield loss of the major crops world-wide (Bray et al. 2000). It was estimated that the world population would reach more than 9 billion and food production needs to be doubled to feed the growing population by the year 2050 (Shabala 2013; Tester and Langridge 2010). To address this problem, plant biologists need to develop stress tolerant varieties, which could sustain their potential yields under these adverse conditions. Abiotic stresses induce a series of changes at morphological, physiological, biochemical and molecular level in plants. Plant adaptation to these stresses depends on their ability to perceive the

external signal and transmitting it through signaling cascades to activate the adaptive responses (Xiong et al. 2002). Understanding these changes is of utmost importance for developing tolerant varieties. With the completion of genome sequencing projects of several model and crop plants, it is now possible to study the change in the genome wide expression of genes responsible for a particular stress or a combination of stresses simultaneously. Such genome wide expression studies using microarray and RNA seq techniques have helped in the identification of a repertoire of genes, which are stress regulated (Hirayama and Shinozaki 2010) . These stress regulated genes need to be functionally characterized for their role in stress tolerance. Although several stress induced genes were characterized through reverse genetic approaches, still a wide gap exists in understanding the complex signaling networks. Although significant increase in the yield and total production has been achieved through conventional breeding approaches, still a wide gap exists between the potential yield and the actual yield realized at the farmer's field. This gap in the actual yield attained under field conditions is mainly due to various biotic and abiotic stresses. In the present work, we focused on salt stress as it is one of the severe problems in the north western agro climatic zone of India due to intensive agriculture and continuous use of ground water for irrigation.

2.4. Salt stress

2.4.1. Effect of salt stress on plant growth and development

Salinity imposes two types of stresses which limit plant growth. Firstly, it increases the osmotic potential of the soil and hampers water uptake by the roots. Secondly, in a longer run, it may enter in the transpiration stream and accumulates in the tissues at toxic levels. This is salt specific or ion excess effect of salinity (Blumwald 2000; Deinlein et al. 2014; Munns 2005; Munns and Tester 2008). These two mechanisms distinguish a biphasic growth response in plants.

Phase 1: This is the initial growth response of the plant to salt stress, which arises due to an increase in osmotic potential of the soil. It results in a reduction in leaf and root growth (Munns et al. 2005). These symptoms mainly arise due water stress.

Phase 2: This phase of response includes gradual deposition of Na and Cl ions to toxic levels in the older leaves. The level of salt present in the cell cytosol is greater than

their ability to compartmentalize it into vacuoles. This will alter the K^+/Na^+ ratio and Na and Cl ion concentrations within the cytosol resulting in inhibition of enzymatic activity, metabolic processes and photosynthesis. Higher levels of salt may deposit in the cell wall and dehydrate the cell (Munns and Tester 2008).

2.4.2. Halophytes Verses Glycophytes

Based on their salt tolerance ability, plants are classified into two groups: halophytes and glycophytes. Halophytes are plants which survive under high salt conditions normally NaCl, but may also contain a variety of other salts such as Na_2SO_4, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCl and Na_2CO_3. Glycophytes on the other hand, lack the mechanism to survive under high salt conditions and are salt sensitive. Although these are two distinct groups, most of the plant species follow a continuum in respect of salt tolerance ranging from highly susceptible species such as rice and chickpea to the most tolerant *Tecticornia* species in halophytes. The high salt tolerance adaptive mechanisms of halophytes have been studied in detail and are attributed due to differences in the physiological and molecular adaptive mechanisms. Several studies have shown that halophyte genes share homology with their glycophytic counterparts, but differ in terms of their expression and function (Barkla et al. 2013; Bartels and Dinakar 2013; Yang et al. 2013). Halophytes have evolved better capacity to adjust the osmotic potential, exclusion and sequestration of excess salts within the cellular organelles, secretion of excess salts through secretary glands through the transporters and maintenance of net K^+ to Na^+ selectivity (Adem et al. 2014; Ashraf and Ali 2008; Bose et al. 2014; Ozgur et al. 2013; Shabala et al. 2014; Shabala and Pottosin 2014).

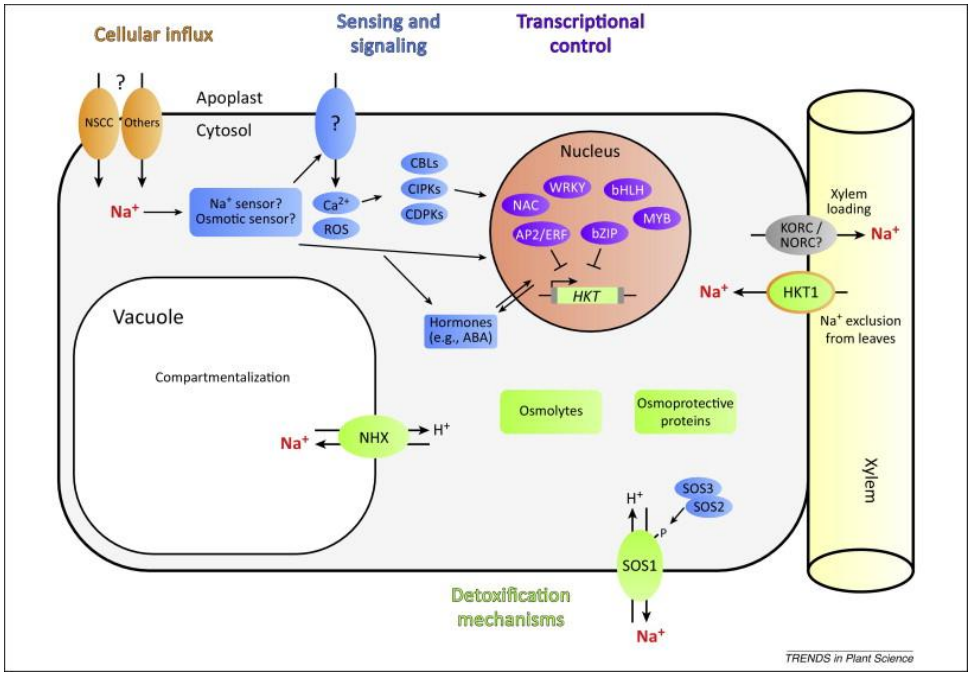
Gene homologs from halophytes have been expressed in the glycophytes, which resulted in improved salt tolerance (Baisakh et al. 2012; Guan et al. 2011; Hamada et al. 2001; Himabindu et al. 2016; Kobayashi et al. 2012; Nah et al. 2009; Oh et al. 2009; Ohta et al. 2002; Zhang et al. 2008; Zhao and Zhang 2006).

2.4.3. Sensory mechanisms of salt stress

Plants have developed unique sensory mechanisms to sense hyperosmotic and ion specific components of salt stress. Understanding these mechanisms of Na^+ entry and translocation to different tissues and within cell compartments is of vital importance to improve crop resistance to salt stress. The overview of Na^+ transport mechanisms

in the plant root cells have been shown in Figure 2.2. Na^+ can enter into roots through multiple Na^+ influx pathways comparising of nutrient channels and transporters. Under normal physiological condition, plants maintain a low cytosolic Na^+ concentration (1-10 mM) (Binzel et al. 1988) and a negative electrochemical gradient across the plasma membrane (Higinbotham 1973). Increase in the extracellular Na^+ ion will establish large electrochemical potential gradient and favours passive transport of Na^+ across the plasma membrane (Blumwald et al. 2000).

Proteins involved in uptake of Na^+ from and its subsequent transport within the the plant cell are present within the plasma membrane. Under saline condition, rapid influx of Na ion across the plasma membrane occurs through the Non-selective cation channels (NSCCs) and later through the high affinity K^+ transporter (HKT1) (Essah et al. 2003; Tester and Davenport 2003). Rice Na^+ transporter OsHKT2;1 has been shown to mediate Na^+ influx into roots under K^+ starvation (Horie et al. 2007).



Source:Deinlein et al. (2014)

Figure 2.2: Overview of cellular Na^+ transport mechanisms and important components of the salt stress response network in plant root cells.

Plants sense the low water potential outside the rhizosphere through membrane transporters. Low water potential changes the turgidity of the cell. This is perceived by the periplasmic domain of the AtHKT transporter and induces the MAPK signaling cascade to alter the gene expression (Chefdor et al. 2006; Urao et al. 1999). Alternatively, cell distortion causes the change in the geometry of mechano-sensitive ion channels resulting in the depolarization of plasma membrane and finally increase in the cytoplasmic Ca^{2+} levels to initiate further signaling to respond to the stimulus (Maathuis 2014). Till date no Na^+ specific sensor has been identified in plants.

2.4.4. Transporters in salt stress

Plants maintain Na^+ ion homeostasis inside the cells through the involvement of ion channels and transporters present across the plasma and endomembrane. In roots, Na^+ enters the plant cell through passive transport due to enormous negative electric potential across the membrane. It may enter through the calcium permeable non selective cation channels (NSSC) and GLR. Most of the Na^+ that enters the root cells is pumped back into the soil through ATP driven Na^+/H^+ antiporters. The intracellular Na^+ is sequestered to vacuoles by tonoplast Na^+/H^+ antiporters. Na^+ reaches the stellar cells by symplastic movement. The net delivery of Na^+ in xylem depends on four components of the transport system (a) influx of Na^+ into outer half of the roots from the rhizosphere (b) Efflux back into the soil solution through active transport (c) Efflux of Na^+ into the inner half of the root to xylem and (d) influx back into these cells from the xylem (Tester and Davenport 2003). HKT transporters are involved in the retrieval of Na^+ from the xylem vessels and appear to be the key players in root to shoot Na^+ partitioning (Byrt et al. 2007; Davenport et al. 2005; Deinlein et al. 2014; Hamamoto et al. 2015; James et al. 2006; Ren et al. 2005; Sunarpi et al. 2005). Arabidopsis HKT1;1 null mutant plants show salt sensitive phenotype with hyperaccumulation of Na^+ in shoots and reduced accumulation in roots (Berthomieu et al. 2003; Maser et al. 2002; Rus et al. 2004). Tissue specific expression of *AtHKT1:1* in the stele tissue enhances salt tolerance (Moller et al. 2009). *AtHKT1;1* expression has been suggested to stimulate the acquisition of K^+ in the xylem vessels via outward-rectifying K^+ channels, resulting in a high K^+/Na^+ ratio in leaves (Ren et al. 2005; Sunarpi et al. 2005). QTL analysis of wheat has identified *Nax1*, *Nax2* and *Kna1* locus which confers salt tolerance (Munns and James 2003). *Nax1* locus contain

TaHKT1;4 gene which was found to contribute to Na^+ removal from xylem in the leaf sheath to protect leaf blades from Na^+ accumulation (Huang et al. 2006).

HKT transporters are negatively regulated by cytokinins via type B response regulator ARR1 and ARR2 (Mason et al. 2010). Exogenous application of cytokinins (CK) reduces the expression of HKT transporters. Transcriptome analysis of a salt tolerant mutant defective CK synthesis showed increased expression of HKT transporters (Nishiyama et al. 2012). Recent studies have identified two transcription factors, ABI4 and AtZIP24 as negative regulators of *AtHKT1;1* expression. The loss of function mutant abi4 shows salt tolerance and reduced Na accumulation. Reduced Na^+ accumulation is correlated with enhanced expression of *AtHKT1;1* expression in *abi4* mutants. Overexpression of *ABI4* decreases the expression of *AtHKT1;1* and results in salt sensitive phenotype (Shkolnik-Inbar et al. 2013). RNAi mediated suppression of AtZIP24 enhances salt tolerance in Arabidopsis by enhancing the expression of *AtHKT1;1* (Yang et al. 2009a).

Class II HKT transporters are known as Na^+/K^+ symporters. This class of transporter has been suggested to contribute to the salt tolerant phenotype due to maintenance of K^+ acquisition through their K^+/Na^+ co-transport under salinity stress. Maintenance of K^+/ Na^+ ratio under salt stress is crucial for plant salt tolerance (Deinlein et al. 2014).

SOS1 is plasma membrane localized Na^+ transporter, which is involved in efflux of major portion of Na^+ ions that enters the cell. Upon entry of excess Na^+ ion inside the cell, a rapid rise in the cytoplasmic calcium takes place. The rise in $[Ca^{2+}]_{cyt}$ is sensed by calcineurin B like protein (CBL4), which was originally identified as SOS3. Ca-SOS3 interaction leads to dimerization of SOS3 that further interacts with a CBL interacting protein kinase (CIPK24) originally named as SOS2 (Halfter et al. 2000; Liu et al. 2000; Liu and Zhu 1998). SOS3/SOS2 complex is targeted to plasma membrane enabling the phosphorylation and activation of membrane bound SOS1 (Qiu et al. 2003; Quintero et al. 2002; Shi et al. 2000).

2.4.5. Compatible solutes

Accumulation of compatible solutes has been used as one of the strategies to survive under abiotic stresses. Compatible solutes are low molecular weight, highly soluble and non-toxic compounds that accumulate at higher concentration in response to

stresses. These molecules help in osmotic adjustment, detoxification of ROS, maintenance of membrane integrity and stabilization of enzymes/ proteins (Ashraf and Foolad 2007; Bohnert and Jensen 1996; Chen and Murata 2002; Yang et al. 2008). These compatible solutes include betaine and related compunds; proline; sugar and sugar alcohols such as trehalose, mannitol and sorbitol. Genetic transformation with the candidate genes involved in the biosynthesis of the various compatible solutes resulted in improved tolerance of the transgenic plants to the abiotic stresses (Chen and Murata 2002; 2011; Hayashi et al. 1997; Karakas et al. 1997; Prasad et al. 2000; Sakamoto et al. 1998; Sakamoto and Murata 2000). Alternatively, exogenous application of glycine betaine or proline has significantly increased drought, salt and temperature stress tolerance (Agboma et al. 1997; Diaz-Zorita et al. 2001; Harinasut et al. 1996; Ma et al. 2006; Makela et al. 1998).

2.4.6. Hormonal control of salt stress

Phytohormones play central roles in the ability of plants to adapt to changing environments, by mediating growth, development, nutrient allocation, and source/sink transitions (Peleg and Blumwald 2011). These compounds are synthesized by plant biosynthetic pathways and may act either at their site of synthesis or are transported to the distant part of the plant body to regulate its growth and development under normal and stress condition. These compounds form an integral part of the plant signalling network. The mechanisms by which these signals are generated and translated into adaptations to counter the unfavourable environment are being intensively studied (Wilkinson et al. 2012). Plant growth regulators include the five classical phytohormones: abscisic acid (ABA), ethylene, cytokinin (CK), auxin (IAA), gibberellin (GA), jasmonate (JA), as well as brassinosteroids (BR), salicylic acid (SA), nitric oxide (NO), and strigolactone (SL), and it is likely that additional growth regulators are yet to be discovered. Studies revealed that phytohormones do not act in isolation but are interrelated by synergistic or antagonistic cross-talks (Peleg et al. 2011).

2.4.6.1. Abscisic acid

Abscisic acid (ABA) is one of the most studied plant hormones, which regulates many physiological and developmental processes including abiotic stress (Finkelstein et al.

2002; Parent et al. 2009; Qin et al. 2011a; Raghavendra et al. 2010). Based on the ABA deficient mutants, ABA biosynthesis pathway has been elucidated. It is now a well known fact that ABA in higher plants is synthesized from C_{40} carotenoid precursor following an indirect pathway (Schwartz et al. 2003; Seo and Koshiba 2002; Xiong and Zhu 2003). Carotenoids are synthesized from the C_5 precursor, isopentenyl pyrophosphate (IPP). IPP is converted to a C_{20} product, geranylgeranyl pyrophosphate (GGPP). Conversion of GGPP to a C_{40} carotenoid phytoene, catalyzed by phytoene synthase (PSY), is the first committed and rate-limiting step in carotenoid synthesis. Subsequently, phytoene is converted to ζ-carotene, lycopene, β-carotene and then to a xanthophyll, zeaxanthin. Phytoene desaturase (PDS) catalyzes the conversion of phytoene to ζ-carotene and is also one of the enzymes dedicated to carotenoid synthesis.

The first step to ABA synthetic pathway from the carotenoids is the two-step epoxidation of zeaxanthin to form all-*trans*-violaxanthin, a reaction being catalyzed by zeaxanthin epoxidase (ZEP) which occurs in plastid (Marin et al. 1996). Next, the xanthophylls, 9-*cis*-violaxanthin and/or 9′-*cis*-neoxanthin are oxidatively cleaved by 9-*cis*-epoxycarotenoid dioxygenase (NCED) and converted into xanthoxin (Schwartz et al. 2003; Tan et al. 1997; Thompson et al. 2000). Further, xanthoxin is transported into cytosol and is converted to ABA through a two-step reaction via ABA aldehyde (Gonzalez-Guzman et al. 2002; Taylor et al. 2000). Conversion of xanthoxin to ABA aldehyde is catalysed by a short-chain alcohol dehydrogenase/reductase (SDR) and final step of ABA biosynthesis is mediated by ABA aldehyde oxidase (AAO) (Seo et al. 2000).

ABA acts as an endogenous messenger in abiotic stress responses in plants and is often called a ‘stress hormone’ (Danquah et al. 2014; Qin et al. 2011a). As the plant perceives the stress signal, ABA synthesis is induced in the vascular tissues and is exported to the distant parts by specific ATP-dependant transporters. The mechanism allows rapid distribution of ABA into neighboring tissues (Raghavendra et al. 2010).

Early ABA signaling events depend upon the function and coordination of three classes of proteins: PYR/PYL/RCAR proposed to function as ABA receptors; Protein Phosphatase 2Cs (PP2Cs), which act as negative regulators, and SNF1-related protein kinase 2 s (SnRKs), which are positive regulators (Danquah et al. 2014; Gosti et al.

1999; Ma et al. 2009; Merlot et al. 2001; Mustilli et al. 2002; Park et al. 2009; Schweighofer et al. 2004; Umezawa et al. 2009; Yoshida et al. 2014; Yoshida et al. 2006). In the absence of ABA, PP2Cs interacts with SnRK and dephosphorylate its kinase activation loop. Dephosphorylation of SnRK2 inhibits it to transmit the signal to downstream targets. Binding of ABA to its receptors PYR/PYL/RCAR promotes the binding of receptors to the catalytic site of PP2C and inhibit its enzymatic activity (Melcher et al. 2010; Miyazono et al. 2009; Nishimura et al. 2009; Santiago et al. 2009).This allows the SnRK2 to autophosphorylate and relay the ABA signal to the downstream effector molecules (Cutler et al. 2010; Hubbard et al. 2010; Umezawa et al. 2010).

ABA plays central role in abiotic stress signaling in plants. It controls the expression of about 10% of the total genes in Arabidopsis (Hoth et al. 2002; Nemhauser et al. 2006) (Hoth et al., 2002; Nemhauser et al., 2006; Seki et al., 2002). Promoters of ABA inducible genes contain *cis* regulatory element ABRE (ABA responsive element) or a combination of ABRE with other elements such as CE1, CE3 and DRE/CRT (Gomez-Porras et al. 2007; Lee et al. 2010; Mishra et al. 2014; Yamaguchi-Shinozaki and Shinozaki 2005; Zhang et al. 2005). ABRE, which contains an ACGT core is recognized by bZIP proteins such as AREB, which induce the transcription of the downstream genes (Choi et al. 2000; Foster et al. 1994; Izawa et al. 1993; Uno et al. 2000).

AREB/ABF are induced by ABA and abiotic stresses such as drought and salinity at vegetative stage (Abdeen et al. 2010; Fujita et al. 2005; Kang et al. 2002; Kim et al. 2004; Uno et al. 2000; Yoshida et al. 2010). AREBS are phosphorylated by the SnRK2, which activates the downstream genes. It has been shown that Ser-Thr protein kinases such as calcium dependant protein kinases (CDPK) also phosphorylate AREB/ABF and thus act as positive regulators in ABA signaling (Zhu et al. 2007).

2.4.6.2. Ethylene

Generally, ethylene biosynthesis and signal transduction enhance plant salt tolerance, while its inhibition leads to increased plant sensitivity to salinity. Like other abiotic stresses, salinity also promotes ethylene biosynthesis by modulating the expression of ethylene biosynthesis genes (Achard et al. 2006; Dong et al. 2011). In plants, ethylene is perceived by a group of membrane-located receptor proteins including ETR1

(ETHYLENE RESPONSE 1), ERS1 (ETHYLENE RESPONSESENSOR 1), ETR2 (ETHYLENE RESPONSE 2), ERS2 (ETHYLENE RESPONSE SENSOR 2), and EIN4 (ETHYLENE INSENSITIVE 4) (Zhao et al. 2011). In the absence of ethylene, these receptors act as negative regulators of the signaling pathway, repressing the ethylene response (Hua and Meyerowitz 1998; Merchante et al. 2013). Briefly, ethylene signaling during abiotic stress starts with the binding of ethylene to the N-terminal of its receptors which results in the deactivation of CONSTITUTIVE TRIPLE RESPONSE1 (CTR1). In the absence of ethylene, CTR1 is involved in the proteasome mediated degradation of EIN2, EIN3 and EIL1. Deactivation of CTR1 allows the cleavage of ETHYLENE INSENSITIVE2 (EIN2), which in turn moves to nucleus and activates EIN3, EIN-3 like1 (EIL1) and EIL2. Activation of EIN3 further activates the downstream transcription factors such as WRKY, AP2/ERF AND NAC s (Ji and Guo 2013; Ju et al. 2015; Kazan 2015; Salehin and Estelle 2015; Wang et al. 2002; Zhang et al. 2016). ERF represents a large family of transcription factors in the plant genome. In Arabidopsis, 124 ERF genes were reported, which are largely divided into two subfamilies (a) DREB and (b) ERF subfamily. Overexpression of several ERF members in the heterologous systems conferred abiotic stress tolerance (Seo et al. 2010; Zhang and Huang 2010; Zhang et al. 2012).

Ethylene signaling pathway has been shown to regulate salt tolerance either positively or negatively. External application of ethylene and its precursor increases salt tolerance by inducing the production of ROS scavengers (Peng et al 2014). Cao et al. (2007) reported that ethylene insensitive mutants (*etr1-1* and *ein2-1*) were more sensitive to salt stress suggesting ethylene signaling may be necessary for plant salt tolerance. Recently, rice SALT TOLERANCE1 (a lectin receptor like kinase) positively regulates salt tolerance by stimulating the activity of the MAPK3/6 protein kinase and ethylene production (Li et al. 2014). Some reports suggest a negative correlation between ACC levels and salt tolerance. Arabidopsis *acs* mutant with reduced levels of endogenous ethylene shows salt tolerance at seed germination stage suggesting it as a negative regulator of salt tolerance. *Arabidopsis* plants overexpressing wheat *ACO1* display elevated ethylene levels, but decreased salinity tolerance (Chen et al. 2014). Ethylene signaling acts in close association in ROS signaling during salt stress responses (Steffens 2014; Xia et al. 2015; Zhang et al. 2016).

2.4.6.3. Jasmonic acid

The role of jasmonates has been well established in the induction of systemic resistance in response to insect and pathogen attacks (Bari and Jones 2009; Mithofer et al. 2014; Wasternack and Hause 2013). However, a lot of accumulating evidence suggests its role in abiotic stress tolerance too. Under non stress condition, plants maintain low levels of Ja-Ile (bioactive form of jasmonic acid), which allows a multimeric protein complex to inhibit JA signaling. But as it encounters an environmental stress such as salinity, levels of JA-Ile get elevated. JA-Ile binds to COI1 and this complex interacts with JAZ proteins leading to SCF mediated ubiquitination. The ubiquitinated JAZ factors were degraded via 26S proteasome pathway. As a result, MYC-type transcription factors were released from the repression of JAZ factors inducing the transcription of early JA-responsive genes (Ismail et al. 2014; Kazan and Manners 2008). High salinity enhances the levels of JA in rice, tomato and *Iris hexagona* (Pedranzani et al. 2003; Tani et al. 2008; Wang et al. 2001). Pretreatment of barley seedlings with methyl jasmonate before salt stress imposition alleviates salt stress induced damage to growth, photosynthetic system and RuBPC activity (Tsonev et al. 1998). Exogenous application of jasmonic acid enhanced salt stress tolerance in wheat seedlings by improving the activities of antioxidant enzymes (Qiu et al. 2014). Overexpression of the wheat (*Triticum aestivum*) TaAOC1 gene, encoding an allene oxide cyclase (AOC) enzyme involved in JA biosynthesis, elevates JA levels and confers salt tolerance in wheat and Arabidopsis, suggesting that JAs positively regulate salt tolerance (Zhao et al. 2014).

2.4.6.4. Brassinosteroids

Brassinosteroids (BRs) represent a group of natural steroid hormones that play critical roles in a wide range of plant developmental processes including cell division and elongation, photomorphogenesis, flower & fruit development, leaf senescence, and various stress responses (Clouse and Sasse 1998; Divi and Krishna 2009; Fahad et al. 2015; Hayat et al. 2010; Khripach et al. 2000; Ozdemir et al. 2004; Vriet et al. 2012). Although the positive role of BRs in promoting tolerance to a wide range of abiotic stresses has been reported, the molecular mechanisms explaining how BRs control stress responses and regulate stress-responsive gene expression in plants are largely unknown.

BRs reduced the salinity-induced inhibition of seed germination and seedling growth in *Oryza sativa* (Anuradha and Rao 2003; Ozdemir et al. 2004). Seed soaking in 24-epibrassinolide prior to germination enhances growth and attenuates the deleterious effects induced by salt stress in pea (Shahid et al. 2011). Studies on *det2-1* and *bri1-1* BR defective mutants display ABA hypersensitive phenotypes during early seed germination raising the possibility of BR interaction with ABA signaling (Steber and McCourt 2001). The salt stress tolerance mediated by brassinosteroids was found to be associated with the induction of stress marker genes, such as *HSP* genes, *RD29A* and *ERD10* (Dhaubhadel et al. 1999; Kagale et al. 2007). BRs-mediated stress tolerance in Arabidopsis was associated with ABA, SA, and ET pathways (Divi et al. 2010). Recent molecular studies propose that BRs act as crucial positive regulators in early seedling development by regulating the signaling outputs of ABA signals (Ryu and Hwang 2013; Zhang et al. 2009).

2.4.6.5. Cytokinin

Over the past decade, the role of cytokinins in the regulation of environmental stress response gained importance in plant biology research (Zwack and Rashotte 2015). Increasing evidence has revealed that CKs play a significant role in the regulation of environmental stress responses, involving intensive interactions and cross talk with abscisic acid (ABA). Environmental stresses, such as drought and salinity, decrease the production and transport of CKs from roots. Exogenous application of CKs increases stomatal aperture and transpiration (Pospisilova et al. 2005) and influences photosynthetic machinery (Chernyad'ev 2009). Cytokinins promote cell division, which is an energy consuming process and is under tight regulation under stress. Mild stress may be associated with a transient increase in the CK levels needed for signaling (Havlova et al. 2008) but, prolonged drought and salt stress are associated with the downregulation cytokinin contents to arrest the plant growth and so as to redistribute the limited resources for defense mechanisms (Albacete et al. 2008; Ghanem et al. 2008; Kudoyarova et al. 2007; Nishiyama et al. 2011; Rashotte et al. 2006). Nishiyama et al. (2011) reported that cytokinin deficient mutants exhibited strong salt tolerant phenotype and ABA hypersensitivity. Constitutive downregulation of CK levels by the overexpression of CKX (cytokinin dehydrogenases) genes or by inactivation of IPT genes results in drought and salt stress-tolerant phenotypes. Generally, CKs and ABA are known to have antagonistic effects (Chang et al. 2003;

Cowan et al. 1999; Gan and Amasino 1995; Zdunek and Lips 2001), but recent studies on CK mutants suggest the existence of an intensive interaction and cross talk between them (Chang et al. 2013; El-Showk et al. 2013). Decrease in the CK is associated with increased sensitivity to ABA and the upregulation of ABA responsive genes (Nishiyama et al. 2011). Exogenous application of ABA in plants leads to downregulation of expression of CK metabolic genes (Nishiyama et al. 2011; Vaseva et al. 2008). Although constitutive overexpression of genes in degradation CK is associated with enlarged root system, it also shows stunted shoot growth. Root specific or stress inducible promoter increased salt and drought stress tolerance in the transgenic plants without affecting shoot growth (Qin et al. 2011b; Qiu et al. 2012; Rivero et al. 2010).

2.4.6.6. Gibberellins

Gibberellins are involved in regulation of plant growth and development at seed germination, leaf expansion, photomorphogenesis, stem elongation and flowering stage (Daviere and Achard 2013; Richards et al. 2001; Sun and Gubler 2004). GA metabolism and signaling pathway are tightly controlled by diverse internal and external factors (Sun and Gubler 2004). Giberellin biosynthesis and signaling are regulated by both biotic and abiotic stresses. Genetic studies with GA mutants have identified DELLA family of proteins as the negative regulators of GA signaling (Sun and Gubler 2004). GA signaling cascade starts with its binding to its receptor that facilitates its interaction with the DELLA proteins. This protein complex then binds SCF E3 ubiquitin ligase resulting in polyubiquitination and finally leading to proteasome-mediated degradation of the DELLA proteins (Claeys et al. 2014; Dill et al. 2004; Griffiths et al. 2007; Nakajima et al. 2006). Exposure to salt stress induced a reduction in endogenous levels of bioactive GAs, which coincided with higher accumulation of DELLA proteins (Achard et al. 2006; Colebrook et al. 2014). Furthermore, a link between DELLA function and survival of salt stress has been identified (Achard et al. 2006; Achard et al. 2008; Magome et al. 2004). Furthermore, GA deficient biosynthetic or signaling mutants displayed enhanced survival of severe salt stress, but the quadruple- DELLA loss-of-function mutant was more susceptible to salt stress (Achard et al. 2006). Exogenous GA3 application, a bioactive GA, in tomato enhanced plant water availability at low salt stress conditions (Maggio et al.

2010). Application of GA_3 alleviates the effects of salt stress in chickpea, soybean and maize seedlings (Hamayun et al. 2010; Kaur et al. 1998; Tuna et al. 2008).

2.5. Glucose tolerance:

Sugars play an important role in plant growth and development by acting as carbon source or as signaling molecules ((Rolland *et al*., 2006; Ramon *et al*., 2008; Bolouri-Moghaddam *et al*., 2010; Smeekens *et al*., 2010; Miao et al 2013). Over the past decade, integrated genetic, cellular, chemical, proteomic and genomic approaches in the reference plant *Arabidopsis thaliana* have begun to unravel the surprisingly broad range of functions and actions of three glucose-modulated master regulators, hexokinase1(HXK1), the energy sensor kinases KIN10/ 11 and the glucose activated target of rapamycin (TOR). *Arabidopsis thaliana* (HXK1), which is a glucose phosphorylating enzyme, has been identified as the glucose sensing protein too. The role of HXK1 as a glucose sensor and signal transducer is independent of its enzymatic function (Jang et al., 1997; Xiao et al., 2000; Harrington and Bush, 2003; Moore et al.).

Glucose plays a negative role in seed germination and early seedling development. It works in close association with plant hormones, particularly ABA (Gibson 2004, Yuan and Wysocka-Diller 2006, Rook et al. 2006, Finkelstein et al. 2008). High levels of exogenous glucose cause ABA accumulation, which delays seed germination and early seedling growth (Arenas-Huertero et al. 2000). Glucose induced ABA accumulation can be induced either by enhancement of ABA biosynthesis genes or suppression of genes involved in its catabolism. In *Arabidopsis*, glucose induced ABA accumulation has been reported to induce ABA biosynthesis genes such as ABA2 and NCED3 (Cheng et al. 2002, Chen et al. 2006) whereas in rice it was mainly due to the suppression of ABA catabolic gene encoding ABA 8′-hydroxylase (Zhu et al 2009). Moreover ABA deficient mutants are found to be glucose insensitive (Arenas-Huertero et al. 2000; Laby et al. 2000). A key link between sugars and ABA perception is exemplified by ABI4, which encodes an AP2 domain transcription factor and is required for normal sugar response during germination and seedling growth (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001).

2.6. Annexins

Annexins represent a multigene family capable of Ca^{2+} dependent and independent membrane binding property. The name of this family of proteins is derived from a Greek word "annex" meaning "bringing/hold together" to describe their principal property of membrane lipid binding. The name also has some historical relevance as some groups working independently discovered them in search of scaffolding or bridging proteins (Gerke and Moss 2002). Initially, several unrelated names were used to describe these proteins like synexin (Creutz et al. 1978), chromo bindins (Creutz et al. 1987), calcimedins (Moore 1984), lipocortin (Flowers 1984) and calpains (Glenny 1987). But subsequent research identified key biochemical, structural and sequence similarities among these independently identified proteins. This led to postulate that proteins identified by several groups in fact belong to a same multigene family who expanded by gene duplications during evolution, and a common name annexin was introduced to describe these proteins.

Annexins have diverse taxonomic distribution covering plants, fungi, protists and invertebrates (Greke and Moss 2002; Hofmann 2004, Moss and Morgan 2004, Mortimer 2008). The first report of plant annexin was made by Boustead et al. (1989) in tomato. Since then, many annexins were reported from different plant species. With the completion of several genome sequencing projects, it becomes clear that annexins represent a multigene family with a variable number of genes encoding for these proteins in different plant species. Arabidopsis genome contains contain eight annexins, ten in rice, thirteen in *Brassica.rapa*.

2.6.1. Plant annexin structure

Plant annexins represent 0.1% of the total protein (Delmer and Potikha 1997) . These proteins have a molecular weight in the range of 32-42 kDa. Structurally, an annexin protein consists of a conserved α helical core and a variable N-terminal region. The core annexin region consists of four domains (I-IV), each domain comprised of 70 amino acids (Fig. 2.3). Each domain consists of five α helices connected by loops. These endonexin sequence K-G-X-G-T-38 - D/E present on each domain confers Ca^{2+} binding ability to annexins. During membrane association, Ca^{2+} ions forms a bridge betwwen the annexin domains and the negatively charged phospholipids. Although plant and animal annexins share a common evolutionary ancestor, there are

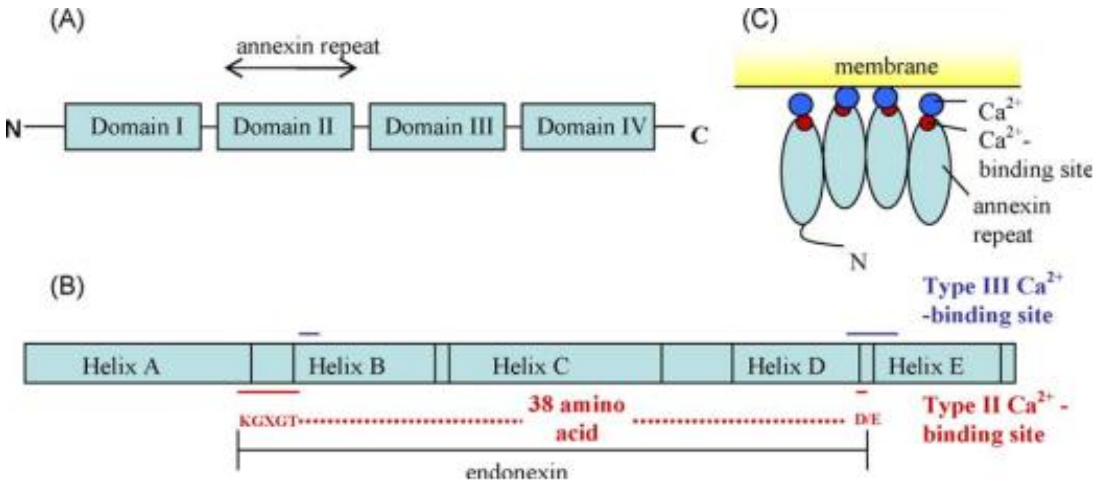
some structural differences among them. Crystal structures reveal that unlike animal counterparts, plant annexins can bind Ca^{2+} only in I and IV annexin domains (Hofmann et al 2000; 2003 and Hu et al 2008).Mammalian annexins represent great variability in the N-terminal region, which is involved in the regulation of conformational stability and protein- protein interactions (Greke and Moss 2002). It is the site of post-translational modifications like phosphorylation, nitrosylation, S-glutathionylation and N-myristoylation (Konopka-Postupolska et al. 2009; Lindermayr et al. 2005; Mortimer et al. 2008). Crystal structure of plant annexins revealed that N-terminal region is conserved in plant annexins and interacts with the annexin core region. Although, the N-terminal region of plant annexins is shorter (~10 amino acids) compared to their animal counterparts.

2.6.2. Ca^{2+} dependent and independent membrane binding of annexins

Annexins (both plants and animals) show Ca^{2+} dependant binding to negatively charged phospholipids such as phosphatidylserine, phosphatidylinositol, and phosphatidic acid (Blackbourn et al., 1991; Balasubramanian et al., 2001). Calcium mediated membrane binding of annexins was attributed by the endonexin sequence (K-G-X-G-T-38 variable residues-D/E) and type III Ca^{2+} binding site. Annexins binding to membranes can be reversed by the addition of Ca^{2+} chelators.

Calcium-bound crystal structure of cotton annexin revealed that it can bind three calcium ions in canonical fashion in the repeats I and IV (Hu et al 2008). Conformational flexibility in the IAB loop region due to presence of a conserved Trp-35 residue which is present in the first repeat plays a crucial role in calcium independent membrane binding of annexins.

Annexins are capable of Ca^{2+} independent membrane binding at neutral pH (Blackbourn et al. 1991; Breton et al. 2000; Hofmann et al. 2000a, 2002; Dabitz et al. 2005). Site directed mutagenesis study in *Capsicum annum* has identified two lysine residues and one tryphtophan residue in the convex side of annexin disc responsible for Ca^{2+} independent binding (Dabitz et al. 2005). Ca^{2+} independent membrane binding suggests it role at membranes under normal condition too.



Source: Laohavisit and Davies (2009)

Figure 2.3: Annexin structure (A) Primary structure of a typical annexin protein with four tandem repeats (I–IV), each containing 70–80 amino acids. (B) A simple diagram showing typical characteristics of one of the annexin repeats. Each repeat has five α- helices (A–E) with loop regions connecting each. The endonexin sequence, type II Ca^{2+}- and type III Ca^{2+}-binding sites are approximately shown in black, red and blue respectively. (C) A simplistic drawing of annexin tertiary structure. Annexin repeats (light blue) form a slightly curved disc-like core domain. The Ca^{2+}-binding sites are exposed at the convex side of the molecule (shown in red), allowing the molecule to attach to a membrane through bound Ca^{2+} ions (dark blue).

2.6.3. Post translational modifications

Annexins undergo post translational modifications depending on the surrounding conditions. They undergo homo- and hetero-dimerization when associated with the membranes (Hofmann et al. 2002). AnnAt1 isolated from Arabidopsis (native system) showed higher peroxidase activity than the purified recombinant protein isolated from a heterologous expression system suggesting that the post translational mechanism necessary for the its function is absent in the heterologous system (Gidrol et al. 2005). Annexins N-terminal chain is the site for post translation changes. Phosphorylation of annexins by kinases has been reported in *Brassica napus*, rice and cotton (Agrawal and Thelen 2006; Rohila et al. 2006; Wang et al. 2013). S-nitrosylation, S-glutathionylation and N-myristoylation of the cysteine residues of annexin indicates its role in distinct signaling pathways (Konopka-Postupolska et al. 2009; Lindermayr et al. 2005; Mortimer et al. 2008).

2.6.4. Peroxidase activity

The first report of peroxidase activity of plant annexins came from a study of Arabidopsis AnnAt1annexin. Heterologous expression of AnnAt1 in H_2O_2 -sensitive *Escherichia coli* ΔoxyR mutant and mammalian cells provides protection under oxidative stress (Gidrol et al 1996; Kush and Sabapathy 2001). Gorecka et al. (2005) expressed AnnAt1 in *E.coli* and *Nicotiana benthamiana* and found a difference in the peroxidase activity of the recombinant proteins. Peroxidase activity of AnnAt1 purified from *N. benthamiana* showed three times higher activity than that of the *E.coli* expressed protein. This finding suggested the possible role of post-translational modifications in peroxidase activity of AnnAt1. Purified soluble annexin proteins display peroxidase activity *in vitro* (Konopka postupolska et al. 2009; Gidrol et al 1996; Gorecka et al. 2005, Jami et al. 2008). Earlier peroxidase activity of plant annexins was attributed due to the presence of a conserved His40 residue present in the first annexin domain, which is highly similar to the~30 amino acid of the horse radish peroxidase (Clark et al. 2001; Gorecka et al 2005). Mutation of His40 to Ala40 abolishes the associated peroxidase activity of AnnAt1 (Gorecka et al 2005). Animal annexins lack this residue and does not possess peroxidase activity. But later on Konopka-Postupolska et al (2009) re-examined of peroxidase activity of native AnnAt1 and mutated His40 annAt1 protein and found comparable peroxidase activity in both forms of protein.

2.6.5. ATPase and GTPase activity

Plant annexins possess ATP and GTP-hydrolysing activity (Calvert et al. 1996; Lim et al. 1998; Mcclung et al. 1994; Qiao et al. 2015; Shin and Brown 1999). Nucleotide binding and hydrolysing property of annexin is proposed to be due to the presence of Walker A motif (GXXXXGKT/S) and GTP binding motif (DXXG). These motifs are present in the fourth domain of annexins and deletion of the fourth domain abolishes its GTPase activity (Shin and Brown 1999). Studies on cotton annexin AnxGh1 revealed that there are two predicted GTP-binding sites in the fourth domain and one of them overlaps with the Ca^{2+} binding site. Thus, there is a possibility that Ca^{2+} and GTP compete with each other for binding to this particular overlapping site. In tomato, Ca^{2+} mediated phospholipid binding impairs GTP hydrolytic activity (Calvert et al. 1996). But, its GTP binding ability persists in actin bound state, suggesting a

role in relocation of annexins within the cell (Calvert et al. 1996). In zucchini (*Cucurbita pepo*), two PM-associated annexins also bind to F-actin in vitro (Hu et al. 2000), which could mean that annexins are part of the apparatus connecting the PM to the cytoskeleton. *In vitro* ATPase/GTPase activity of plant annexins was found to be higher than that of their animal counterparts (Bandorowicz-Pikula, 2003). In a study with maize root protoplasts, Carroll et al. (1998) demonstrated that annexins, Ca^{2+} and GTP together modulate polysaccharide secretion from the cells.

2.6.6. Annexins and abiotic stress

Plant annexins are emerging as the important members involved in stress signaling. This is mainly attributed to their inherent peroxidase activity, Ca^{2+} channel regulating activity and ROS induced expression. Recently, an annexin *AnnAt1* was shown to be involved in OH mediated Ca^{2+} flux changes in the root epidermal cells (Laohavisit et al. 2012; Richards et al. 2014). Changes in the Ca^{2+} flux generate a stimulus-specific Ca^{2+} signature that activates the downstream signaling cascade to elicit stimuli-specific response (Batistic and Kudla 2004; Kudla et al. 2010; Luan et al. 2002; McCormack et al. 2005). Transcriptomic and proteomic studies have identified annexins as one of the highly expressing/abundant proteins during environmental stresses. Salt stress upregulates the expression of *AnnAt1*, *AnnAt4-8* in Arabidopsis (Cantero et al. 2006), *Os08g32970* and *Os09g23160* in rice (Jami et al. 2012), *GmAnn1*, *GmAnn11-12* in soybean (Feng et al. 2013). A proteomic study of NaCl treated soybean hypocotyl tissues revealed the abundance of annexin under salt stress (Sobhanian et al. 2010). In an another root proteomic study with barley cultivars differing in salt tolerance, annexin was found to be one of the abundant protein in the tolerant cultivar (Mostek et al. 2015). Low temperature stress induced the accumulation of rice and wheat annexins (Breton et al. 2000; Hashimoto et al. 2009). Drought stress upregulates the expression of annexins in *Arabidopsi*s (Bianchi et al. 2002), Indian mustard (Jami et al. 2008), Rice (Gorantla et al. 2005; Jami et al. 2012), and *Medicago* (Buitink et al. 2006). Comparative proteome profiles of two rootstock cultivars of citrus with contrasting drought tolerance identified two annexins abundant in the tolerant cultivar (Oliveira et al. 2015). Heat stress enhanced the abundance of annexins in Aabidopsis and *Nelumbo nucifera* (Chu et al. 2012; Wang et al. 2015). A role of these proteins can be postulated in nutritional stress, as boron deficiency upregulated the expression of an annexin in *Brassica napus* (Wang et al. 2010).

Similarly, low potassium stress upregulated the expression of four annexin genes in tobacco seedlings (Lu et al. 2015).AnnAt1 transcripts accumulate in response to phosphate deprivation (Muller et al., 2007). Metal stresses induce the expression of annexins. Zinc affects the expression of *ZmAnx1* and *ZmAnx5* (Zhou et al. 2013); copper affects *AnnAt3* and *AnnAt4* (Weber et al. 2006); cadmium upregulates the expression of *AnnAt*1 and pea root annexin abundance (Konopka-Postupolska et al. 2009; Repetto et al. 2003).

Expression profiling of annexin gene families from Arabidopsis, *Brassica juncea*, *Brassica rapa* , tomato, maize, soybean and peanut has been studied in detail in response to different abiotic inducers and hormones (Cantero et al. 2006; Jami et al. 2009; Yadav et al. 2015; Lu et al. 2012; Feng et al 2013; Zhou et al. 2013; He et al. 2015). This family of proteins is differentially regulated by various abiotic inducers and signaling molecules. Functional characterization of few annexins from model and crop plants proved their role in environmental stress mitigation. Arabidopsis annexin, AnnAt1 overexpression lines confers drought tolerance, while the loss of function (Δ*annAt1)* mutant showed sensitive phenotype (Konopka-Postupolska et al. 2009). Later, it was found that that AnnAt1 and AnnAt4 interact with each other in a Ca^{2+} -dependent manner and function to regulate responses to drought and salt stress (Huh et al. 2010). Ectopic expression of an annexin *AnnBj1* from *B.juncea* in tobacco and cotton conferred multiple stress tolerance (Divya et al. 2010; Jami et al. 2008). Overexpression of *AnnBj3* attenuated the methyl viologen induced oxidative stress in transgenic Arabidopsis (Dalal et al. 2014a). Further, expression of this gene in *Saccharomyces cerevisiae (*ADY1) cells counteracted the thiol-specific antioxidant (TSA1) deficiency in *S. cerevisiae. AnnBj3* protects yeast cells from oxidative stress either by directly detoxifying ROS or positively modulating the endogenous antioxidant system, thereby affecting ROS accumulation (Dalal et al. 2014b). Similarly, Zhang et al. (2015) demonstrated that *GhAnn1* plays an important role in mitigating drought and salt stress by generating overexpression and suppression lines of *GhAnn1*. Constitutive expression of a rice annexin *OsAnn1* imparted heat stress tolerance to the transgenic plants by modulating the production of hydrogen peroxide (Qiao et al. 2015). Potato annexin StAnn1was shown to be involved in mitigating drought stress in transgenic plants (Szalonek et al. 2015). Although, these reports are

available on the role of annexins in stress tolerance, the mechanism underlying the tolerance phenomenon is still not clear.

2.6.7. Role of annexins in plant growth and development

Annexins are ubiquitous proteins. Expression analysis of this family of proteins in different plant species shows their expression is regulated based on the tissue type and developmental stage of the plant (Clark et al.,2001, 2005a; Hoshino et al. 2004; Cantero et al. 2006; Jami et al. 2009: Feng et al. 2013). *A. thaliana* annexins express during seed germination stage (Cantero et al. 2006). *Nelumbo nucifera* annexin *AnnNn*1transcripts were predominantly present at seed development and germination stage; and its ectopic expression in Arabidopsis conferred thermotolerance and germination vigor (Chu et al. 2012). Several annexins show high level of transcripts at the growing tips such as root hairs, pollen tubes, and fern rhizoids (Battey and Blackbourn 1993; Blackbourn et al. 1992; Clark et al. 2005; Clark et al. 2012). Functional characterization of *AnnAt5* through RNAi led to the pollen abortion before maturity (Zhu et al. 2014a; Zhu et al. 2014b). Abundance of some annexins like p35 (maize) and AnnAt1 & AnnAt2 (Arabidopsis) in root elongation zone points towards their role in root growth (Bassani et al. 2004; Carroll et al. 1998) and Arabidopsis (Clark et al. 2005). Immunological studies with Pea and maize annexins revealed that they have high abundance in the young developing vascular tissue and outer cells of root caps producing slime (Clark et al 1992; 1994). This supports the hypothesis postulated by Blackbourn et al. (1991) that annexin like proteins may be involved in the Golgi mediated secretion. Several studies in cotton suggest their role during fiber development (Arpat et al. 2004; Huang et al. 2013; Shin and Brown 1999; Tang et al. 2014; Wang et al. 2010a). Zhao et al. (2010) identified four annexin iso-variants, which get downregulated in the *Ligon lintless* mutant in comparison to wild type of upland cotton. *GhAnn2* preferentially expresses during fiber elongation and functional characterization of this by RNA silencing resulted in thin and shorter fibers in transgenic plants (Tang et al. 2014). Another annexin member from cotton, AnxGb6 regulates fiber elongation through its interaction with actin1 (Huang et al. 2013). AnnGh3 overexpression in Arabidopsis promotes initiation and elongation of leaf trichomes, suggesting its role in fibre cell initiation and elongation in cotton (Li et al. 2013). Certain annexins showed differential expression during fruit development and ripening. Transcriptome studies revealed that transcript levels of a strawberry

(*Fragaria ananassa*) annexin significantly increases during ripening and is regulated by calcium and phytohormones (Chen et al. 2016; Wilkinson et al. 1995).

2.6.8. Reactive Oxygen Species

Plants continuously produce ROS as byproducts of several metabolic processes in different cellular compartments (Apel and Hirt 2004). Excessive production of ROS results in oxidative damage to cellular components, which eventually leads to cell death. However, when produced in a controlled manner, ROS acts as a secondary messenger in signaling (Miller et al. 2008; Suzuki et al. 2012). Under physiological contains, ROS homeostasis is being maintained by different antioxidative defense mechanisms. These antioxidative mechanisms restrict the excessive accumulation of ROS within cell while maintaining an optimum level required for signaling. Environmental stresses such as drought and salinity, increase ROS production in an uncontrolled manner, which perturbs ROS homeostasis and brings an oxidative burst within the cell (Miller et al. 2010; Suzuki et al. 2012). The highly active organelles in the cells with active metabolic pathways like chloroplast, mitochondria and peroxisomes are the major ROS production sites within plant cells (Asada 2006; Mittler et al. 2004). In chloroplast, O_2^- is produced by Mahler reaction and antenna pigments. In addition, singlet oxygen ($^1O_2$) can be produced in PSII by the electron transfer of the excited triplet state of chlorophyll to $^3O_2$, mainly under high light intensities. Peroxisomes generate large quantity of H_2O_2 by the enzymatic activity of glycolate oxidase reaction, fatty acid β-oxidation, and disproportionation of O_2^- ((Bose et al. 2014; Foyer and Noctor 2003; Mittler et al. 2004). Mitochondria are considered the main source of ROS production (H_2O_2) in the non-green tissues such as roots by electron transport chain involving complex I and III. Apart from these major organelles, ROS are also produced at the apoplastic space by NADPH oxidases and cell wall-associated peroxidases (Sagi and Fluhr 2006).

Excessive ROS produced during metabolic pathways are detoxified by various enzymatic and non-enzymatic mechanisms. Enzymatic ROS scavenging enzymes include ascorbate peroxidase (APX), glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT) and peroxiredoxin (Apel and Hirt 2004; Mittler et al. 2004)). Non-enzymatic ROS detoxification processes comprise antioxidants such

as ascorbic acid, glutathione, carotenoids, tocopherols and compatible solutes (Gill and Tuteja 2010; Ozgur et al. 2013). (Gill and Tuteja 2010; Ozgur et al. 2013)

Salt stress results in increased ROS production due to its osmotic and ionic effect (Miller et al. 2010; Xie et al. 2011). Upon abiotic stresses, ROS scavenging enzymes are induced to decrease the concentration of toxic intracellular ROS levels. Annexins have been reported of having an inherent peroxidase activity (Gorecka et al. 2005; Jami et al. 2009a; Konopka-Postupolska et al. 2009). Hence, the annexin-peroxidase connection raises the possibility that annexins exhibit a role in limiting the duration of a plant's exposure to ROS or preventing the accumulation of damaging levels of these agents during stress-induced oxidative burst in plants (Konopka-Postupolska et al. 2009).

2.6.9. ROS and Ca^{2+} signaling

Reactive oxygen species (ROS) and calcium (Ca^{2+}) are connected together to lead to a specific and adaptive response to a given biotic or abiotic stimulus (Gilroy et al. 2014; Mazars et al. 2010; Potocky et al. 2012; Rodriguez-Serrano et al. 2009; Steinhorst and Kudla 2013; Van Breusegem et al. 2008). Several reports suggest a cooperative relation between ROS and Ca^{2+} signal. ROS activates Ca^{2+} channel in the guard and root cells (Demidchik et al. 2007; Mori and Schroeder 2004; Murata et al. 2001; Pei et al. 2000). Alternatively, Ca^{2+} induces cellular ROS accumulation (Kobayashi et al. 2007; Takeda et al. 2008). In plants, respiratory burst oxidase homolog (RBOH) proteins are the most studied ROS-producing enzymes (Suzuki et al. 2011; Marino et al. 2012). These enzymes are integral membrane proteins capable of generating superoxide anions and are rapidly converted in H_2O_2. RBOH proteins possess two Ca^{2+}-binding EF-hands and multiple phosphorylation sites in their N-termini (Baxter et al. 2014; Dubiella et al. 2013; Suzuki et al. 2011). RBOH proteins are activated by Ca^{2+} binding and subsequent phosphorylation of their N terminal region.

In past few years, ROS waves coupled with Ca^{2+} signaling has been highlighted in initiating a rapid systemic signaling in response to abiotic stress (Gilroy et al. 2016; Gilroy et al. 2014; Miller et al. 2009; Mittler et al. 2011).. An external stimulus perceived by a tissue or organ is communicated to the distant parts via cell to cell communication. This signal passes through the different cells in the form of a ROS

wave and spreads through the entire plant at a rate of 8.4 cm/min (Miller et al. 2009). In response to external stimuli, a Ca^{2+} flux is generated in the cytosol, which triggers a signaling cascade to activate the downstream kinases; these kinases in turn phosphorylate the N termini of membrane localized RBOH proteins. Ca^{2+} binding and phosphorylation of RBOH proteins activate them generating ROS in the cell apoplastic region. ROS in the apoplastic region triggers a calcium flux in the nearby cells, which in turn activates their RBOH proteins. This phenomenon of ROS induced calcium flux and calcium induced activation of RBOHs leads to the auto-propagation of the signal to induce a systemic response in the whole plant (Gilroy et al. 2014).

2.6.10. Role of annexins in calcium mediated signalling in plants

Annexins are emerging as one of the important members of calcium signaling in plants. It is mainly due to their calcium binding and channel properties. Plants use $[Ca^{2+}]_{cyt}$ flux as the crucial component of signaling. Increase in the $[Ca^{2+}]_{cyt}$ triggers a signal transduction cascade through the activation of kinases. Proteins involved in generating the Ca^{2+} flux are poorly understood in plants. Much attention has been focused on GLRs and CNGC as the major calcium permeable channels (Dietrich et al. 2010; Dodd et al. 2010; Hedrich 2012). Recently, plant annexins are proposed as one of the key players in Ca^{2+} signaling pathway. The channel property of animal annexins has been studied widely (Gerke et al. 2005; Huber et al. 1990; Kourie and Wood 2000; Rojas et al. 1992), but its counterparts in plants are in the emerging phase.

Plant annexins are able to form Ca^{2+} permeable channels in membrane lipid bilayers (Laohavisit et al. 2010; Laohavisit and Davies 2009; Laohavisit et al. 2012) . These soluble proteins are capable of membrane binding and insertion (Laohavisit and Davies 2011). The most abundant annexin in Arabidopsis, AtANN1, can exist as a plasma membrane protein (Lee et al. 2004). The first report on the ability of annexins to form Ca^{2+} permeable channels came from the study *on Capsicum* annexin, where they are found to generate Ca^{2+} flux across the liposomes (Hofmann et al. 2000). Later, purified *Zea mays* annexins ZmANN33/35 were found to generate a Ca^{2+} flux across the root protoplast plasma membrane of Arabidopsis (Laohavisit et al. 2009). In vitro lipid bilayer experiments demonstrated that maize annexins, ZmANN33/35 could generate Ca^{2+} influxes in slightly acid condition (pH 6.0). Pre-incubation of

ZmANN33/35 with a calcium channel blocker lanthanides such as Gd^{3+} or La^{3+} completely inhibited elevation of $[Ca^{2+}]_{cyt}$ in the protoplasts (Laohavisit et al. 2009). Recently, Richards et al. (2013) have reported that AnnAt1is involved in root $[Ca^{2+}]_{cyt}$ elevation in response to hydrogen peroxide (H_2O_2). Laohavisit et al. (2013) demonstrated that Arabidopsis AtANN1 is involved in mediating ROS induced changes in $[Ca^{2+}]_{cyt}$ in response to NaCl across the plasma membrane of root epidermal protoplasts. Electrophysiological analysis showed absence of NaCl activated Ca^{2+} influx currents in loss of function mutant *Atann1*. Modification of lipid bilayers by ROS has been found to influence the Ca^{2+} transport function of annexin (Laohavisit et al. 2009).

Chapter 3

Materials and Methods

3.1 Plant Materials

Seeds of *Brassica juncea* (L.) Czern & Coss variety Pusa Jai Kisan and *Brassica rapa* var. Pusa gold were procured from National Research Centre for Plant Biotechnology, New Delhi. For tobacco transformation, *Nicotiana tabacum* L. cv Xanthi were used for the present study.

3.2. Chemicals

All the general chemicals used in the present study were procured from Sigma-Aldrich, USA; Himedia, India; Qualigens fine chemicals, India

3.3. Restriction enzymes and markers

All restrictions enzymes and ladders were obtained from Fermentas, Germany.

3.4. Plasmid DNAVectors

- pTZ57R/T was used for cloning the PCR products.
- pRT100 was used for the incorporation of CaMV35S promoter and polyA termination signal in the overexpression construct.
- pCAMBIA2300 binary vector was used for plant transformation.

3.5. Preparation of competent cells of *Escherichia coli*

Competent cells of *E. coli* DH5α were prepared by $CaCl_2$ method. A loop full of frozen cells of *E. coli* DH5α strain was streaked on Luria Agar (LA) plate and incubated at 37 ºC for overnight. Single colony of overnight grown *E. coli* DH5α was inoculated in 10 ml of LB medium and was incubated overnight at 37 °C on a rotary shaker at 200 rpm. Next day, 200 µl of the overnight grown culture was reinoculated in 100 ml of fresh LB broth and incubated at 37 ºC on a rotary shaker at 200 rpm until the O.D reached to 0.3 at 600 nm. Then the *E. coli* culture was cooled by keeping on ice for 20 minutes and transferred to prechilled 50 ml Oakridge tubes. The culture was pelleted down by centrifuging at 4000 rpm for 5 min at 4 °C. After centrifugation, the supernatant was discarded carefully without disturbing the pelleted cells. The pellet was resuspended by adding 20 ml of chilled 0.1 M $CaCl_2$ and incubated on ice for 20 min. After incubation the cells were again pelleted down by centrifuging at 4000 rpm

for 5 min at 4 °C. Again the supernatant was discarded and the pelleted cells were resuspended in ice cold solution of 0.1M $CaCl_2$ and 15% glycerol. The cells were mixed by gentle pipetting and kept in 200 µl aliquots in 1.5 ml sterile tubes. The tubes were immediately frozen in liquid nitrogen and stored in -70 °C deep freezer.

3.6. Transformation of *E.coli* competent cells

Around 50 to 100 ng of the plasmid construct carrying the desired gene was added to *E. coli* competent cells and the cells were incubated on ice for 10 to 30 min. A heat shock was given at 42 ^{0}C in a water bath for 90 seconds and the culture was immediately chilled on ice. The volume was made up to 1.0 mL by adding 800 µl of sterile Luria-Broth (LB) medium. The tubes were then incubated on a rotary shaker at 37 ^{0}C for 1hr. After recovery and growth of the transformed cells, they were plated on LA medium containing selection antibiotics. The plates were incubated for overnight at 37 ^{0}C incubator for the appearance of colonies. Plates with visible colonies were stored at 4 ^{0}C in a refrigerator. Plasmid DNA was isolated from the colonies to check and confirm the transformation.

3.7. Plasmid isolation (miniprep) from *E. coli* (Sambrook and Russell, 2001)

A single colony of *E. coli* cells after transformation was incubated in 10 mL of LB medium with appropriate antibiotics on a rotary shaker at 37 ^{0}C and 200 rpm for 12-16 h. This overnight grown culture was taken in 1.5 mL micro-tube and was centrifuged at 12000 rpm for 60 seconds at 4 ^{0}C. The supernatant was removed and the pellet was suspended in 100 µl sterile ice cold Solution I [25 mM Tris Cl (pH 8.0), 10 mM EDTA [Ethylene Diamine Tetra Acetic Acid (pH 8.0)], 50 mM Glucose, stored at 4 ^{0}C] using a vortex mixer. To the suspension, 150 µl of Solution II [0.2 N NaOH, 1% SDS, freshly prepared and stored at RT] was added. The contents were mixed thoroughly by inversion till the solution became clear. The tubes were incubated on ice for 10 min. After the lysate got chilled, 200 µl of Solution III [3.0 M potassium acetate (pH 4.8), autoclaved and stored at RT] was added. The solution was mixed thoroughly by gentle inversion. The tubes were incubated on ice for a further 5 min. The contents of the tubes were centrifuged at 4 ^{0}C and 12,000 rpm for 10 min in a cooling centrifuge. The supernatant was transferred to a fresh 1.5 mL microtube. From a stock of 10 mg mL^{-1} of RNase, 2-3 µl was added to the lysate and was incubated at 37 ^{0}C in a water bath for 1 hr. The lysate was treated with phenol:

chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) successively and was centrifuged after each treatment at RT and 12,000 rpm for 15 min to separate the aqueous phase from the organic layer. The upper aqueous layer was separated to a new tube without disturbing the middle protein layer. The purified dsDNA was precipitated with two volumes of 100% chilled ethanol or an equal volume of isopropyl alcohol. The mixture was allowed to stand at -20 ^{0}C for 30 min and the DNA was collected at the bottom of the tube by centrifuging at 4 ^{0}C and 12,000 rpm for 10 min. The supernatant was decanted completely and the pellet was rinsed with 1.0 mL of 70% ethanol. The pellet was air dried and dissolved in 30 to 50 µl TE buffer [10 mM Tris HCl and 1.0 mM EDTA (pH 8.0)]. The isolated plasmid DNA was stored at -20 ^{0}C. Plasmid isolations were also carried out using kit (Sigma-aldrich, USA) following the manufacturer's instructions.

3.8. Preparation of *Agrobacterium* competent cells and transformation

Competent cells *of Agrobacterium tumefaciens* EHA105 was prepared as described for *E. coli* except that cells were grown at 28 ^{0}C. Freeze thaw method (Holsters et al., 1978) was used for Agrobacterial competent cells transformation. It was performed by immediate freezing of competent cells in liquid nitrogen after adding plasmid DNA and then followed by incubation in a 37 ^{0}C water bath for 5 min. To this, up to 1 mL of LB medium was added and incubated at 28 ^{0}C for 3-4 h with continuous shaking. The cells were pelleted at 5000 rpm for 5 min and plated on LB agar medium supplemented with rifampicin and the corresponding selectable marker of the plasmid DNA. For long-term storage of the transformed cells, liquid cultures of the cells were stored at -70 ^{0}C after adding sterile 50% glycerol.

3.9. Agarose gel preparation and electrophoresis:

DNA fragments were resolved by using 0.8% agarose gel, prepared by melting 0.8 g agarose (A-9539, Sigma-Aldrich, St. Louis, USA) in 100 mL of 1x TAE buffer [50x TAE: 2.0 M Tris Cl, 1.0 M Acetate, and 100 mM EDTA (pH 8.0)]. From the stock of ethidium bromide (10 mg mL-1) solution, 2 µl was added in melted agarose and the gel was cast in the tray fitted with a proper comb. After the polymerization, they tray was kept inside the electrophoresis tank containing 1x TAE buffer so as to cover the gel. The DNA mixed with 6x loading dye [0.15% bromophenol blue, 0.15% xylene cyanol, 5.0 mM EDTA, 40% sucrose] to a concentration of 1x and was loaded in the

wells created by the comb. The gel was electrophoresed at 70 V for 1 h or till the dye front covered almost 3/4th of the length of the gel. A molecular weight marker was loaded along with the samples for reference.

3.10. Purification of DNA fragments from the agarose gel

After the PCR amplification or restriction digestion of plasmid DNA constructs, the identified DNA bands or plasmid inserts were cut out along with the gel slice, weighed and taken in a micro-tube. GenElute Gel Extraction Kit (Sigma, USA) was used for extracting DNA from agarose gel following manufacturer‘s instructions.

3.11. Ligation

T4 DNA ligase (Fermentas, Germany) was used in various independent experiments during ligation. The reaction mixture was made up in a total volume of 20 µl comprising 2 µl ligation buffer (10X), appropriate volumes (in µl) each of linear insert DNA and digested plasmid DNA, and finally T4 DNA ligase (1-2 U for cohesive ends and 5 U for blunt ends). For cohesive ends, the reaction mixture was incubated for 16 h at 16 ^{0}C and blunt end ligation at 22 ^{0}C overnight.

3.12. RNA isolation

Total RNA from different samples were isolated by using the TRI-Reagent (Sigma-Aldrich, USA), following the manufacturer’s instructions.

3.13. Genomic DNA extraction

CTAB method (Murray and Thompson, 1980): Plant genomic DNA isolation was done from the second or third leaf from the shoot tip of young plants. The leaves were freshly collected, frozen in liquid nitrogen and stored at -70 ^{0}C. The leaf tissue (100-500 mg) was homogenized to fine powder using liquid nitrogen along with a pinch of PVPP (Polyvinyl Polypyrrolidone). About 1.0 mL of CTAB buffer (Cetyl/ Hexadecyltrimethyl Ammonium Bromide) extraction buffer [2% CTAB, 100 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl and 2% β- mercapto ethanol (β-merc)] was taken in 2.0 mL micro tubes and homogenized powder was transferred to the tube, and mixed well to suspend the powder uniformly by repeated inversion of the tubes. The mixtures were incubated at 65 ^{0}C for 1 h with intermittent mixing. After incubation, 0.5 mL of Chloroform: Isoamyl alcohol (24:1) mixture was added

and mixed thoroughly by repeated inversion. The two phases were separated by centrifugation at 14,000 rpm for 15 min. The upper aqueous layer was taken in a fresh 2.0 mL tube. The nucleic acid content was precipitated from the aqueous phase by mixing well an equal volume of isopropyl alcohol and incubating the tubes at -20 ^{0}C for a minimum of 30 min. The tubes were centrifuged at 12,000 rpm for 15 min to sediment the nucleic acids. The solution was decanted completely and 1.0 mL of 75% ethanol was added, and incubated for 10 min at RT. The tubes were centrifuged at 12,000 rpm for 5 min and ethanol was decanted. The pellet was air-dried and dissolved in required volume of TE [10 mM Tris HCl and 1.0 mM EDTA (pH 8.0)] buffer. For further purification, DNA was treated with DNase free-RNase (1mg/mL) for 2 h at 37 ^{0}C. Once again, the sample was treated with phenol: chloroform: isoamyl alcohol (25: 24:1) and twice with chloroform: isoamyl alcohol (24:1) for the removal of any residual protein contamination. Each time the organic phase was mixed thoroughly with the aqueous, centrifuging at 12,000 rpm for 15 min and collecting carefully the upper clear aqueous phase in a fresh tube. Finally, the purified DNA was precipitated by adding 1/10th volume of 3M sodium acetate, (pH 5.2) and one volume of isopropanol followed by centrifugation at 12,000 rpm for 15 min at 4 ^{0}C. The pellet was washed with 70% ethanol, dried and dissolved in TE. Genomic DNA samples were stored at –20 °C for long-term use.

3.14. Quantification of DNA and RNA

The quality and concentration of DNA and samples were examined by agarose gel electrophoresis (ethidium bromide stained) and Nanodrop spectrophotometer (Thermo scientific).

3.15. Polymerase Chain Reaction (PCR)

Consumables from Sigma-Aldrich (USA) and Invitrogen (USA) were used in PCR reactions. PCR reactions were performed on Biorad thermal cycler, USA or Eppendorf Personal Thermal cycler, Germany. PCR conditions were optimized according to the template and primer combinations.

3.16. Preparation of Murashige and Skoog (MS) Media stocks

All MS media stocks were prepared in sterile double distilled water and stored at 4ºC.

Stock A	**20X (1 L)**	
Ammonium nitrate	NH_4NO_3	33g
Potassium nitrate	KNO_3	38g
Calcium chloride	$CaCl_2.2H_2O$	8.8g
Magnesium sulphate	$MgSO_4.7H_2O$	7.4g
Monopotassium phosphate	KH_2PO_4	3.4g
Stock B	**500X (100ml)**	
Boric acid	H_3BO_3	0.310g
Potassium iodide	KI	0.0415g
Sodium molybdate	$NaMoO_2.2H_2O$	0.0125g
Cobalt chloride	$CoCl_2.6H_2O$	0.00125g
Manganese(II) sulphate	$MnSO_4.7H_2O$	1.115g
Zinc sulphate	$ZnSO_4.4H_2O$	0.430g
Copper sulphate	$CuSO_4.5H_2O$	0.00125g
Stock C	**500X (100ml)**	
Ethylenediaminetetraacetic acid ferric salt	$C_{10}H_{12}N_2NaFeO_8$	1.393g
Stock D	**500X (100ml)**	
Thiamine HCl	5mg	
Pyridoxine HCl	25mg	
Niacin	25mg	
Glycine	100mg	
Stock F	**500X (100ml)**	
Myo inositol	5g	

Growth regulators

6 benzylaminopurine (BAP)	1 mg/ml
Naphthalene acetic acid (NAA)	1mg/ml

Preparation of Stock solutions for Southern Blotting

20X SSC (1L)

Tri sodium citrate (0.3M)	88.2g
NaCl (3M)	175.3g

Depurination solution (0.25 M HCl) 200 ml

HCl	4.5 ml
Double distilled water	195.5 ml

Denaturation solution (200 ml)

NaCl (1.5 M)	17.53g
NaOH (0.5 M)	4g

Neutralization solution (200 ml) pH 7.5

NaCl (1.5M)	17.53g
Tris base (1M)	24.2 g

Hybridization buffer

DIG Easy Hybridization buffer (Roche applied science, Germany; catalogue no. 11603558001) was used for pre-hybridization and probe hybridization.

Post Hybridization Wash buffer I (2X SSC + 0.1% SDS) 200 ml

20X SSC	20 ml
10% SDS	2 ml
Double distilled water	up to 200 ml

Post Hybridization Wash buffer II (2X SSC + 0.1% SDS) 200 ml

20X SSC	5 ml
10% SDS	2 ml
Double distilled water	up to 200 ml

Immunological detection

Washing Buffer (200 ml) pH 7.5

Maleic acid (0.1 M)	2.80 g
NaCl (0.15M)	1.74 g
Tween 20 (0.3% v/v)	0.6 ml

Maleic acid buffer (200 ml) pH 7.5

Maleic acid (0.1M)	2.53 g
NaCl (0.15M)	1.75 g

Detection Buffer (100 ml) pH 9.5

Tris-HCl (0.1M)	1.21 g
NaCl (0.1M)	0.58 g

Blocking solution (10X)

The blocking reagent supplied in the DIG DNA Labeling and Detection Kit (Roche applied science, Germany; Cat. no. 11093657910) has been prepared by dissolving it in 1X Maleic acid buffer to prepare a 10X blocking stock solution. The 10X stock solution was stored at 4 ºC. For blocking the membranes, 1X blocking solution has been freshly made by diluting it with Maleic acid buffer

Antibody solution

Anti-Digoxigenin-Alkaline phosphatase conjugate (750 U/ml) was diluted using 1X blocking solution to make 150 mU/ml.

Chapter 4

Generation of *AnnBj2* overexpressing transgenic tobacco plants and their evaluation under salt stress

4.1. Background

In nature, plants are exposed to diverse environmental cues. They have an inbuilt adaptive mechanism to counteract these stresses. These adaptive mechanisms vary from one species to another at morphological, physiological and molecular levels. At molecular level, as the plant encounters a stress, it is perceived by some sensor molecules mostly present on the cell membrane, which activate a signaling cascade through the activation of kinases. The signaling cascade finally synthesizes some proteins or compatible solutes to reestablish the cellular homoeostasis. This ability to respond to a particular stress depends upon the balance between the perception of the signal, activation of the signaling cascade and synthesis of required defense proteins, which either directly acts against the stress or further active the downstream signaling pathway. Most of the biotic and abiotic stress results in elevation of cytosolic $[Ca^{2+}]_{cyt}$, which is sensed by proteins that initiate a Ca^{2+} signaling pathway (McCormack et al. 2005; Kudla et al. 2010).

Annexins form a multigene family with diverse functions. These proteins can bind plasma and endomembranes in a Ca^{2+} and pH dependent manner (Blackbourn et al. 1991; Gerke and Moss 2002). These proteins are ubiquitously present in the cell. Transcriptome and proteome data from several plant species identified annexins as a possible player in plant signaling pathway (Clark et al. 2012). Their expression is regulated by ABA, H_2O_2, MeJa, SA, ethylene, or by the stress inducing chemicals such as NaCl, PEG and sorbitol, which mimic saline, drought or osmotic stress respectively (Cantero et al. 2007; Jami et al. 2009; Jami et al. 2012; He et al. 2015). Gene expression profiling of annexin family demonstrates their differential expression in response to a specific stimuli or stress (Cantero et al. 2006; Jami et al. 2009; Lu et al. 2012; Yadav et al. 2015; Zhou et al. 2013; Xu et al 2016). This suggests their involvement in unique signaling pathway. During past decade, several groups reported the potential role of annexin members in mitigating salt, drought or osmotic stress by genetic manipulation of the model and crop plants. AnnAt1 overexpression in Arabidopsis conferred drought tolerance (Konopka-Postupolska 2009). Studies on cotton annexins provided evidence for its role in fiber elongation (Tang et al. 2014). AnnGh1 provided drought and salinity tolerance to transgenic cotton (Zhang et al. 2015). Rice annexin OsAnn1 conferred heat tolerance by modulating H_2O_2 production

in rice (Qiao et al. 2015). Such functional characterization of annexins supports the hypotheses of involvement of annexins in plant abiotic stress tolerance.

Earlier six annexins from Indian mustard were reported (Jami et al. 2009). Among these, the roles of *AnnBj1* and *AnnBj3* under stress were studied by ectopic expression in heterologous systems. Transgenic plants expressing *AnnBj1* exhibited multiple stress tolerance in tobacco and cotton (Jami et al. 2008 and Divya et al. 2010). *AnnBj3* alleviated oxidative stress in transgenic Arabidopsis and complemented *TSA1* mutant of *Saccharomyces cerevisiae* (Dalal et al. 2014).

In continuation with our previous study, we present here the expression pattern of *B. juncea* annexins at seedling stage (5 d old). Based on our results of expression studies at seedling stage, we studied the function of *AnnBj2* in the in the model system *Nicotiana tabacum* in response to salt stress.

The partial promoter of *AnnBj2* isolated by Jami et al. (2009) revealed the presence of various *cis*-acting elements that are reportedly involved in abiotic stress signaling. It shows the presence of MYB, MYC core and ERD1 elements, which have proven roles in water and dehydration stress (Abe et al. 2003; Abe et al. 1997; Simpson et al. 2003); GT-1 motif involved in pathogen and NaCl-induced gene expression (Park et al. 2004), CCAAT box present in the promoters of heat shock proteins, CuRE (responsive to copper element), W- box motif related to SA-mediated defense response (Maleck et al. 2000; Quinn et al. 2000; Rieping and Schöffl 1992; Rushton et al. 2002). Tissue specific expression of *AnnBj2* and its homolog in Arabidopsisis is higher in roots compared to other tissues. Based on these observations, we anticipated that it might play some role in abiotic stress tolerance or signaling. Therefore, we generated transgenic lines of this tobacco ectopically expressing the gene and analyzed the transgenics for salt tolerance.

4.2. Materials and methods

4.2.1. Plant material

For cloning the *AnnBj2* gene, *B. juncea* variety Pusa Jaikisan was used. Tobacco transformation was done in *Nicotiana tabacum* cv. Xanthi.

4.2.2. Preparation of *AnnBj2* overexpression construct

For constructing the overexpression cassette for *AnnBj2*, it was PCR amplified from the cDNA with *Nco*I and *Xba*I restriction sites in the forward (5'CGGGATCCATGGCGTCTCTCAAAAGTCCC3') and reverse (5'GCTCTAGATCAGACATCCCCATGTCCGAG3') primers respectively. The PCR amplified product was double-digested with these two restriction enzymes and cloned into the corresponding sites of pRT100 vector for the incorporation of CaMv35S promoter and Termination signal. This cassette was released from the pRT100 by *Pst*1 digestion and cloned into the corresponding site of binary vector pCAMBIA2300, which was designated as *AnnBj2*-pCAMBIA2300. The construct was then mobilized in the *Agrobacterium tumefaciens* EHA105 by the standard freeze- thaw method of transformation.

4.2.3. Generation of ectopic expression lines in Tobacco

To generate AnnBj2 overexpressing transgenic tobacco plants, mature leaves from two month old plants grown in green house were used for *Agrobacterium* mediated transformation as described by Horsch et al (1985). Fully expanded leaves were surface sterilized with 0.1% mercuric chloride for 5 minutes followed by washing with sterile double distilled water for 3 times. Sterilized leaves were cut into small pieces and incubated with *Agrobacterium* suspension culture for 30 minutes. The explants were transferred to co cultivation media (MS+2mg/l BAP+0.1mg/l NAA) and kept in the culture room maintained at 26°C±2°C with 16/8, light/dark condition. After two days of co cultivation, explants were transferred to shoot regeneration medium (MS + 2mg/l BAP+0.1mg/l NAA+ 250mg/l cefataxime and 125 mg/l kanamycin. The explants were subcultured after every two weeks. After two subcultures, green shoots developed from the explants and were shifted to shoot elongation media (MS +1BAP+250mg/l cefataxime and 125mg/l kanamycin. Elongated shoots were transferred to rooting medium (MS +0.5mg/l NAA+250mg/l cefataxime and 125mg/l kanamycin). Plantlets with well-developed root system were transferred to soil in small plastic cups and covered with transparent polythene bags to maintain humidity and kept in culture room for acclimatization. Well established plants were shifted to pots and allowed to grow to maturity to set seeds.

4.2.4. Molecular confirmation of the transgenic plants

The transgenic plants raised as described earlier were screened by PCR for the presence of *nptII* and *AnnBj2* transgenes using gene specific primers. Seeds from the transgenic plants were screened by germinating them on ½ MS media supplemented with 150 mg/l kanamycin. After two weeks, the seedlings that remained green were transferred to soil to obtain the T_1 plants. Homozygosity of the transgenic lines was checked by 100% seed germination in the presence of selection marker kanamycin (125 mg/l) in T_2 generation.

4.2.4. Seed germination assay for salt tolerance

Seeds of NS and transgenic lines were surface sterilized with 2.0 % sodium hypochlorite for 5 minutes followed by washing with sterile double distilled water for four times. Then the seeds were kept on germination medium composed of half strength MS salts supplemented with 0, 100 and 200 mM NaCl. The greening of cotyledons was used as the scorable marker for seed germination assay.

4.2.6. Seedling assay

To assess the NaCl and ABA tolerance, 10 d old T_2 seedlings of AnnBj2 OE tobacco transgenic plants grown on ½ MS media were used along with the Null segregant (NS) seedlings germinated in a similar way. The seedlings were transferred to fresh ½ MS media supplemented with 0,100, 200 and 300 mM NaCl. To compare ABA sensitivity of the seedlings 4 and 8µM ABA concentration was used along with ½ MS medium. Data were recorded after 12 d of growth on stress media. Seedling assays were carried out with three technical replicates. The seedling assays were repeated three times to ensure reproducibility of the phenotype.

4.2.7. Chlorophyll content

Total chlorophyll was extracted from the control and NaCl-treated seedlings following Hiscox and Israelstam (1979) protocol. A sample of 100mg was extracted in 4 ml of DMSO according to the protocol described by Hiscox and Israelstam (1979) from the control and NaCl-treated samples. The absorbance was taken at 645 and 663 nm against DMSO as a control.

4.2.8. Proline estimation

Proline content was estimated using the standard procedure described by Bates et al. (1973). For estimation of proline under normal and salt stress condition, seedlings grown in stress media for twelve days were used. 100 mg of samples from mock and NaCl treated seedlings were extracted in 2 ml of 3% of sulposalicylic acid. The homogenate was centrifuged and 100 µl of supernatant was reacted with 100 µl of 3% of sulfosalicylic acid, 200 µl glacial acetic acid and 200 µl acid ninhydrin mixture by boiling at 100°C for 1 hour. The reaction was stopped by keeping on ice and 1.2 ml of toluene was added to the samples. The chromophore containing toluene was transferred to fresh tube. Absorbance of the chromophore was read at 520 nm using toluene as a blank. Proline concentration was determined from a standard curve and expressed as µg g^{-1} FW.

4.2.9. Lipid peroxidation

Lipid peroxidation of the samples was estimated by measuring thiobarbituric acid reactive substances (TBARS) following the protocol described by Heath and Packer (1968). Control and treated samples (100 mg) were homogenized in 0.5 ml of 0.1% TCA and the homogenate was centrifuged at 12,000 rpm (4°C) for 10 minutes. Supernatant (0.5ml) was mixed with 1.5 ml of 0.5% (w/v) TBA in 20% TCA (w/v) and incubated at 95°C for 30 minutes. The reaction was stopped by keeping the tubes on ice followed by centrifugation for 5 minutes at 12,000 rpm (4°C). The absorbance of the resultant supernatant was measured at 532 and 600 nm. OD_{600} values were subtracted from the MDA-TBA complex values at 532 nm and MDA concentration is calculated using the Lambert-Beer law with an extinction coefficient εM= 155 $mM^{-1}cm^{-1}$. Results are presented as nmols MDA g^{-1}FW.

4.2.10. Relative water content

To calculate the relative water content, three-week old seedlings grown on ½ MS medium were transferred to liquid ½ MS medium supplemented with 100 and 200mM NaCl for 72 hours. Fresh weight (W) of the seedlings was taken and immediately and the samples were hydrated completely for twelve hours to obtain turgid weight (TW). Samples were oven dried for 48 hours at 60°C to determine its dry weight (DW). Relative water content was measured using the following equation.

RWC (%) = [(W-DW) / (TW-DW)] x 100

4.2.11. Statistical analysis

All graphs were prepared using SigmaPlot11scientific data analysis and graphing software. Data were analyzed by one way ANOVA (analysis of variance) with Duncan's Multiple Range Test (DMRT) to determine the significant difference between the null and transgenic lines at $p \leq 0.05$ (indicated by single asterisk mark) or $p \leq 0.01$ (indicated by double asterisk mark).

4.3. Results

4.3.1 Tissue-specific expression of *AnnBj2* and its transcriptional induction by NaCl and ABA

In an earlier study, we reported the tissue-specific expression of six annexin genes in six-week-old mustard plants. In continuation to that, we observed the expression pattern of the annexins in this work in the root, hypocotyl and cotyledonary leaf tissues of 5-day old seedlings (Fig4.1A). We observed that transcript levels of *AnnBj1* and *AnnBj2* were most abundant followed by *AnnBj4*, *AnnBj7*, and *AnnBj6*. In our study, we found *AnnBj3* as the least expressing annexin at seedling stage. Although transcript levels of *AnnBj1* and *AnnBj2* are highly abundant at the seedling stage, there was some difference in the pattern of their expression within the tissues. Transcript levels of *AnnBj1* were almost same in all the three tissues, whereas *AnnBj2* has a maximum expression in roots followed by hypocotyl and cotyledonary leaves. We further studied the expression of *AnnBj2* in response to NaCl and ABA at the seedling stage. We checked the expression of all the six annexins in response to NaCl stress (100mM) at seedling stage (5 d old) and found transcripts of *AnnBj1*, *AnnBj2* and *AnnBj4* were strongly upregulated in response to NaCl treatment (Figure 4.1b). Among these three annexins, transcripts of *AnnBj2* and *AnnBj4* were strongly induced as early as within 1 h of treatment, which were maintained up to 3 h and then decreased at 12 h post NaCl treatment. Transcripts of *AnnBj1* increased gradually and reached maximum at 12 h post treatment. Message levels *AnnBj3*, *AnnBj6* and *AnnBj7* did not show any change in response to NaCl treatment (Figure 4.1b). ABA treatment also upregulated *AnnBj2* transcripts within 1 h treatment and the transcript levels were maintained up to 12 h treatment (Figure 4.1c).

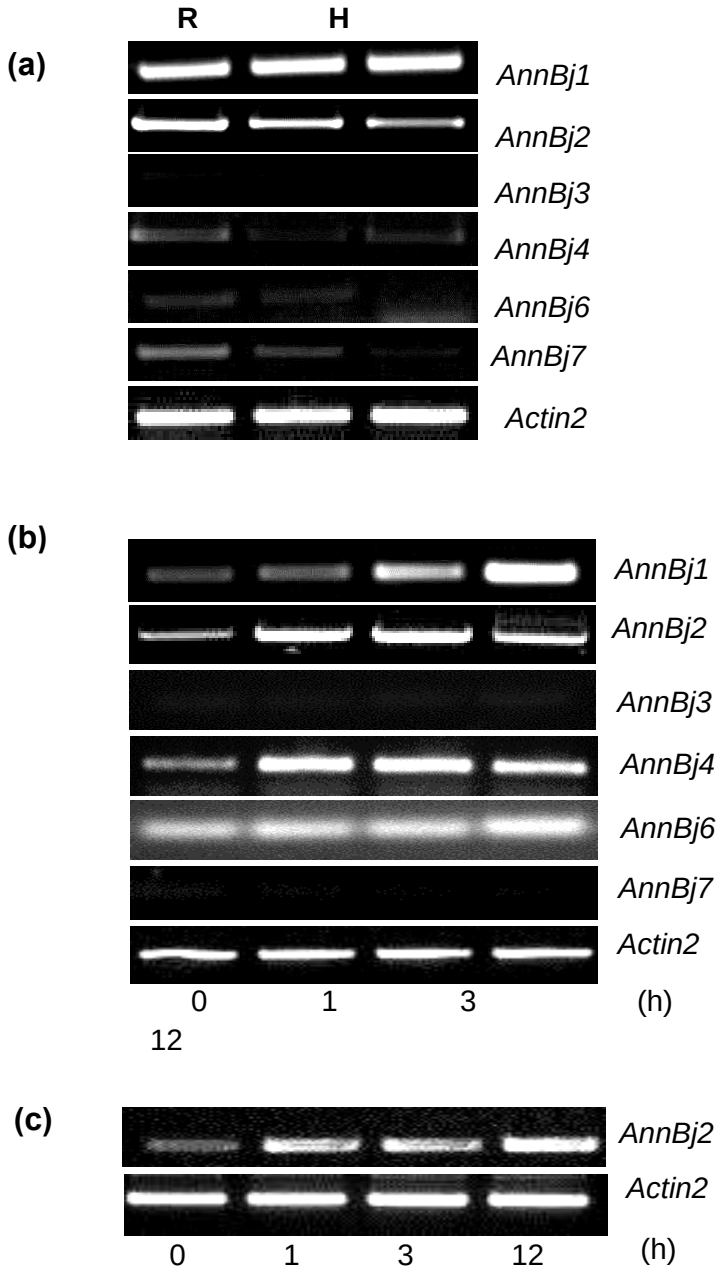
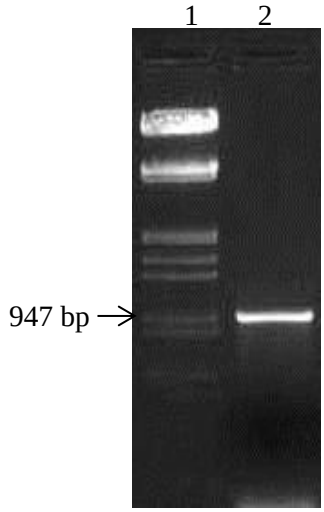


Figure 4.1: (A) Tissue specific expression of annexins in root, hypocotyl and cotyledons of five day old *Brassica juncea* seedlings. (B) Effect of 100Mm NaCl on the expression of annexins at seedling stage (c) Effect of 100µM ABA on expression of *AnnBj2* at seedling stage.

4.3.2. Preparations of overexpression construct *AnnBj2*-pCAMBIA2300

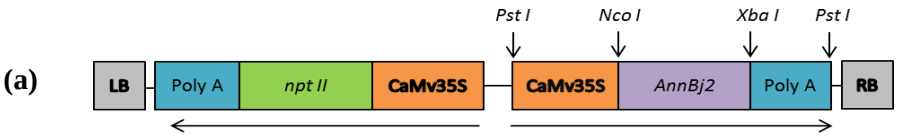
For constructing the constitutive expression cassette for *AnnBj2,* it was PCR amplified from the *B. juncea* cDNA with *Nco*I and *Xba*I restriction sites in the forward (5'CGGGATCCA TGGCGTCTCTCAAAAGTCCC3') and reverse

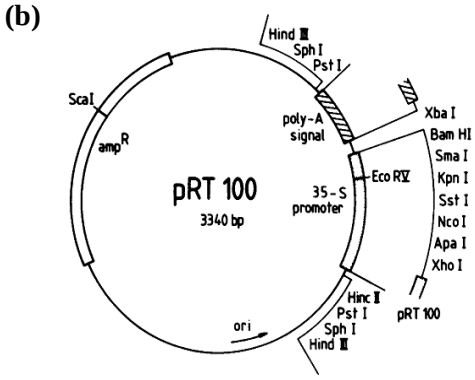
(5'GCTCTAGATCAGACATCCCCATGTC CGAG3') primers respectively. The PCR amplified product was cloned in the pTZ57R/T vector and sequenced using M13 forward and reverse primers. Sequencing of the PCR product showed 100% similarity with *AnnBj2* gene sequence submitted in the NCBI with accession no ABD47519. After confirming the PCR product, it was double digested with the above two restriction enzymes and cloned into the corresponding sites of pRT100 vector for the incorporation of CaMv35S promoter and Polyadenylation signal. This expression cassette was released from the pRT100 by *Pst* restriction digestion. The released fragment consisting of CaMv35S promoter-AnnBj2-polyA is shown in fig 4.2 and cloned into the corresponding site of binary vector pCAMBIA2300 and was designated as *AnnBj2*-pCAMBIA2300. The construct was then mobilized in the *Agrobacterium tumefaciens* EHA105 by freeze and thaw method of transformation.

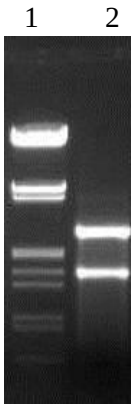


1. *Eco*R1+ *Hin*dIII Lambda DNA marker
2. PCR amplification of *AnnBj2* from cDNA

Figure 4.1: PCR amplification of *AnnBj2* from *B.juncea* cDNA.



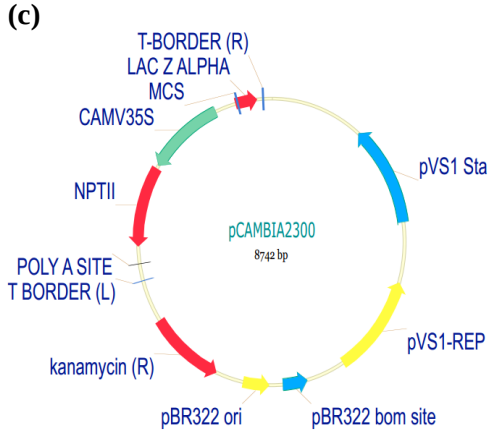


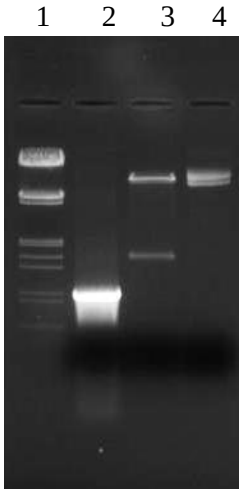


pRT100 vector

35S:*AnnBj2* :polyA fragment (1.6kb)

1. *Eco*R1+ *Hin*dIII Lambda DNA marker
2. Restriction digestion to release **35S:*AnnBj2* :polyA** from pRT100 vector





1. *Eco*R1+ *Hin*dIII Lambda DNA marker
2. PCR amplification of AnnBj2
3. *Pst1* digestion to release fragment
4. Undigested AnnBj2-pCAMBIA2300

Figure 4.2. Preparation of *AnnBj2* overexpression cassette (a) Schematic representation of AnnBj2 overexpression cassette (b) Confirmation of incorporation of CaMv35S promoter and Polyadenylation signal to *AnnBj2* (c) Confirmation of cloning in binary vector pCAMBIA2300.

4.3.3. Generation and molecular analysis of the transgenic tobacco plants expressing *AnnBj2* ectopically

To functionally characterize the role of AnnBj2 in plant abiotic stress tolerance, we generated fourteen independent transgenic lines of tobacco AnnBj2. The genetic transformation of tobacco has been carried out using the standard leaf disc method (Horsch et al. 1985). Kanamycin resistant shoots of tobacco have been rooted to obtain plantlets, which were hardened in the growth room and transferred subsequently to the Green House for further studies (Figure 4.3). These plants were confirmed by PCR using *nptII* and gene specific primers (Figure 4.4). PCR analysis showed the presence of a band corresponding to 700 bp in the transgenic plants and the positive control, which confirms the presence of *nptII* marker gene in the transgenics. The transgenic plants were further confirmed for the presence of *AnnBj2* transgene. As shown in Figure 4.4, the presence of a band corresponding to 950 bp confirms the presence of *AnnBj2* in the transgenic plants. The marker gene (*nptII*) and transgene related amplification products were absent in the negative control (WT).

The transgenic plants were analysed for the expression of *AnnBj2* gene by semi-quantitative RT-PCR, and the high expression plants were selected for further analysis. To generate homozygous population, T_1 transgenic seeds from T_0 plants were germinated on ½ MS plates supplemented with 125 mg/l kanamycin to screen the positive progeny plants, which were further grown in the soil to get the T_1 plants. Homozygosity of the transgenic lines was confirmed by 100% seed germination in the presence of antibiotic kanamycin (125mg/l) in T_2 generation. On the basis of semi-quantitative PCR analysis, OE 4.2, OE 8.2 and OE 14.2 were selected as high expression lines. The expression levels of these lines are shown in Figure 4.4.c by semi quantitative RT PCR.

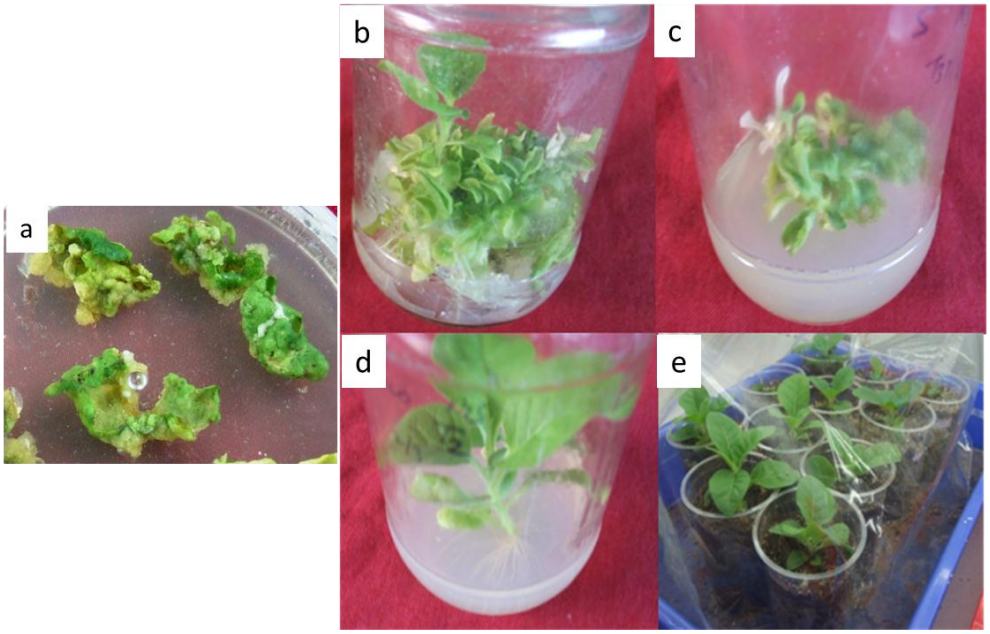


Figure 4.3: Generation of AnnBj2 overexpressing lines in Tobacco through Agrobacterium mediated transformation (a) Explants after one week of transfer to shoot regeneration medium (b) shoot regeneration medium (c) Shoot elongation medium (d) Rooting medium (e) Plantlets transferred to soil for acclimatization.

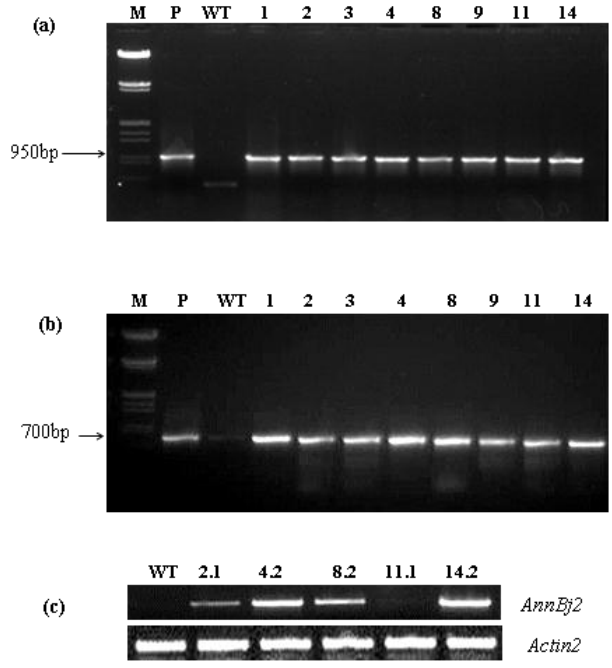


Fig4.4: PCR confirmation of the transgenic plants (a) Amplification of *AnnBj2* gene (b) Amplification of *nptII* marker gene (c) Semi quantitative RT- PCR to identify the expression levels of *AnnBj2* in the transgenic plants.

4.3.4 Ectopic expression of *AnnBj2* enhances salinity tolerance of tobacco seedlings at germination and seedling stage

AnnBj2 tobacco transgenic seeds (T_2 generation) were used to assess the salinity tolerance at seed germination stage. Under 200mM NaCl stress, we observed enhanced seed germination rate in *AnnBj2* expressing transgenic lines (OE 4.2, OE 8.2 and OE 14.2) compared to that of the NS line. After six days of germination, there was no phenotypic difference in the root lengths of NS and *AnnBj2* tobacco seedlings germinated on ½ MS medium (without stress). But under 200 mM NaCl stress, longer roots were observed in the transgenic lines compared to that of NS seedlings. Effect of NaCl on seed germination is shown in Fig 4.5.

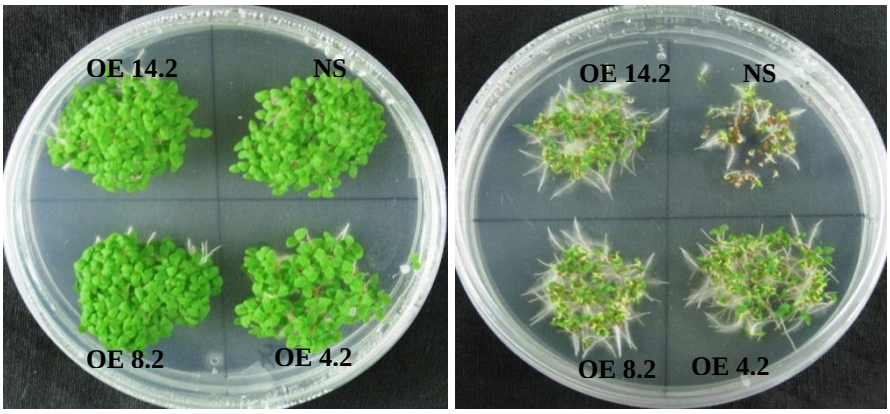


Fig4.5: Seed germination assay for salt tolerance. Photographs were taken 7 d after seeds were kept for germination

Further, we checked salinity tolerance at seedling stage. For this, we used one-week old NS and AnnBj2 overexpressing tobacco T_2 lines OE 4.2, OE 8.2 and OE 14.2 grown on ½ MS medium. We present here the seedling growth after twelve days of seedling transfer to stress media (**Fig 4.6**.). As shown in the figure, we observed an increase in root length of the transgenic lines when transferred to 100 mM NaCl stress medium but NS maintained the root length as under control condition. As we increased the NaCl concentration to 200 mM, a severe reduction in the root length was observed in the NS seedlings whereas the transgenic lines have longer root length comparatively (Fig 4.7). Biomass accumulation in terms of fresh weight was calculated after twelve

days of NaCl stress. Under 100 mM NaCl stress,~1.7 fold increase in the fresh weight of AnnBj2 transgenic tobacco lines was observed compared to that of the NS seedlings. The difference further increased to~2.0 fold, when the NaCl concentration was increased to 200 mM. The difference in the fresh weight of the seedlings after stress is shown in (Fig 4.8).

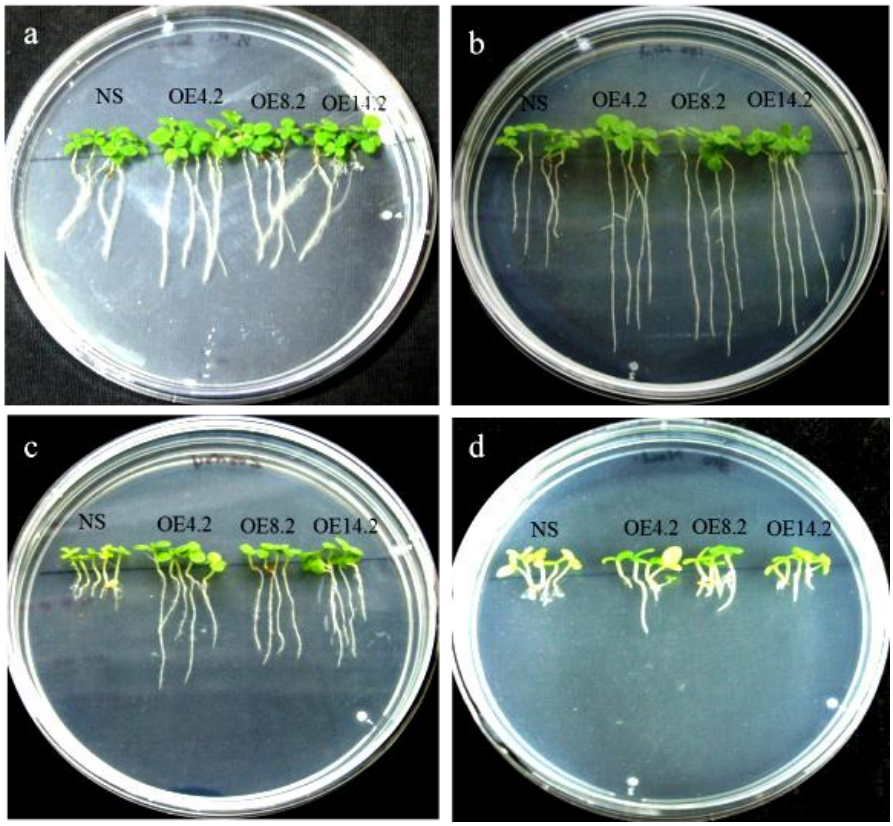


Fig4.6: Seedling assay for salinity tolerance (a) 0 mM NaCl (b) 100 mM NaCl (c) 200 mM NaCl (d) 300 mM NaCl. 10 d old seedlings grown on ½ MS media were transferred to different concentrations of NaCl supplemented ½ MS medium. Photographs were taken after 15 days of growth on stress medium.

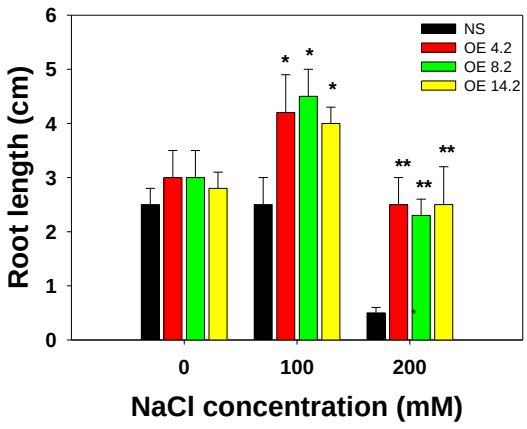


Fig4.7: Effect of NaCl stress on root length of NS and AnnBj2 transgenic lines. Data recorded after 12 days of growth on NaCl stress medium.

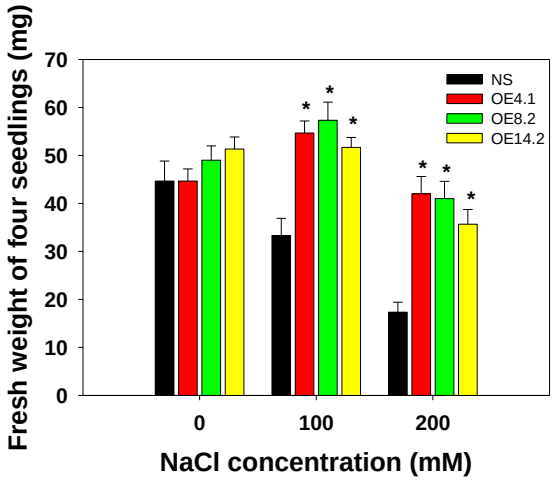


Fig 4.8: Biomass accumulation of NS and AnnBj2 transgenic seedlings under salt stress.

4.3.5 AnnBj2 expressing tobacco transgenic lines retained higher chlorophyll, proline and lower MDA levels under NaCl stress

After twelve days of seedlings growth in NaCl stress medium, chlorophyll, proline and MDA levels were estimated. Tobacco transgenic lines expressing AnnBj2 maintained higher chlorophyll content than the NS line under all the three concentrations of NaCl used in our study as shown in Fig 4.9a. Under 100 Mm NaCl stress, 10% decrease in the total chlorophyll content was observed whereas the transgenic lines maintained similar chlorophyll content as under control conditions (without NaCl treatment). The difference in the chlorophyll content between the null and transgenic lines further increased with the increase in the NaCl stress. Proline content of the null and transgenic lines were estimated following Bates et al. (1973). Under control condition (0 NaCl), we did not find any significant difference in the proline content of null and AnnBj2 OE tobacco transgenic lines. But with the increase in the NaCl concentration, relative increase in the proline content was higher in the transgenic lines compared to that of NS line. Under 100 mM NaCl treatment, proline content increased up to 2.5 fold in the AnnBj2OE tobacco seedlings whereas it was 1.6 fold increase in NS seedlings. When the NaCl concentration was increased further to 200 mM,~ a four-fold increase in proline content was observed in AnnBj2OE tobacco seedlings whereas it was~2.6 fold in NS seedlings (Fig4.9b).

MDA levels were also measured under control and stress condition to estimate the lipid peroxidation, which is used an indicator of the level of membrane damage. In tobacco, we did not find any significant difference in the MDA levels of NS and OE lines under control condition. But with the increase in NaCl concentration to 100 and 200mM in growth media, significant differences in the MDA content of the NS and AnnBj2 OE seedlings were observed. AnnBj2 OE seedlings showed comparatively lower levels MDA relative to that of NS seedlings as shown in Fig 4.9c. After twelve days of treatment, when NS seedlings were transferred to 100mM NaCl stress, they showed~2 fold increase in MDA levels compared to their counterparts grown under control condition (0 NaCl stress). In contrast to this, AnnBj2 expressing transgenic lines showed no difference in the MDA contents under similar conditions. With further increase in the NaCl concentration to 200mM,~4fold increase MDA level was observed in NS whereas~3 fold was observed in AnnBj2 OE lines as compared to the MDA level of the similar seedlings grown under control condition (without NaCl stress).

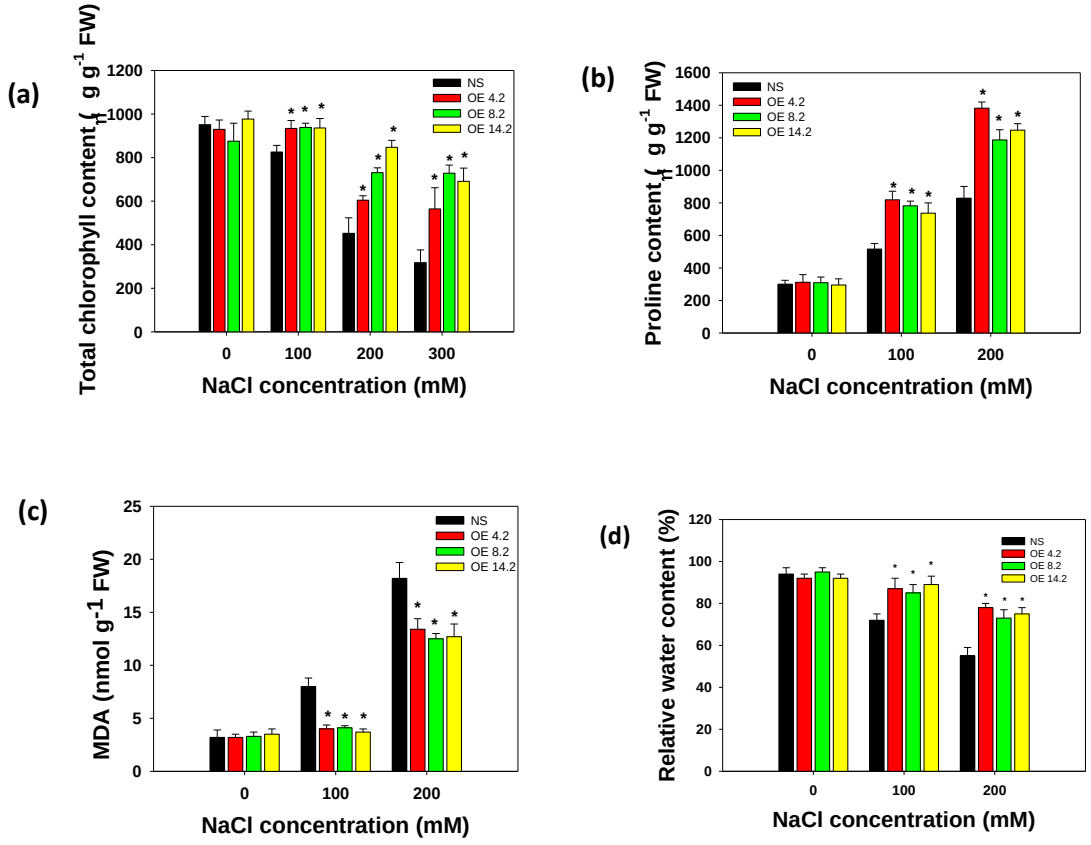


Fig4.9: Biochemical analysis of AnnBj2 OE tobacco lines (a) Total chlorophyll content (b) Proline content (c) MDA content (d) Relative water content

4.3.6 AnnBj2 transgenic lines retain higher relative water content under NaCl stress

Relative water content (RWC) is considered as a measure of plant water status as well as an osmotic adjustment under abiotic stresses. To assess RWC in AnnBj2 OE transgenics, three week old seedlings were subjected to different NaCl (100 and 200 mM) concentrations. We found that there was no significant difference in the RWC of NS and AnnBj2OE seedlings without treatment. When RWC was calculated after NaCl stress treatments, a significant reduction in the RWC of NS was observed compared to that of the AnnBj2 OE 2.2 and OE 3.3. The treatment with 200 mM NaCl stress reduced the RWC of NS line to 58±2% whereas the transgenic lines maintained it at 75-78% level (Fig 4.9d)

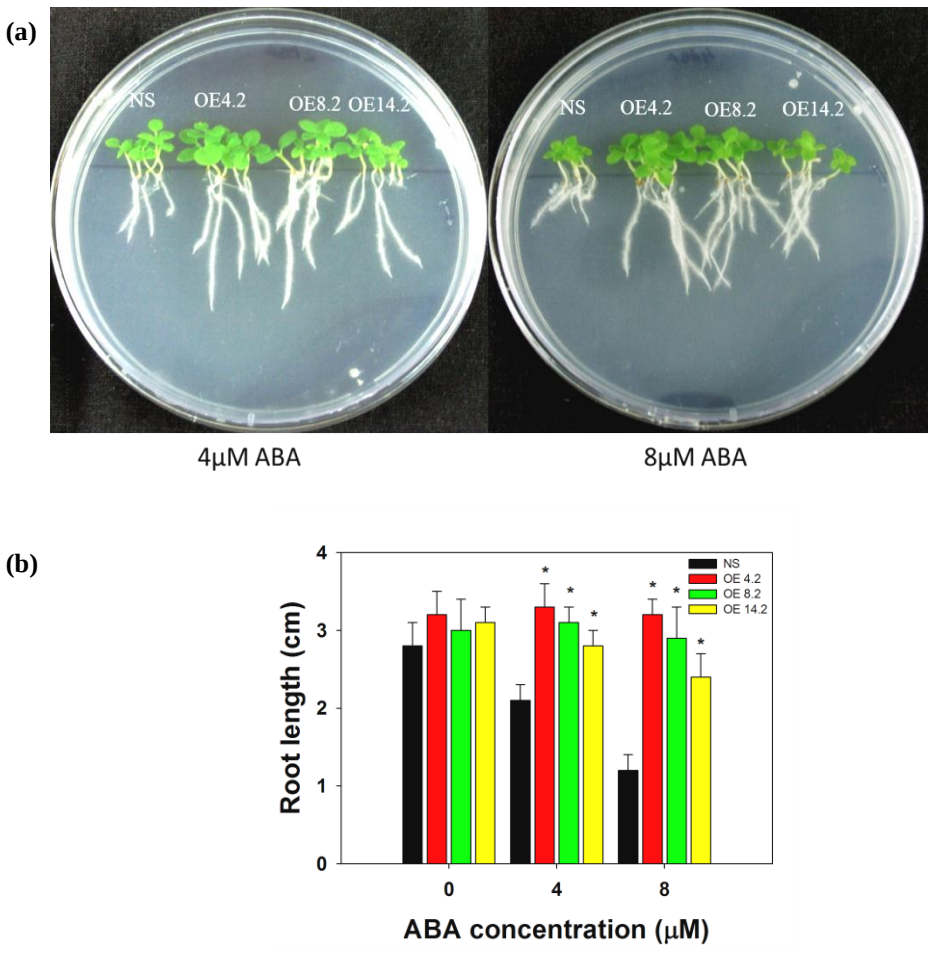


Fig4.10: Effect of ABA on NS and AnnBj2 OE lines at seedling stage (a) Response of AnnBj2 OE transgenic tobacco lines in response to 4 and 8 µM ABA (b) Root length. Data recorded after 12 days of growth on ABA stress medium.

4.3.7 AnnBj2 tobacco transgenic seedlings show reduce sensitivity to ABA

Further, we compared the effect of ABA on NS and AnnBj2 expressing tobacco lines at seedling stage. For this, we used T_2 tobacco seedlings (one week old) and transferred them to ½ MS media supplemented with 4 and 8µM of ABA. With the increase in ABA concentration, a gradual decrease in the root length of the NS seedlings was observed whereas AnnBj2 OE seedlings continue their normal root growth. Phenotypic difference in the root length has been shown in Fig 4.10a. After twelve days of growth, root lengths of the seedlings were measured by a ruler and are shown in the Fig4.10b.

Chapter 5

Genetic transformation of *B. juncea* with *AnnBj2* and evaluation of its role in salt stress

5.1 Background

Salinity limits crop productivity. India is one of the biggest consumers of vegetable oil with more than half of its demand being met mainly through imports. Mustard is the second most important oilseed crop next to groundnut, contributing about 32% of the total oilseed production in the country. It is grown in the north western region of India, where soil salinity is emerging as a severe problem for crop cultivation. Soil salinization of this region is due to intensive agriculture and continuous use of ground water for irrigation. Under these circumstances, there is an urgent need to develop salt tolerant varieties to increase food production. Conventional breeding methods fail to develop abiotic stress tolerant crops due to complex nature of the abiotic stress (multigene trait) and limitation of the transferring the favorable alleles from the available genetic resources. Moreover, QTL transfer has been associated with some undesirable genes. Genetic engineering can be used as a viable option to introduce specific genes responsible for imparting tolerance to develop tolerant crops suitable for cultivation under these harsh environments.

Annexins are multifunctionl proteins with diverse function, proteins with potential channel forming activity, peroxidase activity or as an intermediate in the signaling pathway. These proteins are regulated transcriptionally by ABA, ET, SA, MeJa and H_2O_2, but their functional mechanism in plants in not well understood (Mortimer et al. 2008; Clark et al. 2012).

Previously, several groups have shown that annexins are important for plant growth and development and this family of proteins are recently gaining importance for their role in imparting stress tolerance to plants. Overexpression of cotton annexins *GhAnn1* conferred salt and drought tolerance (Zhang et al 2015). Rice annexin OsANN1 confers thermotolerance (Qiao et al 2015).

The ectopic expression of *AnnBj2* in tobacco conferred salinity tolerance to the transgenic tobacco plants as described in chapter 4. Comparative biochemical analysis of the transgenic plants and null line revealed higher chlorophyll retention and proline synthesis in the transgenic plants. Membrane damage in terms of lipid peroxidation was lower in the transgenic lines. *AnnBj2* showed relatively higher expression in the roots compared to the other tissues at seedling stage and its overexpression in tobacco helps in maintaining longer roots under NaCl stress.

In the present investigation, we transformed *B. juncea* with *AnnBj2* through A*grobacterium*- mediated transformation and analysed its role in salt tolerance in transgenic mustard. The role of *AnnBj2* in maintaining physiological and biochemical parameters were analyzed. Further, we studied the expression of some important genes involved in ABA metabolism and signaling at seed germination stage to elucidate the molecular mechanism in conferring salt tolerance and ABA insensitive phenotype.

5.2 Materials and methods

5.2.1 Plant Materials

Seeds of Indian Mustard variety Pusa Jai Kisan were procured from National Research Centre for Plant Biotechnology, New Delhi.

5.2.2 Generation of *AnnBj2* overexpression lines of *Brassica juncea*

To generate *AnnBj2* overexpression lines, both cotyledonary petiole and hypocotyl explants were used from the one week old *Brassica juncea* seedlings grown on half strength MS medium. The explants were kept on preculture medium for 2 days before being used for the transformation. Agrobacterium strain harboring the overexpression construct was grown in LB medium supplemented with the necessary antibiotics until the $O.D_{600}$ reached 0.5 at 28°C. The cells were harvested and resuspended in liquid MS medium and the final O.D was adjusted to 0.2. After co-cultivation, the explants were washed with sterile double distilled water containing 500mg/l cefotaxime. After the antibiotic wash, the explants were dried on tissue paper and kept on the post-culture medium (MS + 2mg/l BAP, 0.05mg/l NAA, 1g/l casein acid hydrolysate, 3mg/l silver nitrate and 500mg/l cefotaxime) for 5 days and then transferred to the shoot induction medium, which is same as the post-culture medium, but augmented with 15 mg/l kanamycin for selecting the transformed shoots. The regenerated green shoots were transferred to the shoot elongation medium. Healthy shoots were shifted to the rooting medium containing 0.5 mg/l NAA. The plantlets with well-developed roots were transferred to the soil and vermiculite mix (1:3) and acclimatized in the culture room before shifting to the green house.

5.2.3 Molecular confirmation of transgenic plants

The transgenic plants raised as described earlier were screened by PCR for the presence of *nptII* and *AnnBj2* transgenes using gene specific primers. Seeds from the transgenic plants were screened by germinating them on ½ MS media supplemented with 150 mg/l kanamycin. After two weeks, the seedlings that remained green were transferred to soil to obtain the T_1 plants. Southern hybridization was performed to estimate copy number and stable integration of transgenes in the transgenic mustard lines in T_2 generation.

For Southern blotting, 10µg of DNA was digested with 50 units of the restriction enzyme *Eco*RI, which has a single site within the T-DNA region of AnnBj2-pCAMBA2300 construct and blots were probed with DIG-labeled *nptII* gene (700bp) following the manufacturer's protocol (DIG DNA Labeling and Detection kit, Cat no. 11093657910, Roche Biochemicals, Germany).

5.2.4 RNA isolation and semi-quantitative and quantitative RT- PCR

Total RNA was isolated using Trizol reagent (Sigma-Aldrich) and 2µg of the total RNAs were reverse transcribed to the first strand of complementary DNA with MLV-Reverse transcriptase (Sigma-Aldrich) and oligo dT following the manufacturer's protocol. The resultant cDNA were diluted 2.5 times and 1 µl of diluted cDNA was used as a template in a total 10µl reaction volume for amplification using program; initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95◦C for 15 s, annealing at 58◦C for 30 s and extension at 72◦C for 30 s. Primers used for the Real-Time PCR are listed in the table 5.1. *B. juncea* actin2 and EF1α were used as reference genes for normalizing the gene expression levels. The relative gene expression was analyzed using the $\Delta\Delta C_T$ method. The expression levels of the *AnnBj2* in the putative transgenic lines were determined by semi-quantitative RT-PCR to identify the high expression lines. *Actin* was used as the internal control. The qRT-PCR was performed using 2× Fast start SYBR green PCR master mix (Roche GmbH, Germany) in a 96 well plate using Realplex Ep4 system (Eppendorf GmbH, Germany).

5.2.5 Seed germination assays

Seeds of wild type and transgenic lines OE2.2 and OE3.3 were surface sterilized with sodium hypochlorite for 15 minutes followed by washing with sterile double distilled water four times. Then, the seeds were kept on germination media comprising half strength MS salts supplemented with 0, 100, 200 and 300 mM NaCl. Germination bottles were kept in dark for one day and then shifted to the culture room condition with 16/8, dark/ light conditions at 26±2°C. Protrusion of the radicle from the seed coat was used as a scorable marker for germination. Data was recorded on daily basis. The germination assays were done with three technical replicates of 25 seeds each to ensure reproducibility of the data.

5.2.6 Chlorophyll content

Total chlorophyll was extracted from the control and NaCl-treated seedlings following Hiscox and Israelstam (1979) protocol. A sample of 100mg is extracted in 4 ml of DMSO according to the protocol described by Hiscox and Israelstam (1979) from the control and NaCl-treated samples. The absorbance was taken at 645 and 663 nm against DMSO as a control.

5.2.7 Proline estimation

Proline content was estimated using the standard procedure described by Bates et al. (1973). 100 mg of leaf samples from mock and NaCl treated plants (2 months old) were extracted in 2 ml of 3% of sulposalicylic acid. The homogenate was centrifuged and 100 µl of supernatant was reacted with 100 µl of 3% of sulfosalicylic acid, 200 µl glacial acetic acid and 200 µl acid ninhydrin mixture by boiling at 100°C for 1 hour. The reaction was stopped by keeping on ice and 1.2 ml of toluene was added to the samples. The chromophore containing toluene was transferred to fresh tube. Absorbance of the chromophore was read at 520 nm using toluene as a blank. Proline concentration was determined from a standard curve and expressed as µg g-1 FW.

5.2.8 Lipid peroxidation

Lipid peroxidation of the samples was estimated by measuring thiobarbituric acid reactive substances (TBARS) following the protocol described by Heath and Packer (1968). Briefly, 100 mg tissue samples were homogenized in 0.5 ml of 0.1% TCA and

the homogenate was centrifuged at 12,000 rpm (4°C) for 10 min. The supernatant (0.5ml) was mixed with 1.5 ml of 0.5% (w/v) TBA in 20% TCA (w/v) and incubated at 95°C for 30 minutes. The reaction was stopped by keeping the tubes on ice followed by centrifugation for 5 minutes at 12,000rpm (4°C). The absorbance of the resultant supernatant was measured at 532 and 600 nm. The OD600 values were subtracted from the MDA-TBA complex values at 532 nm, and MDA concentration is calculated using the Lambert-Beer law with an extinction coefficient εM= 155 $mM^{-1}cm^{-1}$. Results were presented as nmol MDA $g^{-1}FW$.

5.2.9 Relative water content:

To calculate the relative water content, one-week old seedlings grown on ½ MS medium were transferred to liquid ½ MS medium supplemented with 100 and 200mM NaCl for 72 hours. Fresh weight (W) of the seedlings was taken and immediately the samples were hydrated completely for twelve hours to obtain turgid weight (TW). Samples were then oven dried for 48 hours at 60°C to determine its dry weight (DW). Relative water content was measured using the following equation.

RWC (%) = [(W-DW) / (TW-DW)] x 100

5.2.10 Pot level soil experiment:

To study the salinity tolerance in pot grown plants in the green house, Zhang et al. (2001) method of salt treatment was followed with minor modifications. Thirty seeds of each genotype were sown in the pots (30 cm diameter in size), each of which was fed with 200 ml of 100 mM NaCl solution prior to sowing. After germination, the seedlings were watered with 100 ml of 100mM NaCl once in a week and and with 200 ml of tap water every alternate day. The control set of plants were watered with the same volume of water without any NaCl treatment. After two months of growth; leaf samples were collected to estimate chlorophyll, proline and MDA contents. Seed germination percentage in the pots was calculated after five days of sowing.

5.2.11 Ion estimations

To check the Na^+, K^+ and Ca^{2+} accumulation in the shoots, freshly harvested leaves were washed properly with deionised water to clean the leaf surface and dried in hot air oven at 65°C for four days. The samples of 100 mg (DW) of air dried leaf tissues

were acid digested with a mixture of nitric acid (HNO3) and perchloric acid (HClO4) (3:1, v/v) mixture following the protocol of Munns et al. (2010) with minor modifications. Ionic estimations were done using Atomic absorption spectrophotometer (Perkin Elmer).

5.2.12 Gene expression analysis

To study the gene expression of salt stress marker genes, 5 d old seedlings of control and AnnBj2 OE lines were treated with 100 mM NaCl solution for six hours and the samples were quick frozen for RNA isolation. To study the gene expressions at seed germination stage, sterilized seeds of control and AnnBj2 OE lines were kept on ½ MS+ 8µM ABA to undergo germination. After 24 h of incubation, seeds were harvested for RNA isolation. Total RNA was isolated using Trizol reagent (Sigma-Aldrich) as per the manufacturer's instructions, and 2µg of the total RNA was reverse transcribed to get the first strand of complementary DNA with MLV-Reverse transcriptase (Sigma-Aldrich) and oligo dT primer following the manufacturer's protocol. The resultant cDNA samples were diluted 2.5 X and 1 µl of diluted cDNA was used as a template in a total 10 µl reaction volume for the PCR amplification using the following program- initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95◦C for 15 s, annealing at 58◦C for 30 s and extension at 72◦C for 30 s. Primers used for the Real-Time PCR are listed in Table1. The qRT-PCR was performed using 2× Fast start SYBR green PCR master mix (Roche GmbH, Germany) in a 96 well plate using Realplex Ep4 system (Eppendorf GmbH, Germany). *B. juncea actin2* and *EF1α* were used as reference genes. The relative gene expression was analyzed using the Livaks $\Delta\Delta C_T$ method. The expression levels of *AnnBj2* in putative transgenic lines were determined by semi-quantitative RT-PCR to identify the high expression lines. *Actin2* was used as the internal control.

5.2.13 Statistical analysis

All graphs were prepared using SigmaPlot11scientific data analysis and graphing software. Data were analyzed by one way ANOVA (analysis of variance) with Duncan's Multiple Range Test (DMRT) to determine the significant difference between the null and transgenic lines at $p \leq 0.05$ (indicated by single asterisk mark) or $p \leq 0.01$ (indicated by double asterisk mark).

Table 1: List of primers used in the study

Primer	Sequence
DREB2B F DREB2B R	CCGTTGCGGATTATGGTTGG TCCCGTTCTGGTCTTCATCC
RAB18 F RAB18 R	GGAAAAGTTGCCAGGCCATC TAATGATGACCACCACCACCG
ERF5 F ERF5 R	TCTAACCGAAACCCGCCTTC TTTCCCCCACGGTCTTTGTC
SnRK2.2 F SnRK2.2 R	CCAGGATGTCGCCAGCTTAT CTCCGGCTCTTGGAACTGAC
ABI3F ABI3R	ATCTCAACTACCGGCGATGG ACGTCGCTTTGCTTCAACAC
ABI4 F ABI4 R	TCCCTCACCAACAAACTCAGAT GCCACCTCGTGATGAAACGA
ABI5F ABI5R	CCGTCTAGTGTTATCCCCGC CACCATCGCCATTTGCTGTC
NCED6 F NCED6 R	GGTCACCGGTTATCTACGACA TCACCGTCCTCGGCTATCT
Cyp707A2 F Cyp707A2 R	CTCAACCTACCTGGCACACT TGAATCGCTCAAACCGTTGC
AAO3 F AAO3 R	GAGTTCAGGCTCGGTTCACA CTGGTGATGACTCGGACGTT
AnnBj2 F AnnBj2 R	CGGGATCCATGGCGTCTCTCAAAAGTCCC GCTCTAGATCAGACATCCCCATGTCCGAG
NptII F NptII R	TAAAGCACGAGGAAGCGGTC GATGGATTGCACGCAGGTTC
CaMv35s F	ACGACACTCTCGTCTACTC
P5CS1 F P5CS1 R	GCAGAATGGTGTGCTAAACGAG TACAATTTCAACGGTGCAGGC
SOS1 F SOS1 R	AGTTTCTCAAGACAAGCAACACA ATGATCTCTGGAGCTGGTGC
Actin2 F Actin2 R	TACTCGTTCACCACGACAGC AAAGAACCTCGGGACAACGG
EFIαF EF1αR	TGAAGAATGGTGATGCTGGT CTTCTTCACTGCGGCCTTG

5.3 Results

5.3.1 Generation of *AnnBj2* overexpression lines of Mustard

To generate *AnnBj2* overexpression lines, both cotyledonary petiole and hypocotyl explants were used from the one week old mustard seedlings grown on half strength

Murashige and Skoog (MS) medium without any growth regulator. The explants were kept on preculture medium (MS + 2.0 mg/l BAP, 0.05 mg/l NAA, 3.0 mg/l silver nitrate) for two days before the co-cultivation experiments in genetic transformation. Agrobacterium strain harboring the overexpression construct was grown in Luria-Bertani (LB) medium supplemented with the necessary antibiotics until the O.D reached 0.5 at 28°C. The cells were then pelleted at 5000 rpm (4°C) and resuspended in liquid MS medium and the final $O.D_{600}$ was adjusted to 0.2. The suspension culture was supplemented with 200µM acetosyringone. The explants were dipped in the suspension culture and placed on co cultivation medium (MS+ 2.0 BAP+ 0.05 NAA+ 1.0 g/l casein acid hydrolysate + 3.0 mg/l silver nitrate). After co-cultivation for 48 h, the explants were washed with sterile double distilled water containing 500mg/l cefotaxime. After washing, the explants were dried on sterile tissue paper and kept on the post-culture medium (MS + 2.0 mg/l BAP, 0.05 mg/l NAA, 1.0 g/l casein acid hydrolysate, 3.0 mg/l silver nitrate and 500 mg/l cefotaxime) for five days following which they were transferred to the shoot induction- selection medium, which is same as the post-culture medium, but augmented with 15mg/l kanamycin ws used for selecting the transformed shoots. In the shoot induction media, some explants turned brown and were discarded during the subculturing. The explants started producing multiple shoots in the 3^{rd} week on the regeneration medium. Among the two explants used, cotyledonary petiole was found to more responsive to the regeneration. The untransformed shoots showed retarded growth and got bleached on sub culturing to the fresh regeneration medium. The regenerated shoots that remained green and showed normal growth were transferred to the shoot elongation medium (MS + 1.0 mg/l BAP + 3.0 mg/l silver nitrate + 500mg/l cefotaxime). Healthy shoots were shifted to the rooting medium containing 0.5 mg/l NAA. The plantlets with well-developed roots were transferred to the soil and vermiculite mix (1:3) and acclimatized in the culture room before shifting to the green house. The various stages of generation of AnnBj2 overexpressing transgenic lines were shown in Figure 5.1.

In the T_1 generation, we screened the seeds of the transgenic lines to identify the null segregants.

Figure 5.1: Transformation and regeneration of *B. juncea* (a) & (b) Hypocotyl and cotyledonary petiole kept on preculture medium, (c) & (d) regeneration of shoots from the explants after 3 weeks of transformation (e) growth of shoots on the shoot elongation medium (f) well developed shoots transferred to rooting medium (f) *in vitro* developed plantlets transferred to soil for acclimatization

5.3.2 Molecular confirmation of the transgenic plants

The putative transgenic plants obtained by following the protocol as described in Materials and methods were screened by PCR for the presence of marker and transgene. Gel electrophoresis of PCR products from the transgenic samples showed the presence of a 700 bp using the *nptII* gene specific primers, whereas it is absent in the negative control. This confirms the presence of *nptII* marker gene in the transgenic lines and is shown in Figure 5.2a. Subsequently, we checked for the presence of the AnnBj2 transgene in the transgenic lines using CaMv35S forward and AnnBj2 reverse primers. The presence of a band corresponding to the size of 1350 bp in the transgenic lines confirmed the presence of the *AnnBj2* transgene (Figure 5.2b).

On the basis of semi-quantitative PCR analysis, OE 2.2, OE 3.3 and OE 5.5 were selected as high expression lines. The expression levels of these lines are shown in Figure 5.2c by semi quantitative RT PCR.

Southern blotting was performed to check the stable integration and copy number of the T-DNA in the transgenic lines. Southern analysis showed that most of the transgenic mustard OE lines have single independent integration. Some plants with multiple insertions were obtained, but were not included in the further experiments. Null segregant (NS) line did not show any hybridization signal (Figure 5.2d).

To generate homozygous population, T_1 transgenic seeds from T_0 plants were germinated on ½ MS plates supplemented with 150 mg/l kanamycin to screen the positive plants, which were further grown in the soil to get the T_1 plants. Homozygosity of the transgenic lines was confirmed by 100% seed germination in the presence of antibiotic kanamycin (150mg/l) in T_2 generation.

Null segregants were identified by germinating the seeds of of the transgenic lines on ½ MS supplemented with 150 mg/l kanamycin. The seeds which failed to germinate or showed delayed germination were transferred to fresh ½ MS medium and allowed to grow for 10 days. These seedlings were transferred to soil and screened by PCR for the presence of *nptII* marker gene. The seedlings which did not show the presence of marker gene were identified as nulls.

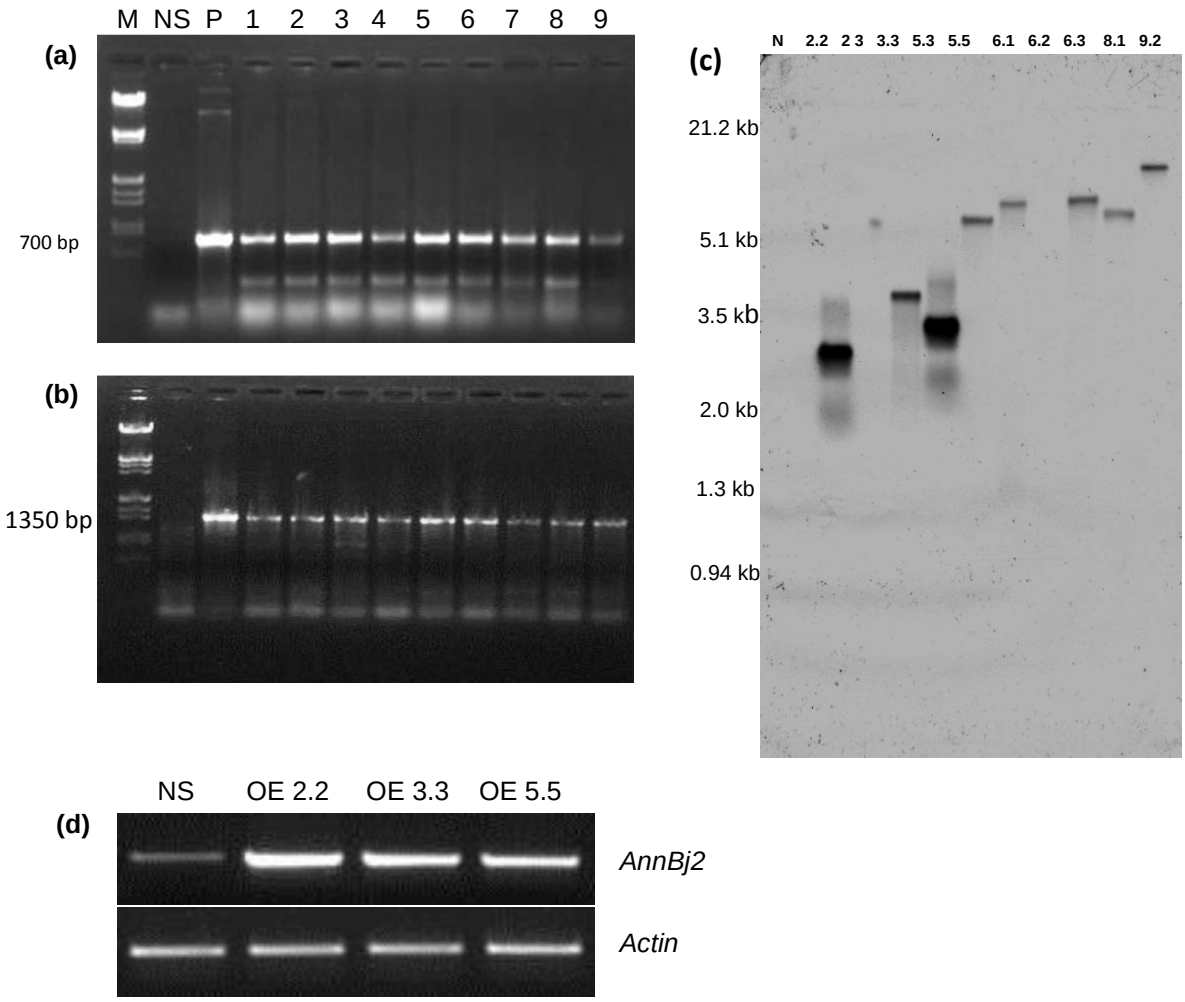


Figure 5.2: Molecular confirmation of *B. juncea* AnnBj2 OE plants (a) PCR confirmation of AnnBj2 OE plants using CaMv35S forward and *AnnBj2* gene specific reverse primer (b) PCR amplification of *nptII* marker gene (c) Southern analysis of T_2 transgenic mustard plants for transgene copy number using the restriction enzyme *Eco*RI, which has a single restriction site on the T-DNA and probing with the PCR amplified *nptII* (700bp) (d) Semi-quantative RT-PCR analysis of AnnBj2 OE plants at T_2 generation. P represents positive control (AnnBj2-pCAMBIA2300 plasmid), NS represents null segregant

5.3.3. Salinity tolerance of mustard plants overexpressing *AnnBj2* at seed germination stage

Seed germination is one of the critical stages prone to salinity. Mustard seeds of null (NS) and *AnnBj2* transgenic lines (OE 2.2, OE 3.3 and OE 5.5) were germinated on media supplemented with different NaCl concentrations and the germination pattern was monitored daily. NS seeds are highly susceptible to NaCl stress. With an increase in NaCl concentration in the germination medium, a gradual decrease in the seed germination pattern was observed. But, the decrease in the germination rates was higher in the NS seeds compared to the *AnnBj2* transgenic lines OE 2.2, OE 3.3 and OE 5.5 (Figure 5.3). The transgenic lines (OE 2.2. OE 3.3 and OE 5.5) germinated faster than NS at all the NaCl concentrations used in our study. Under 100 mM NaCl stress, OE 2.2 and OE 3.3 maintain almost similar seed germination as under control conditions (70-80%) whereas severe reduction was observed in NS seeds (40%). With 200 mM, only 5% of NS seeds germinated as against 50-60% germination in OE 2.2 and OE 3.3. . Phenotypic differences in the growth of seedlings, and root and shoot length after six days of germination were shown in Figure 5.4.

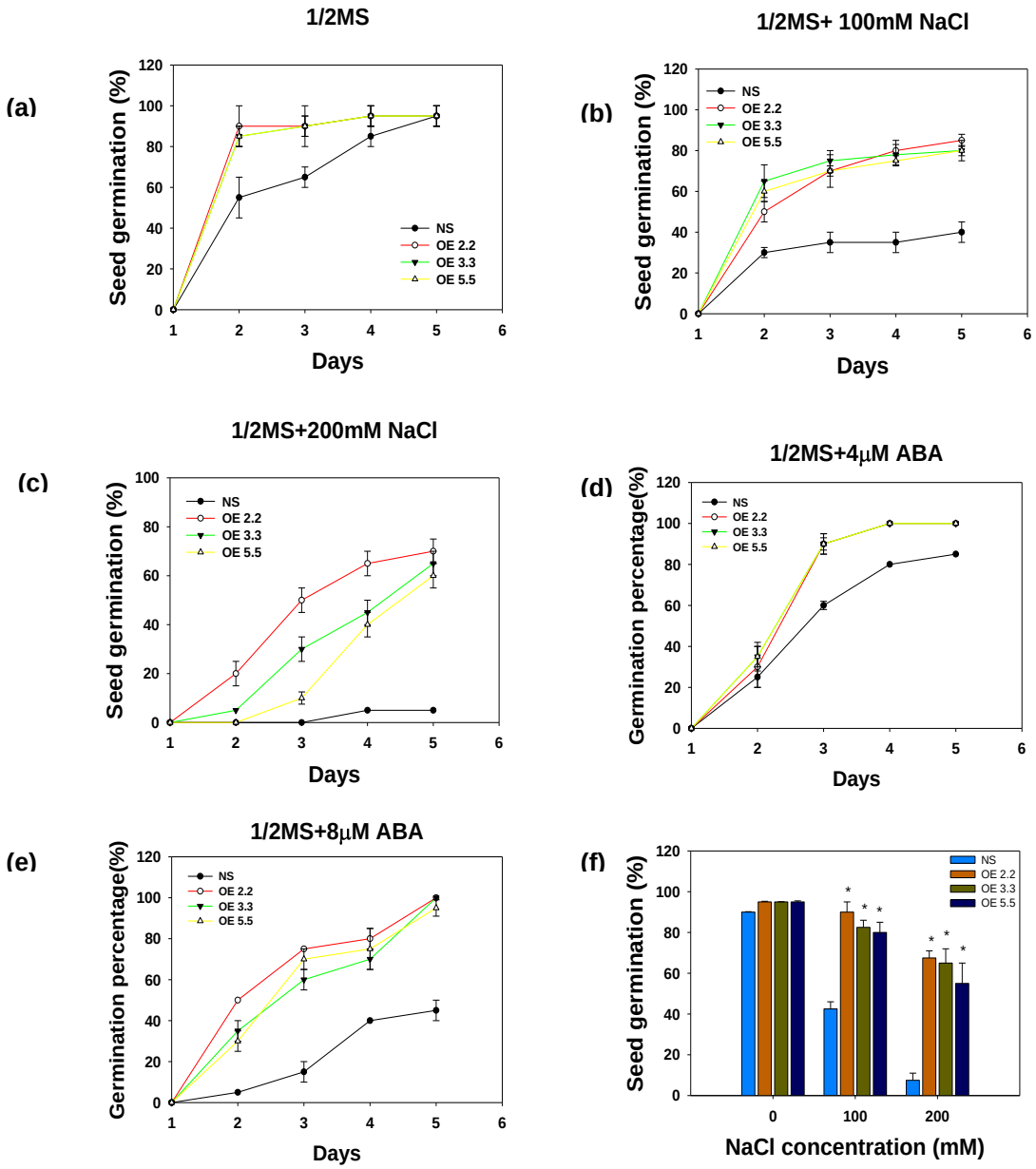


Figure 5.3: Seed germination pattern of Null (NS) and AnnBj2 OE *B. juncea* lines. (A) 0 NaCl (B) 100 mM NaCl (C) 200 mM NaCl (D) 4 µM ABA (E) 8 µM ABA (F) Seed germination percentage was calculated after 5 d under different concentrations of NaCl. Seed germination percentages were recorded daily over 5 d time period. Experiments were carried out with three technical replicates and repeated at least three times. Statistical analysis was done by one way ANOVA to determine the significant difference between null and the transgenic lines. Single asterisk (*) indicates $p \leq 0.05$, double asterisk represents $p \leq 0.01$

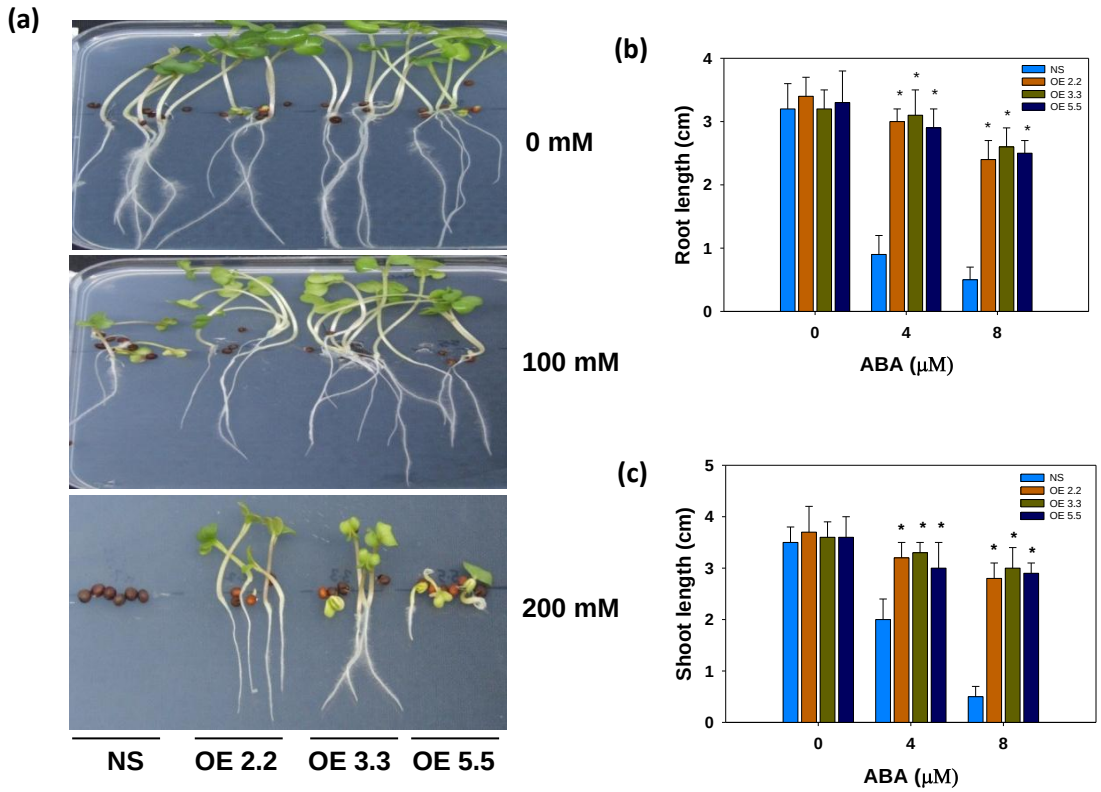
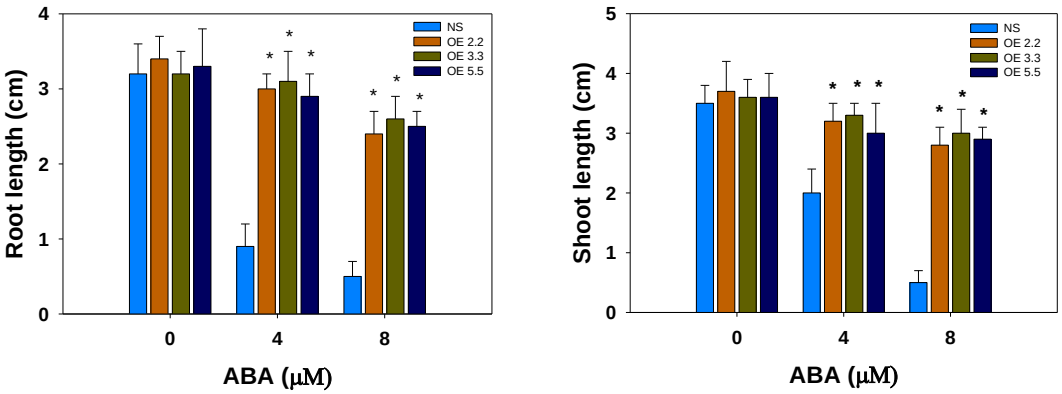


Figure 5.4: i*n vitro* salinity tolerance of control and AnnBj2 OE *B. juncea* lines. (A) Growth responses of control and AnnBj2 OE transgenic seedlings (B) Mean root length (C) Mean shoot length. Data recorded after 5 d germination. Data were analyzed by one way ANOVA to determine the significant difference between the null and transgenic lines. Single asterisk (*) indicates p ≤0.05 and double asterisk (**) represents p ≤0.01.

5.3.4. *AnnBj2* overexpressing Mustard transgenic lines show reduced sensitivity to ABA at seed germination stage.

ABA plays an inhibitory role in seed germination and the expression of AnnBj2 was induced by ABA. We found that seed germination percentage of NS seeds was severely affected with an increase in the ABA concentration. In contrast to this, seed germination of AnnBj2 OE 2.2, OE 3.3 and OE 5.5 showed reduced sensitivity to ABA (Figure 5.5.). Day wise germination pattern revealed 3rd d as the critical point where major difference in seed germination percentage was observed between the genotypes. At the end of 3rd day, 90% of the AnnBj2 OE seeds germinated in contrast to 60% of the NS seeds in the presence of 4 µM ABA. The difference in the seed germination percentage became more pronounced with an increase in the ABA concentration to 8 µM, with only 10% of NS seeds germination in contrast to 50-60% germination in AnnBj2OE lines (Figure 5.3d &e).

Figure 5.5: Effect of ABA on root and shoot growth of null and AnnBj2 OE transgenic lines



Response of null (NS) and AnnBj2 transgenic seeds to ABA at seed germination stage. Experiment was carried out with three technical replicates to ensure reproducibility of data(a) Mean shoot length (b) Mean root length. Data shown here are recorded after 5 d germination.

5.3.5. *AnnBj2* overexpressing transgenic lines of Mustard show reduced sensitivity to glucose

ABA insensitive phenotype is often associated with glucose insensitive phenotype (Rook et al. 2006; Ljung et al. 2015). To study the effect of glucose on seed germination and seedling growth of AnnBj2 overexpressing transgenic lines of mustard, seeds were germinated in the presence of 5% glucose and their seed germination and seedling growth were monitored up to 7 d. Cotyledon greening and expansion were used in assessing glucose tolerance. Glucose showed a detrimental effect on the root growth and cotyledon greening and expansion in NS compared to that of the *AnnBj2* transgenic lines OE 2.2, OE 3.3 and OE 5.5 (Figure 5.6). The growth of seedlings after 5 d has been shown in Fig.

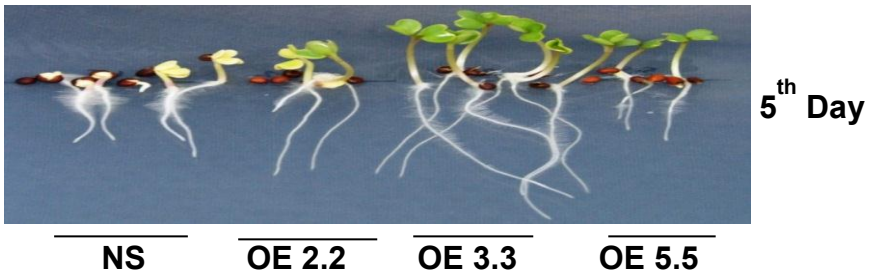


Figure 5.6: Response of NS and AnnBj2 transgenic lines on 5% glucouse at seed germination stage.

5.3.6. *AnnBj2* overexpressing mustard seedlings maintain higher relative water content under NaCl stress

Relative water content (RWC) is considered as an indicator of plant water status as well as an osmotic adjustment under abiotic stresses. To assess RWC in AnnBj2 OE transgenics, one week old seedlings were subjected to different NaCl concentrations. We found that there was no significant difference in the RWC of NS and AnnBj2OE seedlings without any treatment. When RWC was calculated after NaCl stress treatments, a significant reduction in the RWC of NS was observed compared to that

of the AnnBj2 OE 2.2 and OE 3.3. The NaCl stress (200 mM) reduced the RWC of NS line to 58±2% whereas the transgenic lines maintained it at 75-78% (Figure 5.7).

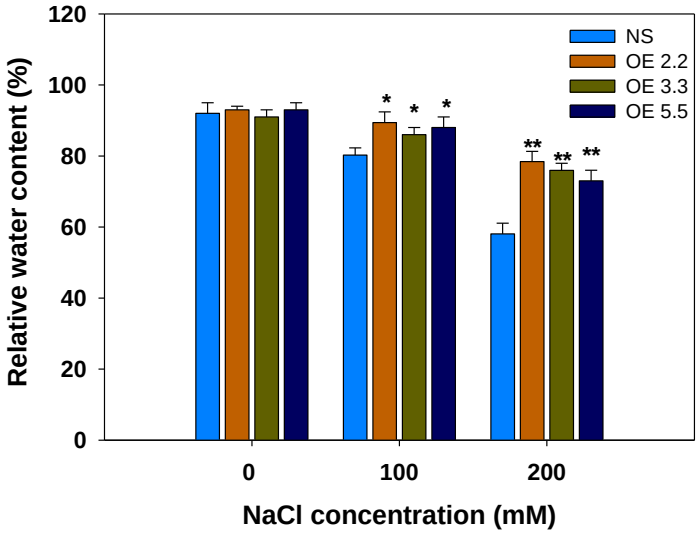


Fig 5.7: Relative water content of Null and transgenic seedlings under salt stress. Data were analyzed by one way ANOVA with DMRT to determine the significant difference between null and the transgenic lines with $p \leq 0.05$ (indicated by single asterisk mark).

5.3.7 AnnBj2 overexpressing transgenic *B. juncea* plants show salinity tolerance at whole plant level

Transgenic lines overexpressing *AnnBj2* showed higher seed germination percentage in pot experiments, when treated with NaCl solution compared to that of the control (NS) genotype. Salt treatment reduced the seed germination percentage of the NS to 40% whereas the transgenic lines OE2.2 and OE 3.3 maintained 80±6 and 63 ±5% respectively (Figure 5.8b). Further, a difference in the growth and survival of control and transgenic lines was observed under NaCl treatment. NS line had stunted growth compared to *AnnBj2* transgenic lines (Figure 5.8a). Transgenic line OE 2.2 showed vigorous growth both in unstressed and stressed condition compared to others. After two months of growth, leaf samples were collected from the non-stressed and stressed samples to compare the chlorophyll, proline and MDA contents. Total chlorophyll content of NS and AnnBj2 transgenic lines were almost similar under non-stressed condition; but under salt stress, transgenic lines retained two-fold (approx.) higher

total chlorophylls compared to that of controls (Figure 5.9a). Under NaCl stress, proline content of transgenic lines was comparatively higher than that of the NS plants (Figure 5.9b). Lipid peroxidation levels as estimated by measuring MDA levels also revealed higher membrane damage in the controls compared to the transgenic lines (Figure 5.9c).

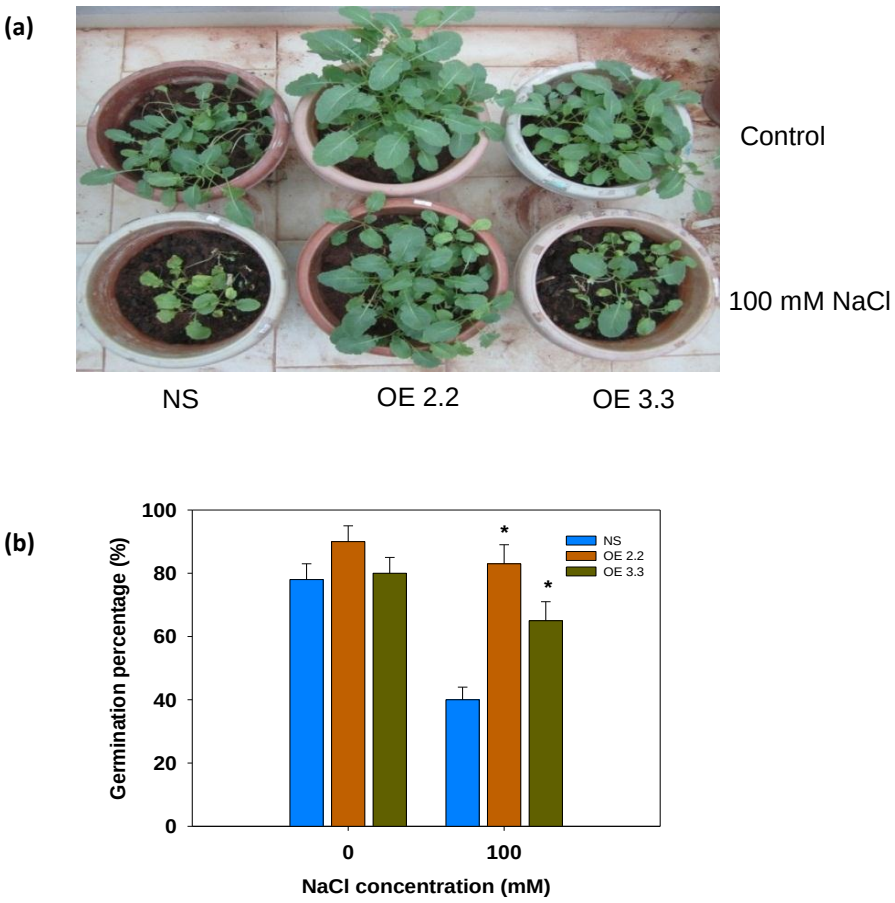


Figure 5.8: Performance of NS and AnnBj2 transgenic lines under salt stress in pots. (a) Phenotypic difference in the growth of the plants (b) Seed germination in NaCl treated soil.

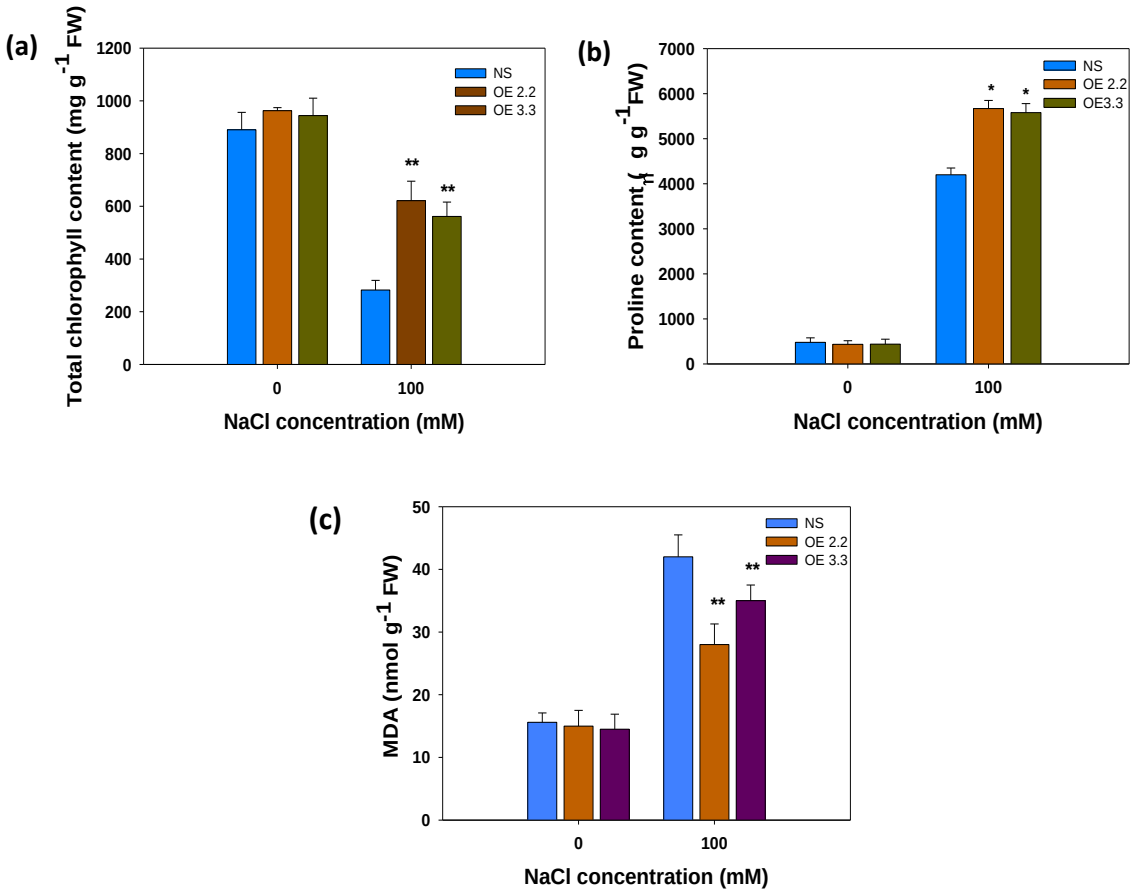


Figure 5.9: Biochemical analysis of the transgenic plants under salt stress (a) total chlorophyll content (b) proline content (c) MDA content. Data were analyzed by one way ANOVA with DMRT to determine the significant difference between null and the transgenic lines with $p \leq 0.05$ (indicated by single asterisk mark).

5.3.8 Ion estimation

Salt stress is known to disrupt ion homeostasis in plants. To gain an insight into how *AnnBj2* overexpression affects the ion content under NaCl stress, we measured the Na^+, K^+ and Ca^{2+} ion accumulation in the leaves of two-month-old NS and AnnBj2 OE lines (OE2.2 and OE 3.3) under control and salt stressed conditions (Fig 5.10). We observed an increase in the Na^+ ion content and a decrease in the K^+ ion content in all the genotypes under NaCl stress when compared to the control condition. But this relative increase in Na^+ ion content or decrease in K^+ ion content differed among the NS and OE lines. We did not find any significant difference in the Na^+/ K^+ ion content between control and OE lines under control conditions. However, sodium ion

accumulation under salt stress was higher in control (58mg/g DW) compared to that of the *AnnBj2* OE lines (32 and 40 mg/g DW). The OE lines (OE 2.2 and OE 3.3) maintained a higher K^+ content than the NS line. There was a decrease in K^+/Na^+ ratio of both control and OE lines under NaCl stress; OE 2.2 maintained highest K^+/Na^+ (1.25) whereas the NS exhibited the lowest (0.43). We observed, a decrease in the Ca^{2+} of all three genotypes under salt stress compared to that of the control condition, but the relative decrease is significantly less in the AnnBj2 OE lines.

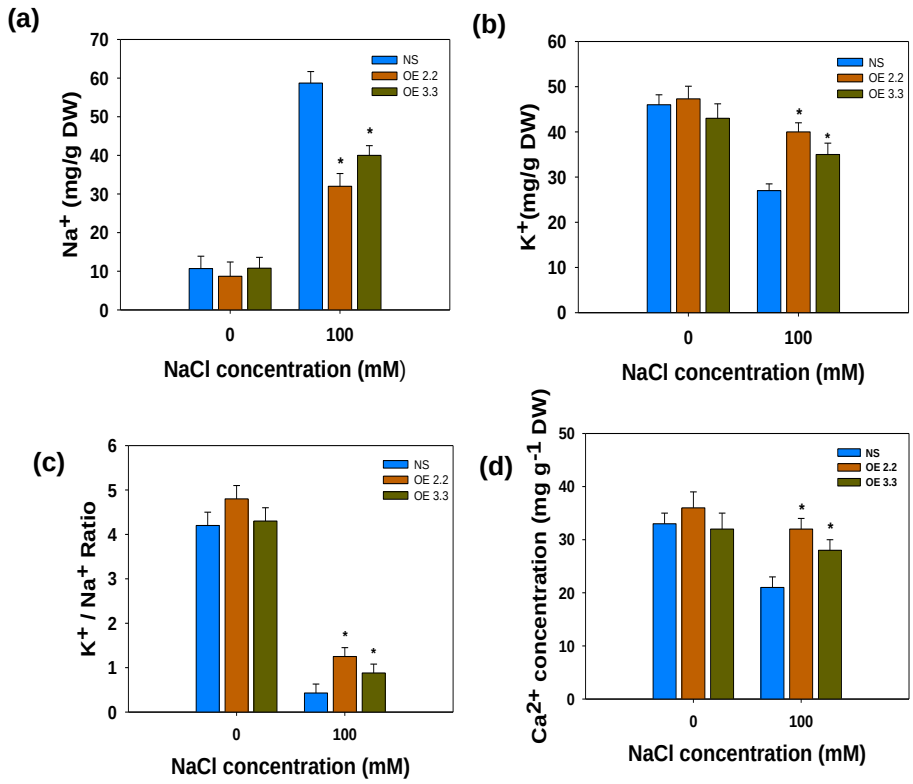


Figure 5.10: Ion estimation from the leaves of NS and AnnBj2 OE plants harvested after eight weeks of treatment NaCl treatment (a) Na^+ content (b) K^+ content (c) K^+/ Na^+ ratio (d) Ca^{2+} content. Error bar represents ± S.D (n=3). Data were analyzed by one way ANOVA with DMRT to determine the significant difference between null and the transgenic lines with $p \leq 0.05$ (indicated by single asterisk mark).

5.3.9. Altered expression pattern of ABA signal related genes at seed germination stage

We observed that the constitutive expression of *AnnBj2* in mustard reduced ABA sensitivity at the seed germination stage. To gain further insight into the role of *AnnBj2* in ABA signaling, we studied the relative expression of some key genes

(*ABI3*, *ABI4*, *ABI5*, *AAO3*, *NCED6* and *CYP707A2*) controlling seed germination with ABA treatment. We did not find any significant difference between control and AnnBj2 OE lines in the expression of two key genes involved in ABA biosynthesis, *AAO3* (Abscisic Aldehyde Oxidase3) and *NCED6* (9-Cis-Epoxycarotenoid Dioxygenase6). The expression level of *CYP707A2* (Cytochrome P450, Family 707, Subfamily A, Polypeptide2), which is involved in ABA degradation was expressed to significantly higher levels in both the AnnBj2 OE lines compared to control (Fig 5.11). ABI4 (Abscisic acid insensitive 4), is a positive regulator of ABA biosynthesis at seed germination stage. We found a significant reduction in the expression level of *ABI4* and *ABI5* in both OE 2.2 and OE 3.3 compared to the control line (Fig 5.11). Expression of *ABI3* did not show any significant differences between control and the transgenic lines.

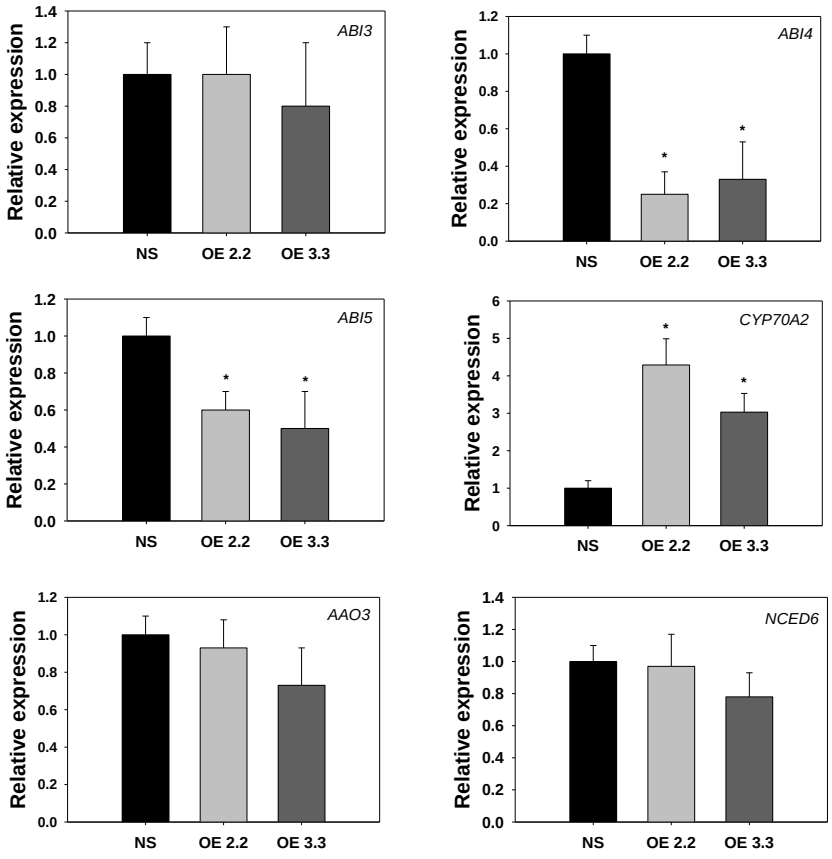


Figure 5.11: Expression of genes involved in ABA regulation during seed germination. Sterilized seeds were kept on ½ MS media supplemented with 8 µM ABA for 24 hours under 16/8 light/dark period. After 24 h, seeds were collected and used for RNA isolation to study the relative expression of genes involved in seed germination. The transcript levels were normalized using reference gene *actin2*. Expressions levels were represented using Livak's $\Delta\Delta C_T$ method. Error bar represents mean ±SD (n=3). Data were analyzed by one way

ANOVA with DMRT to determine the statistical difference between null and the transgenic lines at $p \leq 0.05$ (indicated by single * mark)

5.3.10 Overexpression of *AnnBj2* in mustard activates the expression of both ABA dependent and ABA independent stress marker genes

We compared the expression of ABA dependant and ABA independent stress marker genes in control and AnnBj2 OE transgenic lines of mustard in mock and salt treated samples. We found the relative abundance of the transcripts of *ERF5*, *RAB18*, *DREB2B* were higher in the AnnBj2 OE lines (AnnBj2OE 2.2 and AnnBj2 OE 3.3) compared to control seedlings (Figure 5.12). *RD29A* does not show any significant difference between the control and AnnBj2OE lines.

Amino acid sequence alignment of AnnAt2 and AnnBj2 using CLUSTAL O (1.2.1)

```
CLUSTAL O(1.2.1) multiple sequence alignment


AnnAt2      MASLKVPSNVPLPEDDAEQLHKAFSGWGTNEKLIISILAHRNAAQRSLIRSVYAATYNED
AnnBj2      MASLKVPSNVPLPEDDAEQLHKAFSGWGTNEKLIISILAHRNSAQRSLIRSVYAATYNED
            ****************************************:*****************

AnnAt2      LLKALDKELSSDFERAVMLWTLDPPERDAYLAKESTKMFTKNNWVLVEIACTRPALELIK
AnnBj2      LLKALDKELSSDFERAVMLWTLDPAERDAYLAKESTKMFTKNNWVLVEIACTRSALELFK
            ********************** *************************** ****:*

AnnAt2      VKQAYQARYKKSIEEDVAQHTSGDLRKLLLPLVSTFRYEGDDVNMMLARSEAKILHEKVS
AnnBj2      VKQAYQARYKKSLEEDVAQHTSGDLRKLLLPLVSTFRYEGDDVNMMLARSEAKLLHEKVS
            ************:****************************************:******

AnnAt2      EKSYSDDDFIRILTTRSKAQLGATLNHYNNEYGNAINKNLKEESDDNDYMKLLRAVITCL
AnnBj2      EKAYSDDDFIRILTTRSKAQLGATLNHYNNEYGNAINKNLKEDSD-DDYLKLLRAAITCL
            **:**************************************:** :**:*****.****

AnnAt2      TYPEKHFEKVLRLSINKMGTDEWGLTRVVTTRTEVDMERIKEEYQRRNSIPLDRAIAKDT
AnnBj2      TYPEKHFEKVLRLAINKMGTDEWGLTRVVTTRTEVDMERIKEEYQRRNSIPLDRAVAKDT
            *************:****************************************:****

AnnAt2      SGDYEDMLVALLGHGDA
AnnBj2      SGDYEDMLVALLGHGDV
            ****************.
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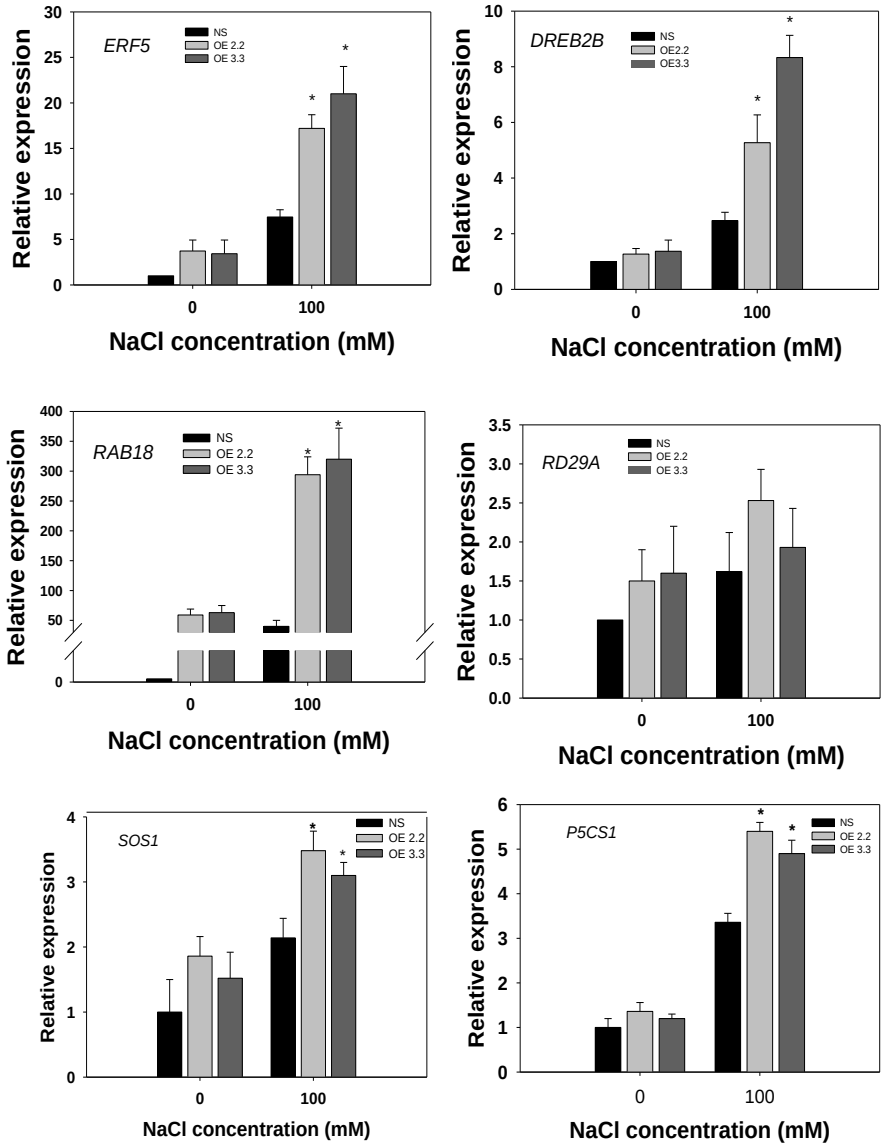

Figure 5.12: Expression of salt stress induced marker genes in control and AnnBj2 OE lines. 5 d old seedlings were treated either with mock or 100 mM NaCl for six hours and the samples were immediately quick frozen for RNA isolation. The relative expression of the *ERF5*, *DREB2B*, *RAB18*, *RD29A*, *SOS1* and *P5CS1* were normalized using the reference gene *actin2*. Expression levels were represented using Livak's $\Delta\Delta C_T$ method. The experiment was repeated thrice with three technical replicates. Error bar represents mean ±SE (n=3).Data were analyzed by one way ANOVA with DMRT to determine the statistical difference between null and the transgenic lines at $p \leq 0.05$ (indicated by single * mark)

5.3.11 *AnnBj2* transgenic lines did not show tolerance to mannitol and polyethylene glycol (PEG) stresses

To check the performance of *AnnBj2* transgenic lines in other stresses such as osmotic and drought stress, we performed seedling assay and monitored the growth of the

seedlings for 7 days. Three day old seedlings grown on ½ MS medium were transferred to 150 mM mannitol or 5% PEG containing ½ MS media. After 7 d of growth on mannitol or PEG stress media, no phenotypic difference were observed between NS and AnnBj2 OE lines (Figure 5.13), whereas NS seedlings showed severe reduction in the root growth on 100 mM NaCl stress medium in comparison to the AnnBj2 OE lines, which showed better root growth comparatively.

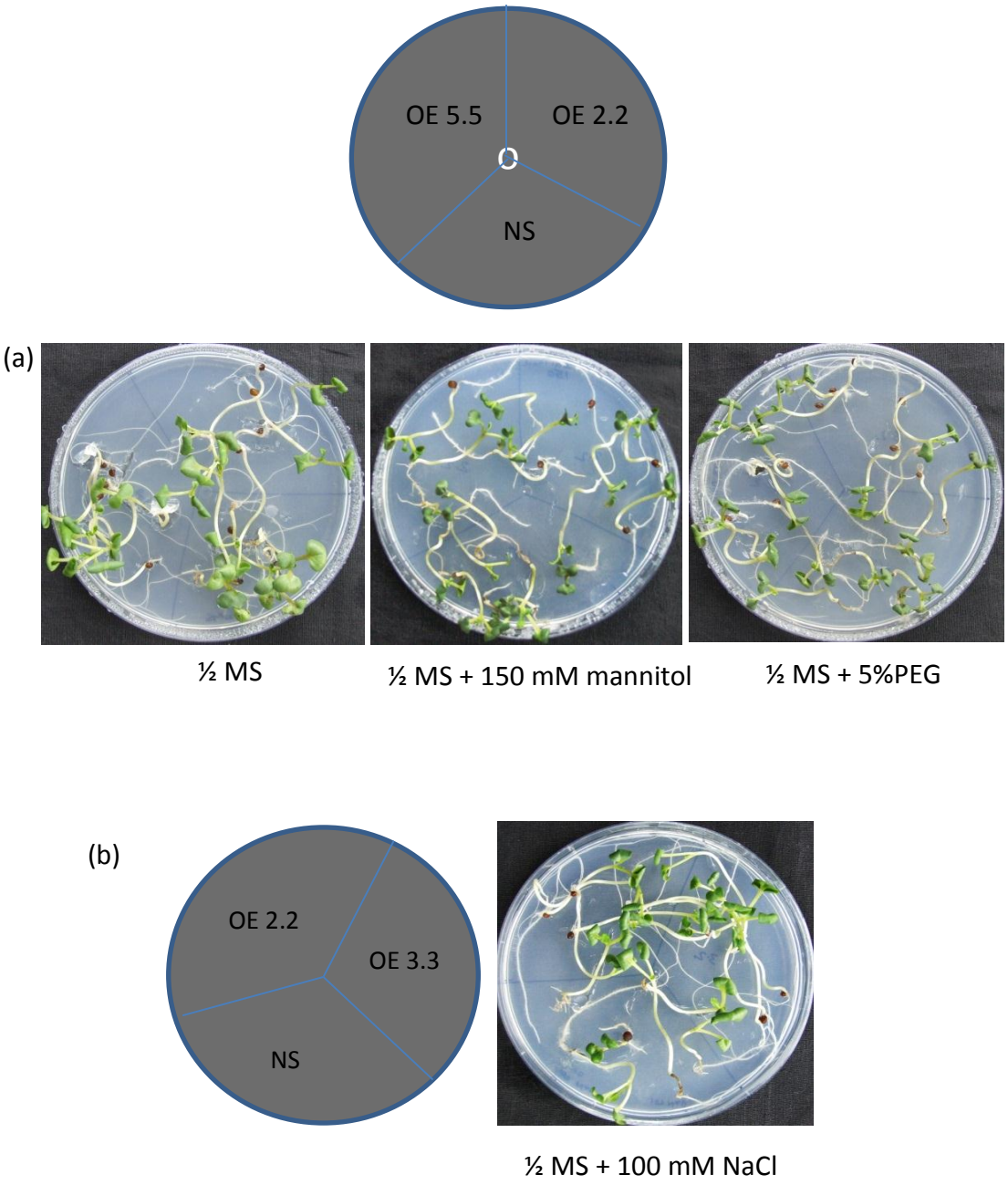

Figure 5.13: Response of NS and AnnBj2OE seedlings to mannitol, PEG and NaCl stress.

Chapter 6

Expression analysis of annexin homologs in *Brassica rapa* in response to different signaling molecules

6.1. Introduction

Chinese cabbage, *Brassica rapa* (2n=20) is an important vegetable crop cultivated worldwide. Its genome has been sequenced and assembled (Wang et al 2011). With the completion of genome sequencing of this crop species, genome wide identification and expression analysis of several gene families involved in plant growth and development under environmental stresses have been initiated by several groups (Duan et al. 2015; Huang et al. 2015; Kayum et al. 2015; Liang et al. 2015; Lu et al. 2015; Song et al. 2013; Tang et al. 2014; Tao et al. 2014; Wang et al. 2015). Genome reorganization is a rapid and common process associated with polyploidization, in which the duplicated gene copies are either deleted or retained for new, complementary and/or extra function (Barker et al. 2012).

Annexins are multifunctional ubiquitous proteins. In plants they play crucial roles in growth, development and stress responses (Clark et al. 2012; Mortimer et al. 2008). Earlier, the expression profiling of annexins has been reported in Arabidopsis and different crop plants. Elucidation of the transcript analysis of the complete gene family in response to stress inducers and signaling molecules helps in the identification of the candidate genes that can be further characterized for crop improvement through genetic manipulation.

Annexin expression is spatially and temporally regulated. Their expression patterns vary with tissue and developmental stages. Transcriptomic studies showed that the trascripts of these proteins are regulated by ABA, ET, SA, MeJa and auxins suggesting their role in plant signaling pathways. Apart from expression profiling studies, proteomic studies have identified annexins as stress regulated proteins. Abiotic stresses such as drought, salinity, osmotic stress, heavy metals, heat and cold results in the upregulation of one or more member of the annexin family. Overexpression of some annexins conferred stress tolerance in the transgenic plants while their loss of function resulted in susceptibility (Dalal et al. 2014a; Jami et al. 2008; Konopka-Postupolska et al. 2009; Qiao et al. 2015; Szalonek et al. 2015; Zhang et al. 2015).

Phylogenetic analysis of *B. rapa* annexins in comparison with other members of Brassicaceae family (Arabidopsis and mustard) revealed that they share a common ancestry. Based on sequence similarity, Bra036764, Bra034402, and Bra039578 are

found to be close to AnnAt1 of Arabidopsis and AnnBj1 of mustard, *Brasscia juncea*; Bra024346, Bra031890 showed similarity with AnnBj2 and AnnAt2 of mustard and Arabidopsis respectively; Bra000091 and Bra017102 are grouped with AnnBj3 and AnnAt3 of mustard and Arabidopsis respectively; Bra017103 and Bra000090 showed similarity with AnnBj4 and AnnAt4 of mustard and Arabidopsis respectively; Bra033961 showed sequence similarity with AnnAt5 of Arabidopsis; Bra009049 was found similar to AnnBj6 and AnnAt6 of mustard and Arabidopsis respectively, Bra009048 was found similar to AnnBj7 and AnnAt7 of mustard and Arabidopsis respectively, and Bra008892 was found similar to AnnAt8 of Arabidopsis.

The present study is aimed at the expression analysis of rapa annexins in response to abiotic stress inducers and identification of the potential candidate gene(s), which could be used for the improvement of *B. rapa* tolerance through genetic manipulation. Further, we also compared the expression of *AnnBj2* to its homologs *in B. rapa*.

6.2. Material and Methods

6.2.1. Plant material and growth conditions

B. rapa cv YID-1 (Pusa Gold) seeds, obtained from National Research Center on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi, India, were used in this study. Seeds were germinated on moistened blotting paper. Three days old seedlings were used for RNA isolation after NaCl treatment. One month old plants in the green house were used for tissue specific RNA isolation. Growth room temperature of 24 ± 2°C and 16/8 light/dark photoperiod were used for plant growth.

6.2.2. Sequence retrieval of *B. rapa* annexins

Annexin sequences in the genome of *B. rapa* were retrieved from the *B. rapa* genome database (BrGDB). Annexins in *B. juncea* and *A. thaliana* were searched using the Phytozome v10.0 GBrowse database (http:// www.phytozome.net/) (Goodstein et al., 2012) and The *Arabidopsis thaliana* Information Resource (TAIR) (https://www.arabidopsis.org/).

6.2.3. RNA isolation and quantitative RT-PCR

Total RNA was isolated from whole seedlings and different tissues by TRIzol method following manufacturer's instructions (Sigma-Aldrich Corporation, USA). cDNA was

prepared by using total RNA (2 µg), dNTPs, oligo-dT primer and MMLV Reverse transcriptase (Clontech, Takara Biotech, Japan) following the manufacturer's instructions. The resultant cDNAs were diluted 2.5 times (1:2.5) with RNase-free water and 1 µl of diluted cDNA was used in a total volume of 10 µl for determining the relative expression of *B. rapa* annexin genes using 2 × Fast start SYBR green PCR master mix (Roche GmbH, Germany). *B. rapa* ubiquitin was used as a reference gene. Real time PCR was performed in a 96 well plate using Realplex Ep4 (Eppendorf GmbH, Germany). Relative gene expression was calculated according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). For expression analysis under salt stress, expression levels of annexins were normalized with ubiquitin. Expression of annexins at 0 h was used as calibrator to determine the relative expression of the individual annexins with time. Expression levels of annexins were normalized with *B. rapa* ubiquitin.

Gene primers used for the real time expression analysis of *B. rapa* annexins

Gene ID	Primer name	Primer sequence
Bra034402	Bra034402 For	CGAGGTGAACATGACATTGGC
	Bra034402 Rev	CGTGCTCATCTTGGTAGCGA
Bra024346	Bra024346 For	GGTGACCTCCGCAAGCTATT
	Bra024346 Rev	TGGTTGAGAGTAGCACCGAG
Bra017102	Bra017102 For	CTATTGAGGCCGGTATGCTG
	Bra017102 Rev	CATCCATCAACATCCTTATCTAACG
Bra000090	Bra000090 For	ACAAGAAGCAAACTCCATCTCG
	Bra000090 Rev	ATACACGGAGGGTTTGAGCA
Bra033961	Bra033961 For	CTCACCTGGTCGCTCTTAGA
	Bra033961 Rev	CGCCGTGTCATCTGTTCCTA
Bra009049	Bra009049 For	GGGAACAAACGAGGGGATGA
	Bra009049 Rev	CAGCTCTCTCAAAGTCACCGG
Bra009048	Bra009048 For	CTTGGCTCACAGAAATGCCG
	Bra009048 Rev	AACATCACAGCTCGCTCGAA
Bra008892	Bra008892 For	TGCTTGCATGAGATCCCCTG
	Bra008892 Rev	CCTGATGTCGCCGGTAGTAC
Bra031890	Br031890 For	ACGGAAACGCTATTAACAAGCACT
	Br031890 Rev	CCCACTCATCAGTCCCCATTT
Bra000091	Br000091 for	TGCAAAGTTGCTGGTGACATTAG
	Br000091 Rev	CAAAGGTTGCTCTGAGCTGG

Bra017103	Br017103 For	TTCTTGCTCACACTGCTATCCA
	Br017103 Rev	CATGTCACCAACGACAAGAACT
Bra036764	Br036764 For	GCTTCGACTCTCGACATACACT
	Br036764 Rev	TACCCCATCCTTCAAAGGCG
Bra039578	Br039578 For	TTGTCCACAAGGAGCAAACCA
	Br039578 Rev	ACCTCAACAGCCCTAGGAACTT
BraActin7	BraActin7 For	TCCCTCAGCACTTTCCAACA
	BraActin7 Rev	ACTCAACACCACGAACGAGA
BraUbiquitin	Bra ubiquitin for	GATACTTGCGGCGGAGAAGA
	Bra ubiquitin rev	TGTCGATGGTGTCACTGCTC

6.3. Results

6.3.1. Differential expression of *B. rapa* annexins in response to hormone and stress treatments

Expression patterns of thirteen annexins were studied in response to different signaling molecules and abiotic stress inducers. The expression of each annexin at 0 h was used as calibrator to determine its relative expression on temporal scale. We found differential expression pattern of *B. rapa* annexins in response to signaling molecules (ABA, MeJa, and SA) and stress treatments (H_2O_2, MV). The expression patterns of annexins are discussed here.

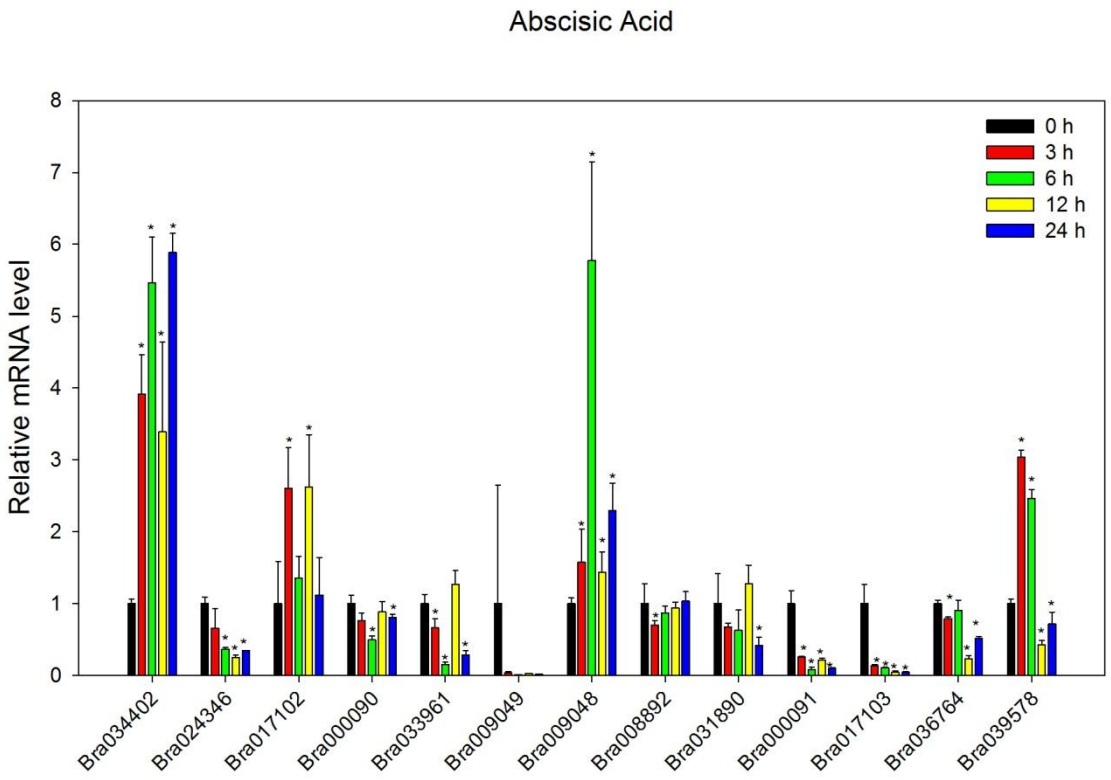

Figure 6.1. Effect of Abscisic acid (ABA) on expression of *B. rapa* annexins. Three day-old *B. rapa* seedlings were treated with 100 µM ABA and the samples were collected after 0, 3, 6, 12 and 24 h of treatment. Expression of annexins at 0 h was used as calibrator to determine the relative expression of the individual annexins with time. Expression levels of annexins were normalized with ubiquitin. Error bar represents the standard error of three biological replicates. The qRT-PCR experiments represent the mean of n= 3 ± SE, for each biological replicate (n) three technical replicates were used. Asterisks represent a statistically significant difference ($P \leq 0.05$) in mRNA level as compared to that of the 0 h.

Treatment of ABA upregulated the expression of Bra034402, Bra024346, Bra009048 and Bra039578 whereas it downregulated Bra024346, Bra009049, Bra000091, Bra017103. Transcript levels of Bra000090, Bra008892, Bra031890 and Bra036764 remain nearly unchanged. Expression of Bra034402 and Bra009048 showed similar pattern of expression; their transcript levels increased after 3hours of ABA treatment, which further increased up to 6 hours and then decreased after12 hours of treatment with a rebound again after 24 hours of treatment. Transcript levels of Bra024346, Bra009049, Bra000091 and Bra017103 decreased gradually within 3 hours of treatment.

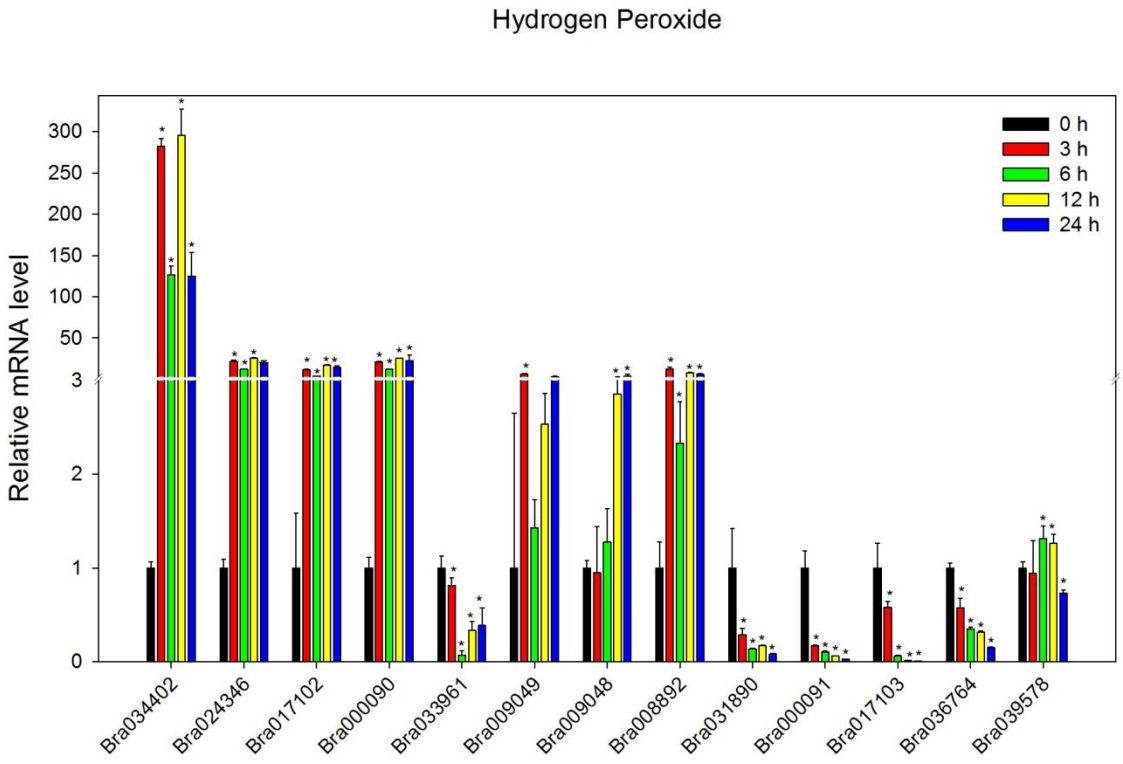

Figure 6.2: Expression of *B. rapa* annexins under Hydrogen peroxide (H_2O_2) stress treatments. Three day-old *B. rapa* seedlings were treated with 10 mM H_2O_2 and the samples were collected after 0, 3, 6, 12 and 24 h of treatment. Expression levels of annexins were normalized with ubiquitin. Expression of annexins at 0 h was used as calibrator to determine the relative expression of the individual annexins with time. Error bar represents the standard error of three replicates. The qRT-PCR experiments represent the mean of n=3±SE, for each biological replicate (n) three technical replicates were used. Asterisks represent a statistically significant difference ($P \leq 0.05$) in mRNA level as compared to that of the 0 h.

H_2O_2 upregulated Bra034402, Bra024346, Bra000090, Bra009048, Bra008892 whereas it downregulated Bra033961, Bra031890, Bra000091, Bra017103 and Bra036764. We observed a difference in the pattern of upregulation of annexin members. Message levels of Bra034402 and Bra009049 increased within 3 h of treatment, which then showed a decrease in transcript levels at 6 h of treatment followed by a rebound again after 12 h of treatment. Unlike Bra034402 and Bra009049, transcript levels of Bra024346, Bra017102 and Bra000090 increased within 3 h of treatment and were maintained up to 24 h of treatment.

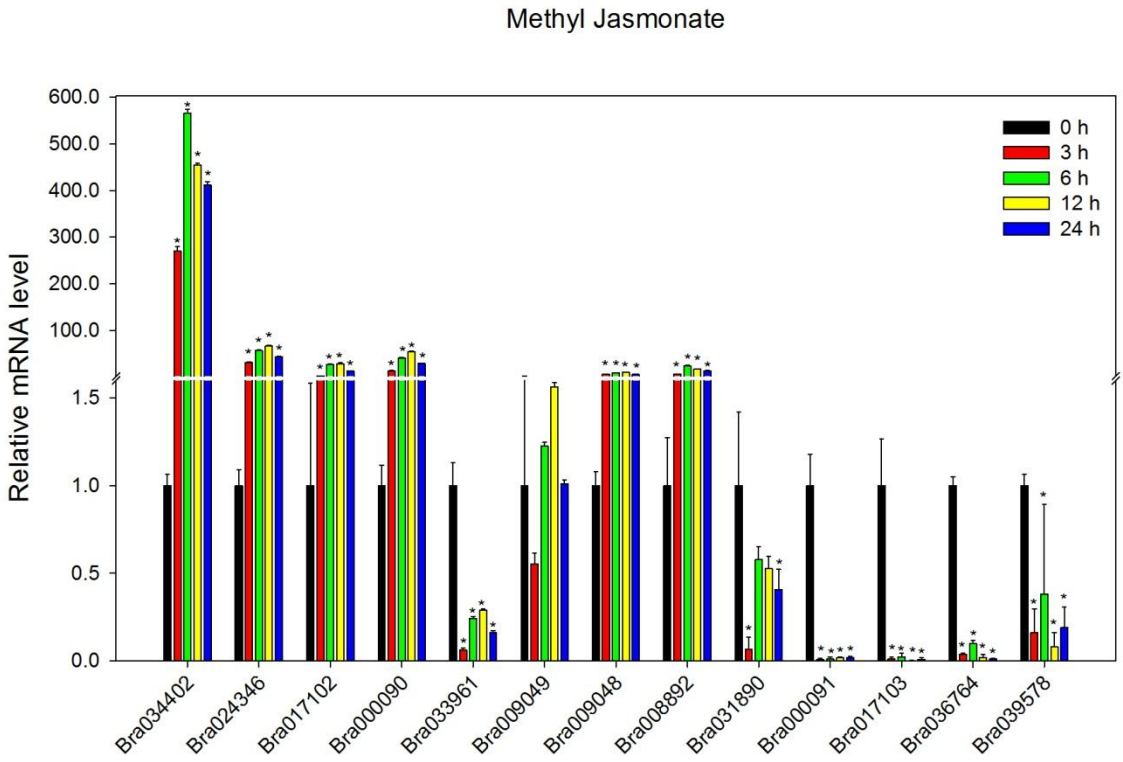

Figure 6.3: Expression of *B. rapa* annexins under methyl jasmonate stress treatments. Three day-old *B. rapa* seedlings were treated with 10 µM MV and the samples were collected after 0, 3, 6, 12 and 24 h of treatment. Expression levels of annexins were normalized with ubiquitin. Expression of annexins at 0 h was used as calibrator to determine the relative expression of the individual annexins with time. Error bar represents the standard error of three replicates. The qRT-PCR experiments represent the mean of n = 3 ± SE, for each biological replicate (n) three technical replicates were used. Asterisks represent a statistically significant difference ($P \leq 0.05$) in mRNA level as compared to that of the 0 h.

Methyl jasmonate upregulated the expression of Bra034402, Bra024346, Bra017102, Bra000090, Bra009048, and Bra008892 whereas it downregulated Bra033961, Bra031890, Bra000091, Bra017103, Bra036764 and Bra039578. Transcript levels of Bra034402 showed 290 fold (approx.) increases within 3 h of treatment, which further increased to 590 folds after 6 h of treatment, which then decreased to 450 fold at 12 h after treatment. Bra024346, Bra017102, Bra000090, Bra009048 and Bra008892 showed similar pattern of expression in response to methyl jasmonate treatment and their transcript levels were maintained upto 24 h of treatment

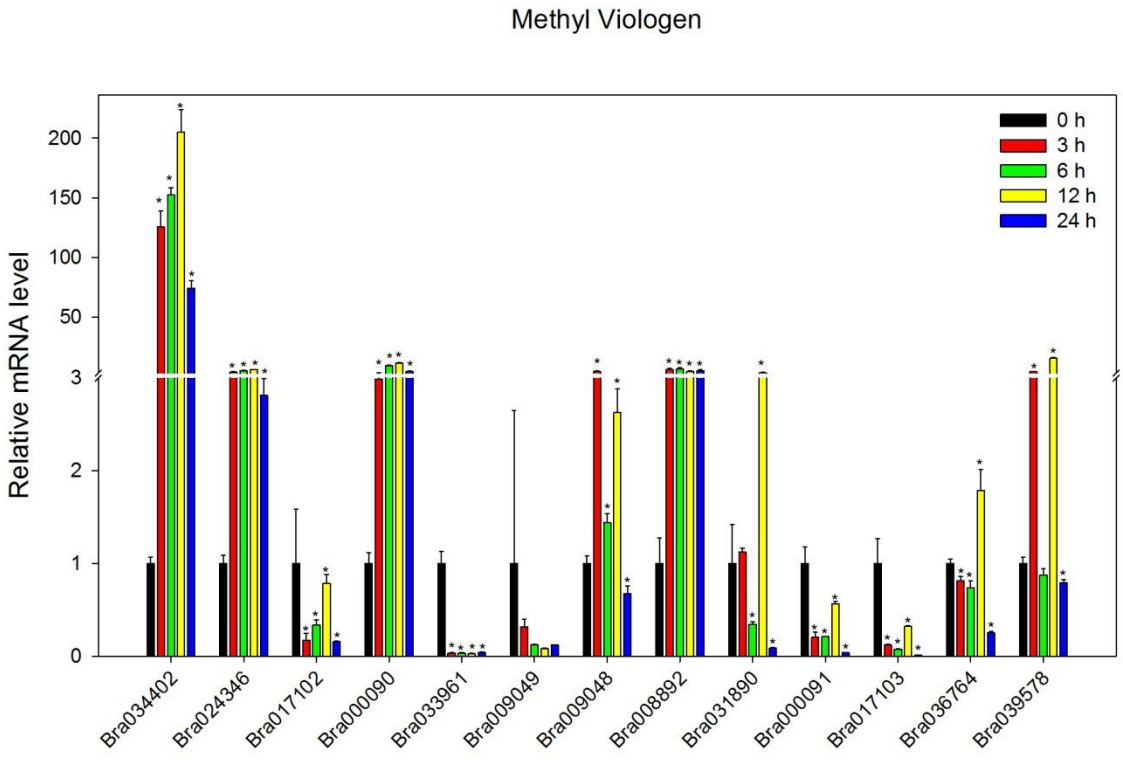

Figure 6.4: Expression of *B. rapa* annexins under methyl viologen (MV) stress treatments. Three day-old *B. rapa* seedlings were treated with 10 µM MV and the samples were collected after 0, 3, 6, 12 and 24 h of treatment. Expression levels of annexins were normalized with ubiquitin. Expression of annexins at 0 h was used as calibrator to determine the relative expression of the individual annexins with time. Error bar represents the standard error of three replicates. The qRT-PCR experiments represent the mean of n = 3 ± SE, for each biological replicate (n) three technical replicates were used. Asterisks represent a statistically significant difference ($P \leq 0.05$) in mRNA level as compared to that of the 0 h.

Methyl viologen (MV) treatment upregulated the expression of Bra034402, Bra024346, Bra000090, Bra009048 and Bra008892 whereas it downregulated the expression of Bra017102, Bra033961, Bra009049, Bra000091, and Bra017103. Expression of Bra017102 that was upregulated in all the other treatments showed decreased transcript levels in response to MV treatment. Bra031890 and Bra036764 were induced to lower levels after 12 h of treatment.

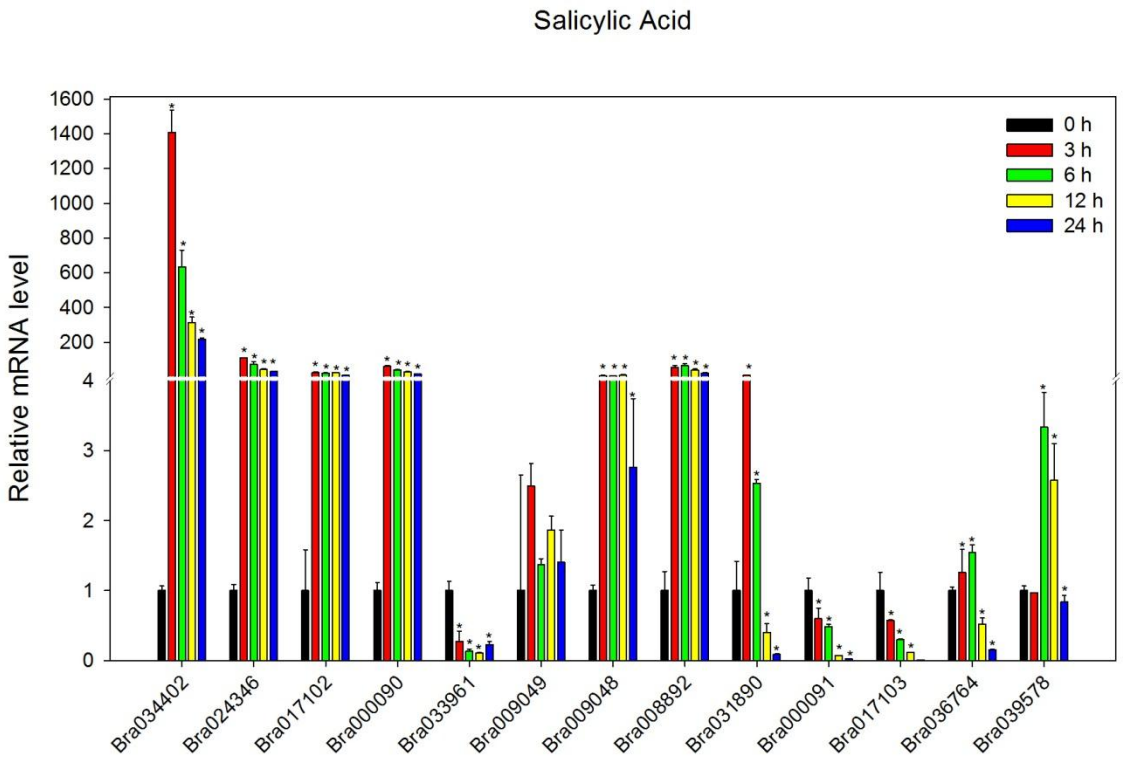

Figure 6.5: Effect of Salicylic acid (SA) on expression of *B. rapa* annexins. Three day-old *B. rapa* seedlings were treated with 100 µM SA and the samples were collected after 0, 3, 6, 12 and 24 h of treatment. Expression of annexins at 0 h was used as calibrator to determine the relative expression of the individual annexins with time. Expression levels of annexins were normalized with ubiquitin. Error bar represents the standard error of three biological replicates. The qRT-PCR experiments represent the mean of n= 3 ± SE, for each biological replicate (n) three technical replicates were used. Asterisks represent a statistically significant difference ($p \leq 0.05$) in mRNA level as compared to that of the 0 h.

Salicylic acid treatment upregulated Bra034402, Bra024346, Bra017102, Bra000090, Bra009048, and Bra008892 whereas it downregulated the expression of Bra033961, Bra000091, and Bra017103. Transcript levels of Bra036764 did not show much difference with SA treatment. Bra034402 showed the highest level of expression (1400 fold) within 3 h of SA treatment and then the transcript levels gradually decreased upto 24 h of treatment. But unlike Bra034402, transcript levels of Bra024346, Bra017102, Bra000090 and Bra008892 showed increase in the transcript within 3 h of treatment and were maintained upto 24 h of treatment.

Among all the annexins, transcript levels of Bra034402 showed the highest increase in response to all the treatments, although the pattern of expression differed with various treatments.

6.3.2. Comparison of Annexin2 from *B. juncea* (*AnnBj2*) and *B. rapa* (Bra024346)

We compared the protein sequence of annexin2 from *B. juncea* and *B. rapa* using ClustalW pairwise alignment tool (http://www.genome.jp/tools-bin/clustalw). They share 98% similarity at amino acid level and 97% at nucleotide level. Both annexins have same number of amino acids (316) with similar deduced molecular weight (36KDa).

Similarity at nucleotide level

```
AnnBj2          ATGGCGTCTCTCAAAGTCCCGAGCAATGTTCCTCTCCCCGAAGACGACGCCGAGCAACTC
Bra024346       ATGGCGTCTCTAAAAGTCCCGAGCAATGTCCCTCTCCCCGAAGACGACGCCGAGCAACTC
                *********** ***************** ******************************

AnnBj2          CACAAGGCTTTTTCAGGATGGGGTACCAACGAGAAGCTGATCATATCAATCCTAGCTCAC
Bra024346       CACAAGGCTTTCTCAGGATGGGGTACGAACGAGAAGCTGATCATATCAATCCTAGCTCAC
                *********** ************** ********************************

AnnBj2          AGGAACTCTGCACAACGCAGCTTGATCCGCAGCGTTTATGCCGCTACTTACAATGAGGAT
Bra024346       AGAAACTCTGCACAACGCAGCTTGATCCGCAGCGTTTACGCCGCTACTTACAATGAGGAT
                ** ********************************** *********************

AnnBj2          CTCCTCAAGGCTTTAGACAAAGAGCTTTCTAGCGACTTTGAGAGAGCTGTGATGTTGTGG
Bra024346       CTCCTCAAGGCTTTAGACAAAGAGCTCTCTAGCGACTTTGAGAGAGCTGTGATGTTGTGG
                ************************** *********************************

AnnBj2          ACTCTTGATCCAGCGGAGAGAGATGCTTATCTGGCTAAAGAATCCACCAAAATGTTCACC
Bra024346       ACGCTTGATCCAGCGGAGAGAGATGCTTATCTGGCCAAAGAATCCACCAAAATGTTCACC
                ** ******************************** ************************

AnnBj2          AAGAACAATTGGGTTCTTGTTGAAATCGCTTGCACAAGGTCTGCTCTTGAGCTTTTCAAG
Bra024346       AAGAACAACTGGGTTCTCGTCGAGATCGCTTGCACAAGGTCTGCTGTTGAGCTTTTTAAG
                ******** ******** ** ** ********************** ********** ***

AnnBj2          GTCAAGCAAGCTTACCAGGCTCGCTACAAGAAATCACTCGAGGAAGATGTTGCTCAACAC
Bra024346       GTCAAGCAAGCTTACCAAGCTCGCTACAAGAAATCGCTCGAGGAAGATGTTGCTCAACAC
                ***************** ***************** ************************

AnnBj2          ACATCTGGTGACCTTCGTAAGCTCTTGCTTCCTCTTGTTAGCACTTTCAGGTACGAAGGA
Bra024346       ACTTCCGGTGACCTCCGCAAGCTATTGCTTCCTCTGGTTAGCACTTTCAGGTACGAAGGA
                ** ** ******** ** ***** ************ ************************

AnnBj2          GATGATGTGAACATGATGCTTGCAAGATCTGAAGCTAAGTTACTTCACGAGAAGGTCTCA
Bra024346       GACGATGTGAACATGATGCTCGCAAGATCTGAAGCCAAGTTGCTTCACGAGAAGGTCTCA
                ** ***************** ************** ***** ******************

AnnBj2          GAGAAAGCTTACAGCGATGATGACTTCATCAGAATCTTGACAACAAGAAGCAAAGCACAG
```

```
Bra024346       GAGAAAGCTTTCAACGATGATGACTTCATCAGAATCTTGACCACAAGAAGCAAAGCACAG
                ********** ** ************************** ******************

AnnBj2          CTCGGTGCTACTCTCAACCACTACAACAATGAGTATGGAAACGCCATCAACAAGAACTTG
Bra024346       CTCGGTGCTACTCTCAACCACTACAACAATGAGCATGGAAACTCCATCAACAAGAACTTG
                ********************************* ******** ******************

AnnBj2          AAGGAAGATTCAGATGATGATTACCTGAAACTACTAAGAGCTGCAATCACCTGTTTGACA
Bra024346       AAGGAAGGTTCAGATGATGATTACCTGAAACTACTTAGAGCTGCAATCACGTGTTTGACA
                ******* *************************** ************** *********

AnnBj2          TACCCTGAGAAGCATTTTGAGAAGGTTCTGCGTCTAGCAATCAACAAAATGGGAACAGAC
Bra024346       TACCCTGAGAAGCATTTTGAGAAGGTTCTGCGTCTCGCAATCAACAAAATGGGAACTGAC
                ********************************** ******************** ***

AnnBj2          GAGTGGGGACTAACCCGAGTCGTGACTACACGAACTGAAGTTGACATGGAACGCATCAAA
Bra024346       GAGTGGGGACTAACCAGAGTCGTGACTACACGAACTGAAGTTGACATGGAACGCATCAAA
                *************** ******************************************

AnnBj2          GAGGAGTATCAGCGAAGGAACAGCATTCCTTTGGACCGTGCCGTCGCTAAAGACACTTCT
Bra024346       GAGGAGTATCAGCGTAGGAACAGCGTTCCTTTGGACCGTGCCGTTGCTAAAGACACTTCT
                ************** ********* ******************* ***************

AnnBj2          GGTGACTATGAGGACATGCTTGTTGCTCTTCTCGGACATGGGGATGTCTGA
Bra024346       GGTGATTATGAGGACATGCTTGTTGCTCTTCTTGGACATGGGGATGTCTGA
                ***** ************************** ******************
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Similarity at amino acid level

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AnnBj2          MASLKVPSNVPLPEDDAEQLHKAFSGWGTNEKLIISILAHRNSAQRSLIRSVYAATYNED
Bra024346       MASLKVPSNVPLPEDDAEQLHKAFSGWGTNEKLIISILAHRNSAQRSLIRSVYAATYNED
                ************************************************************

AnnBj2          LLKALDKELSSDFERAVMLWTLDPAERDAYLAKESTKMFTKNNWVLVEIACTRSALELFK
Bra024346       LLKALDKELSSDFERAVMLWTLDPAERDAYLAKESTKMFTKNNWVLVEIACTRSAVELFK
                ************************************************:***********

AnnBj2          VKQAYQARYKKSLEEDVAQHTSGDLRKLLLPLVSTFRYEGDDVNMMLARSEAKLLHEKVS
Bra024346       VKQAYQARYKKSLEEDVAQHTSGDLRKLLLPLVSTFRYEGDDVNMMLARSEAKLLHEKVS
                ************************************************************

AnnBj2          EKAYSDDDFIRILTTRSKAQLGATLNHYNNEYGNAINKNLKEDSDDDYLKLLRAAITCLT
Bra024346       EKAFNDDDFIRILTTRSKAQLGATLNHYNNEHGNSINKNLKEGSDDDYLKLLRAAITCLT
                ***:.*************************:**:*******.*****************

AnnBj2          YPEKHFEKVLRLAINKMGTDEWGLTRVVTTRTEVDMERIKEEYQRRNSIPLDRAVAKDTS
Bra024346       YPEKHFEKVLRLAINKMGTDEWGLTRVVTTRTEVDMERIKEEYQRRNSVPLDRAVAKDTS
                ************************************************:***********

AnnBj2          GDYEDMLVALLGHGDV
Bra024346       GDYEDMLVALLGHGDV
                ****************
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Figure 6.6: Pairwise sequence alignment of *AnnBj2* and its homolog Bra024346.

We compared the expression pattern of *AnnBj2* to that of its homolog (Bra024346) in *B.rapa*. At tissue level, both these genes have higher transcript levels in root compared to other tissues. Both *AnnBj2* and Bra024346 were upregulated by NaCl,

MeJa, and SA whereas ABA and H_2O_2 stress showed difference in their expression pattern. ABA upregulated *AnnBj2* whereas it downregulated Bra024346. H_2O_2 upregulates Bra024346 as early as 3 h after treatment and the transcripts were maintained up to 24 h of treatment but *AnnBj2* transcripts first decrease within 3 h and then rebound at 6 h after treatment.

```
ATACGTCTAA TTAAAATAAC ATAGTGTTAT TTAAAACTAT TCATATTGAT TATAAAATAA AT
AACAGAAT

                      BOX-4
+ AATTAAAACT AAAATATTAA TAAATTATTA TAATATATTT ATTTTATAAT ATTTATTTTA
TAATATTTAT

+ ATGCATGCAT AATCACGGAA AAATCACTTA GTATTTAATT AAATTCCAAT ACTAACAACG
CCATTAAGTA

+ AACCATGGAC TCATCATCGT CCTCGCTTTC CACATCGTAT CTCAGTATTT CTATAATATA
ATAAAAATAA

                                                  BOX-W1
+ TTTAATTGAA AAACAGTTTG ATTTTGGACT TATTCTGAAT TGACCAAGTC CAAAGAACGT
GTTCACATTC

                  TC-repeat
+ TGCTTATTCG CAAGTTTCTC CATCTCAGCG CACGTTTTAC ATCACATTTA TTGACAATGT
ACCGATCAAT

+ TATTTACATT TATGCCTGTA TACAATTAAC TTTTCTCTTC GAAATTGAAT TGTAGCAAAA
AAGAAGACTT

      Box1
+ GTGTTTCAAA AAAAAAAAAA AAGAAGACTT TTATATTATA AAAACTTAAC TAGGTATCAT
ATCCCTCCTA

                                        ARE-ELEMENT
+ TATCTCTTTT TGTAATAATG ATTTTAATTA CAGGCTGGTT TGAGAAAAAA AACAATTGAA
ATAGTGATTA

                                  WUN-motif
+ CTTGAATTAG TTTTATACAT TTTCAATCAT TACTAACCTT TAAATTTAAA ATTTAAAGTA
TACAAATATA

                                  TC-repeat
+ AATGTCACAG AAAAAGCCAT TTCCTCTTTT TTTCTTCAAG ACAGCTGTTA AATGATTTAT
TAGGACAATA

+ TATGTGTCCA TATATTGAAT TATTTTTAGA TTTAATACTA AATGCTTAGA GATTACTTTC
TTTCTTTTTT

+ TTTTTGTAAC ACAGGGATTA CTTTCTTTAT ATTCATAAAA TAACTTTTGA GATTTCCCGT
AACGTTATTT

+ AAGTGAGAAG ACGTGCATCA ACCTTCTTCA TCTCATTACA ACGAAAAAAA CACAACAACA
CAGAGAGATC
```

Figure 6.7: *cis- acting* elements present in promoter region of *Bra024346*

Box-1: light responsive element; Box-4: light responsiveness; Box W1: fungal elicitor responsive element; TC- repeat: defense and stress responsiveness; WUN motif: wound responsiveness; ARE element: involved in anaerobic induction

We compared the promoter region of Bra024346 and the partial promoter of *AnnBj2*, previously reported by Jami et al. (2009). We found the presence of some common *cis*- acting elements such as W-Box (involved in SA mediated defense response), TC-rich repeat and light responsive elements. Bra024346 does not show the presence of ABRE element and accordingly its expression was not induced by ABA treatment. Both Bra024346 and *AnnBj2* were induced by SA and MeJa treatments, and accordingly they showed the presence of some *cis* acting elements involved plant defense responses.

Chapter 7

Discussion

Discussion

Constitutive expression of *AnnBj2* confers salt tolerance and ABA insensitivity to the transgenic tobacco and mustard seedlings

Annexins are calcium dependent membrane binding proteins. Recent studies revealed their role in plant growth and development. Ectopic expression studies of several annexins showed that they play a protective role in plants while the loss of function of some annexins compromised with their survival when challenged with drought, salt, heat or oxidative stresses (Dalal et al. 2014a; Jami et al. 2008; Konopka-Postupolska et al. 2009; Qiao et al. 2015; Szalonek et al. 2015; Zhang et al. 2015). In *B. juncea* six annexins were reported among which the role of *AnnBj1*and *AnnBj3* has been studied by ectopically expressing them in tobacco, cotton and Arabidopsis (Dalal et al. 2014a; Dalal et al. 2014b; Divya et al. 2010; Jami et al. 2008). In the present study, we focused on another member *AnnBj2* that is strongly upregulated by the signaling molecules ABA, MeJa, SA and Ethephon. Tissue specific expression study has revealed that *AnnBj1* and *AnnBj2* are the two most abundant annexins at seedling stage. Among these two, *AnnBj1* has similar expressions in roots, stem and cotyledonary leaf whereas *AnnBj2* has higher expression in roots compared to other tissues at the seedling stage and is induced by ABA and NaCl treatments. This prompted us to study the involvement of *AnnBj2* in amelioration of abiotic stresses, particularly salt and drought as these stresses are perceived through roots. To study its function, we constitutively expressed it in model system tobacco and in the native system Indian Mustard. We observed that *AnnBj2* did not impart multiple stress tolerance except salinity tolerance, while *AnnBj1* has been shown to impart tolerance to several stresses (Jami et al. 2008, Divya et al. 2010) where as its counterpart in Arabidopsis, AnnAt1 also showed similar functions (Konopka-Postupolska et al. 2009) . Hence, we mainly focused on the characterization of the *AnnBj2* expressing tobacco and mustard plants under salt stress.

AnnBj2 from *B. juncea* shared 97% similarity with its Arabidopsis homolog AnnAt2 at amino acid level and the expression pattern of *AnnBj2* is analogous to that of its homologs in *Arabidopsis* and *Brassica rapa* (Cantero et al. 2006; Clark et al. 2001; Yadav et al. 2015) Earlier reports suggested that the tissue specific expression of certain annexins may be associated with their specific functions (Clark et al. 2005; Tang et al. 2014; Zhu et al. 2014). The contrasting difference in the induction of

AnnBj2 in two different tissues (leaf and root) supports the importance of tissue-specific expression of annexins. The proteomic data of its homolog in Arabidopsis (AnnAt2) also confirmed its upregulation under salt stress (Jia et al. 2015). Higher expression of *AnnBj2* in roots together with its upregulation by NaCl and ABA prompted us to study its role in salt stress, which is perceived through roots. *AnnBj2* OE lines of tobacco and mustard exhibited better root growth than controls under salt stress. Laohavisit et al. (2013) reported earlier the role of AnnAt1 in the salinity-induced root cell adaptation. This study shows that AnnBj2 is also involved in maintaining sustained root growth in salt treatments.

Salt stress leads to the disruption of osmotic and ionic homeostasis of cells (Deinlein et al. 2014; Munns and Tester 2008; Zhu 2003) and to the excessive production of ROS, which are detrimental to the plant (Apel and Hirt 2004; Moller et al. 2007). Proline and lipid peroxidation levels are important indicators of plant oxidative stress (Ashraf and Foolad 2007; Szabados and Savoure 2010; Yoshiba et al. 1995). It was previously reported that the overexpression of *P5CS* gene, a rate-limiting enzyme in biosynthesis of proline enhances osmotic stress tolerance of transgenic plants (Kishor et al. 1995). In our study, we found that constitutive expression of *AnnBj2* enhanced the proline content in transgenic tobacco and mustard under NaCl stress. Further, the expression of *P5CS1* was higher in the transgenics than the control seedlings. Lipid peroxidation was found to be significantly lower in the transgenics than the controls. Verslues and Bray (2006) reported that the *abi4* mutants of Arabidopsis exhibited increased proline accumulation under high water potential and insensitivity to ABA indicating that *ABI4* is a part of a mechanism to restrict proline accumulation when carbohydrate content is low. The *AnnBj2* constitutive expression in tobacco and mustard lead to enhanced proline accumulation under NaCl stress by modulating the expression of *P5CS1* with with significantly downregulated expression of *ABI4*, which is in line with previous findings

Reports show a strong correlation between salt exclusion and salt tolerance in many plant species (Blumwald 2000; Deinlein et al. 2014; Roy et al. 2014). In the present study, we found that *AnnBj2* OE mustard lines also exhibited comparatively less Na^+ accumulation and maintained higher K^+/ Na^+ ratio than the controls under salt stress. Maintenance of higher K^+/Na^+ ratio in the cytoplasm is correlated with salinity tolerance (Adem et al. 2014; Munns and Tester 2008; Zhu 2003). AnnAt1 was earlier

reported to restrict the entry of Na^+ fluxes across the root epidermal cells, and the knock out mutant *annAt1* exhibited higher Na^+ influx when challenged with NaCl stress (Laohavisit et al. 2013). Recently, Jia et al. (2015) reported that SCF E3 ligase overexpression restricted Na^+ ion accumulation in the plant tissues and played a positive role in response to salt stress. SCF overexpression upregulates AnnAt1, AnnAt2, and AnnAt3 at the protein level.

Gene expression analysis under salt stress in the present study revealed that *AnnBj2* OE lines have relatively higher transcript levels of *SOS1* compared to the control. *SOS1* encodes a Na^+/H^+ exchanger on the plasma membrane that modulates the movement of ions across the cell membrane (Shi et al. 2000). This suggests that AnnBj2 appears to restrict the entry of Na^+ inside the cells by stabilizing the integrity of membrane-bound SOS1 Na^+ antiporter. Ca^{2+} plays a protective role in alleviating salt stress by competing with Na^+ at plasma membrane (Cramer et al. 1985; Lynch and Lauchli 1985). More recently, Wang et al. (2015) found a significant decrease in the Ca^{2+} content of *annAt1* loss of function mutants. The significantly increased levels of Ca^{2+} in *AnnBj2* OE lines are also in line with the earlier findings. The A*nnBj2*OE lines 2.2, OE 3.3and OE 5.5 of mustard showed enhanced seed germination pattern even in the absence of any stress. The role of annexins in seed germination and vigour was previously reported (Chu et al. 2012; Huh et al. 2010).

The mechanism underlying the involvement of annexins in mitigating salt and other abiotic stresses remains largely unknown. To elucidate the molecular mechanism of AnnBj2 in conferring salt tolerance and ABA-insensitive phenotype, we studied the expression of the genes, which regulate seed germination process at the transcription level. We have observed enhanced seed germination of *AnnBj2* transgenic seeds under control, salt, and ABA stress conditions. During seed germination, NCED6 catalyzes the cleavage of 9-cis-neoxanthin to xanthoxin, the rate limiting step in ABA biosynthesis (Seo et al. 2009) and *CYP707A2*, which encodes an ABA 8- hydroxylase that catalyzes the hydroxylation of ABA for its conversion into phaseic and dihydro-phaseic acids (Kushiro et al. 2004). The relative expressions of these two genes regulate the ABA content of the seeds during imbibition (Nambara and Marion-Poll 2005; Nonogaki 2015). During germination, ABA content of the seeds decreases while there is an increase in GA level (Ogawa et al. 2003; Weitbrecht et al. 2011). Our results show that AnnBj2 OE lines have increased transcript levels of *CYP707A2*

involved in ABA degradation, while there was no significant difference in ABA biosynthesis gene, *NCED6*. Also, *ABI4*, which is a positive regulator of seed dormancy, has decreased transcript levels in the transgenic plants.

We observed reduced sensitivity of *AnnBj2* OE lines to ABA at seed germination stage suggesting that the annexin acts as a negative regulator of ABA signaling at seed germination stage. ABA plays an inhibitory role in seed germination process (Finkelstein et al. 2002; Rajjou et al. 2012). ABI4 is a key factor that regulates the balance between GA and ABA. It regulates seed dormancy positively and regulates the greening of the cotyledons negatively in seed germination (Shu et al. 2013). It represses *CYP707A1* expression by directly binding to the *ABI4* binding elements in its promoter (Shu et al. 2013). In *AnnBj2* transgenics, we observed significantly reduced *ABI4* levels compared to controls, which exhibited higher *ABI4* transcript levels. Arabidopsis mutants of *ABI4* germinate on inhibitory concentrations of sodium chloride, sucrose, ABA and others (Arenas-Huertero et al. 2000; Finkelstein 1994; Huijser et al. 2000; Quesada et al. 2000).

Finkelstein et al. (2011) demonstrated that ABI4 represses seed germination in the presence of ABA, whereas Shu et al. (2013) reported that higher ABI4 levels in controls lead to reduced seed germination, particularly under salt stress and the transgenics showing low *ABI4* levels exhibited enhanced seed germination and cotyledon greening. Also, Shkolnik-Inbar et al. (2013) demonstrated that *ABI4* downregulated the expression of the sodium antiporter *HKT1;1* in roots affecting salt tolerance. Although the loss of *ABI4* had little effect on root growth, ectopic *ABI4* expression resulted in hypersensitivity to root growth inhibition by exogenous application of ABA. The germination and growth of the plants expressing *ABI4* is completely blocked. The *abi4* mutants germinated on higher concentration of ABA and *ABI4* is strongly expressed in mature seeds from globular stage embryo on wards. High levels of *ABI4* resulted in hypersensitivity to ABA-induced root inhibition and inhibited root growth even in the absence of ABA (Soderman et al. 2000). ABI4 has also been shown to bind to the ABI4 binding sites (ABE) in the promoter of *HKT1;1* downregulating its expression. Arabidopsis *San5* mutant is tolerant to salt and ABA and carries an extremely hypomorphic or null allele of *ABI4* (Quesada et al. 2000). Thus, *ABI4* upregulation resulted in salt-sensitive phenotype. In the present case, *ABI4* overexpression in the control was associated with salt-sensitive phenotype and a

salt tolerant phenotype was exhibited by the AnnBj2 transgenic plants with significantly downregulated *ABI4* transcript levels.

AnnBj2 transgenic plants exhibited enhanced levels of *CYP707A2* with reduced expression of *ABI4* in a significant contrast to controls, which showed high-level expression of *ABI4* and significantly reduced transcript levels of *CYP707A2*. Kushiro et al. (2004) demonstrated that all *CYP707A* genes get upregulated in drought stress treatments and subsequent rehydration of the treated plants. In *Glycine max* also, most of the *CYP707A* genes get upregulated in drought stress and salinity. Overexpression of *CYP707A1* in *atcyp707a2* insertion mutant resulted in its decreased sensitivity to ABA showing that it functions as an ABA hydroxylase (Zheng et al. 2012). The constitutive expression of *CYP707A3* restored growth retardation induced by the exogenous application of ABA with increased transpiration and reduced endogenous ABA levels in both turgid and dehydrated plants (Umezawa et al. 2006).

It has also been shown that the ABA-insensitive mutants exhibit glucose insensitive phenotype. The residual ABA levels in the mutants are evidently reduced to alter the response to sugars (Huijser et al. 2000). Teng et al. (2008) showed that delay of germination (*DOG1*) mutants of Arabidopsis has elevated levels of *ABI4* mRNA and these plants were sensitive to glucose. Interestingly, the transgenic mustard plants expressing *AnnBj2* with significantly reduced *ABI4* transcripts also exhibit glucose insensitive phenotype and enhanced expression of *ABI4* in control resulted in glucose sensitivity.

In our study, we found that the expression of *AnnBj2* conferred ABA insensitivity at seed germination stage and salt tolerance at seedling and adult stage. It is a well-known fact that the accumulation of ABA induces stomatal closure in leaves to maintain water balance during stress conditions (Cutler et al. 2010). Previously, several groups have reported the constitutive expression of certain genes like *WRKY20*, *GmbZIP62*, *GsSKP21* and *OsPP108* conferred ABA insensitivity at seed germination stage but results in abiotic stress tolerance such as drought, salt and alkalinity (Liao et al. 2008; Liu et al. 2015; Luo et al. 2013; Singh et al. 2015). This suggests that ABA signaling differs at seed germination and stomatal closure.

These data suggest that AnnBj2 may be involved in the regulation of ABA content of the seeds during seed germination by modulating the expression of ABA biosynthesis

and catabolism genes. The highly reduced *ABI4* transcripts could be related to enhanced salt tolerance in transgenic plants. Similarly, *ABI4* downregulates the Sodium antiporters, which results in reduced salt tolerance. Thus, the reduced *ABI4* associated with the constitutive expression of *AnnBj2* can be linked to the strong salt tolerant phenotype exhibited by the *AnnBj2* transgenic plants. These transgenic plants exhibited ABA insensitivity and strong salt tolerance phenotype with tolerance to no other abiotic stress treatments.

The AnnBj2 mustard transgenics exhibited strong expression of ABA responsive genes like *RAB18* and genes like *DREB2B*, *ERF5* that have been shown to act in ABA- dependent and -independent pathway (respectively). This phenomenon suggests that the salt tolerance phenotype observed in the mustard transgenics is controlled by both ABA-dependent and ABA-independent pathways, which may cooperatively cross talk with each other. *RD29A* is a possible convergence point in the cross talk as its promoter carries both ABRE and DRE/CRT elements (Yamaguchi-Shinozaki and Shinozaki 2005). ABA dependent and independent stress signaling might not occur as a parallel process, but can cooperate and converge in activating stress genes. The enhanced expression of *RD29A* could be because of the activation of either ABRE or DRE elements (Ishitani et al. 1997). Enhanced expression of *RAB18* has been observed in the salt tolerance observed in *LcSAINS1* expressing Arabidopsis and rice plants (Li et al. 2013). Reduced expression of *RAB18* and *RD29A* has been explained as the operation of ABA-independent pathway in *OSPP2C* expressing Arabidopsis transgenics (Singh et al. 2015). Recent studies suggest that there is a cross talk between ABA-dependent and ABA-independent pathways in abiotic stress and the global transcriptional network activated in response to osmotic stress is not specifically involved in any one of the two pathways, but is controlled cooperatively (Yoshida et al. 2014).

In conclusion, the constitutive expression of *AnnBj2* is found to be associated with the significantly enhanced expression of ABA-hydroxylase gene (*CYP707A2*) and significant downregulation of the *ABI4* when compared to the control and the strong salt tolerance phenotype exhibited by the transgenic plants can be correlated with the modulation of these two genes.

Expression analysis of *Brassica rapa* annexins

Yadav et al. (2015) annotated the different members of the annexin gene family of *B.rapa*. In this investigation, we have analyzed the expression of *B. rapa* annexins in response to various abiotic stress inducers and hormone treatments. Similar expression profiling of the annexins was reported earlier in Arabidopsis (Cantero et al. 2006), mustard (Jami et al. 2009), rice (Jami et al. 2012). tomato (Lu et al. 2012) and wheat (Xu et al. 2016). In our study, we found that the members of annexin family showed differential expression pattern to the same treatment, some are highly upregulated, while a few are downregulated. Such differential expression pattern supports the previous reports on functional non-redundancy of annexins in different signaling pathways (Cantero et al., 2006; Jami et al., 2012; Clark et al., 2012). ABA upregulated the expression of Bra034402, Bra017102, Bra009048 and Bra039578. This is consistent with the previous reports, which suggest the involvement of some members of annexins in ABA dependent stress responses (Jami et al 2009; Lu et al 2012). Promoter region of *AnnAt1*, *AnnBj1*, *Os08g32970*, and all annexins of tomato except *AnnSl9* carried the ABA responsive *cis*-acting binding element (ABRE), which further supports the regulation by ABA.

MeJa and SA play crucial role in plant defense response pathway. Both these hormones can induce ROS production in response to pathogen attack (Mur et al. 2006; Zhang and Xing 2008). In the present study, annexins Bra034402, Bra024346, Bra017102, Bra000090 and Bra008892were found to be upregulated by the MeJa and SA. Although transcriptional regulation of annexins was reported by these hormones, their role during plant defense response against pathogens is not explored well. In tobacco BY-2 cells, an annexin *AnnNt12* is induced by abiotic stress treatments as well as infection by the bacteria such as *Rhodococcus fascians* and *Pseudomonas syringae* (Vandeputte et al. 2007). In another study on the ectopic expression of *AnnBj1* in tobacco, Jami et al. (2008) reported an associated upregulation of disease resistance related genes in tobacco with resistance against a phytopathogen.

Oxidative stress induced by H_2O_2 significantly increased the transcript levels of Bra034402, Bra024346, Bra017102 and Bra000090. The mRNA levels of annexins from Arabidopsis and mustard were induced in response to treatment with H_2O_2. Previously, heterologous expression of *AnnAt1*in *Escherichia coli* ΔoxyR mutant

rescues it from peroxide mediated oxidative stress (Gidrol et al., 1996). Similarly, the mustard annexin, AnnBj1 also exhibited in vitro peroxidase activity. Structural analysis of AnnGh1 from cotton revealed that the presence of an unusual sulfur cluster that formed the molecular basis for oxidative stress response (Hu et al. 2008).

The differential expression pattern of *B.rapa* annexins can be linked to the presence of various cis acting elements in the promoter region of these genes. *In silico* analysis of the promoter region of these genes showed the presence of MYB binding site involved in drought inducibility, HSE (heat stress responsive element), LTRE (low temperature responsive element), TCA-element involved in Salicylic Acid responsiveness, TC-rich repeats involved in defense and stress responsiveness, TGACG and CGTCA-motif involved in MeJa-responsiveness, BOXW1 (Fungal elicitor responsive element), ERE (ethylene responsive element), ABRE involved in the ABA responsiveness.

AnnBj2 shared 98% similarity with its *B. rapa* homolog Bra024346 at amino acid level. We compared the expression pattern of *AnnBj2* to that of its homolog (Bra024346) in *B.rapa*. At tissue level both these genes have higher transcript levels in root compared to other tissues. Both *AnnBj2* and Bra024346 were upregulated by NaCl, MeJa, and SA whereas ABA and H_2O_2 stress showed difference in their expression pattern. ABA upregulates *AnnBj2* whereas it downregulates Bra024346. H_2O_2 upregulates Bra024346 and the transcripts were maintained upto 24 h of treatment but *AnnBj2* transcripts first decrease within 3 h and then rebounds at 6 h after treatment. The contrasting difference in the response of these two homologs to ABA may be due to difference in their *cis* acting elements.

These dates would provide fundamental knowledge and useful information for further functional characterization of *B. rapa* annexins.

Summary and Conclusion

Summary and conclusion

In the present work, we focused on the functional characterization of an annexin *AnnBj2* from *Brassica juncea* by constitutively expressing it in the native and heterologous systems. Tissue-specific expression showed that *AnnBj1* & *AnnBj2* were the most abundant annexins at the seedling stage. The expression of these two annexins differred at tissue level at seedling stage. While *AnnBj1* has similar expression levels in roots, stem, and cotyledonary petiole, *AnnBj2* has relatively higher expression in roots compared to other tissues. Further, the expression of *AnnBj2* is strongly induced by NaCl and ABA treatment. To study its role in abiotic stresses, we generated several overexpression lines of *AnnBj2* in tobacco and mustard through *Agrobacterium* mediated transformation. The transgenic lines were confirmed by genomic DNA PCR, semi quantitative RT PCR and Southern hybridization. Phenotypic confirmation of the transgenic nature of the plant progenies was performed by seed germination assay in the presence of selective agent, kanamycin (15 mg/ml). Null segregant (NS) were used as control to assess the abiotic stress tolerance of the transgenic lines.

Constitutive expression of *AnnBj2* in mustard and tobacco conferred salt tolerance to the transgenic plants. We have not observed tolerance in the transgenic plants for other abiotic and oxidative stresses. Salt tolerance of transgenic plants was associated with higher chlorophyll retention, proline accumulation and lower levels of lipid peroxidation compared to that of the NS line. Relative water content of the transgenic seedlings was found to be higher than the NS line. Ion estimations were done to check the effect of constitutive expression of *AnnBj2* on the accumulation of Na^+, K^+ and Ca^{2+} in the leaf tissues. We checked the response of the *AnnBj2* transgenic lines to exogenous application of ABA and sugar (glucose) at seed germination stage. *AnnBj2* transgenic seeds showed reduced sensitivity to ABA and glucose at seed germination. Further we studied the expression of important genes involved in ABA metabolism and signaling at seed germination stage and observed that *AnnBj2* overexpression modulated the expression *CYP707A2*, *ABI4* and *ABI5*, which have proven roles in salt tolerance and ABA insensitivity. Gene expression studies under salt stress revealed the modulation of the expression of different stress marker genes, which are shown to be operated in ABA dependent and ABA independent mechanisms.

The third objective of the present work describes the expression analysis of annexin homologs in *Brassica rapa*. Treatment of ABA, MeJa, SA, H_2O_2 and MV differentially regulated the expression of annexin family members of *B. rapa*. Among all annexins, transcript levels of Bra034402 showed the highest increase in response to all the treatments. Comparision of *AnnBj2* and its homolog Bra024346 in *B. rapa* revealed that although they share 98% similarity at amino acid level, they respond differently to ABA. This may be due to difference in their *cis*- acting elements in the promoters. Promoter sequence of Bra024346 lacks ABRE element.

In conclusion, we have studied the role of AnnBj2 in salt stress by overexpressing it in mustard, which is the first report of its functional characterization in the native system. Our results suggest the improved performance of *AnnBj2* transgenic mustard plants under salt stress by maintaining physiological indices and ion homeostasis needed for growth under salt stress. Gene expression studies suggest the possible function of this gene in regulating the ABA content of seeds by regulating the expression of genes involved in ABA metabolism conferring them ABA and glucose insensitivity. Gene expression of annexin homologs in *B. rapa* identified Bra034402 as the most highly induced annexin members in response to all the stress treatments used in our study. Further characterization of this gene is needed for its role in stress tolerance.

Bibliography

Abdeen A, Schnell J, Miki B (2010) Transcriptome analysis reveals absence of unintended effects in drought-tolerant transgenic plants overexpressing the transcription factor ABF3. BMC genomics 11

Abebe T, Guenzi AC, Martin B, Cushman JC (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. Plant Physiol 131: 1748-1755

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng JR, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science 311: 91-94

Achard P, Gong F, Cheminant S, Alioua M, Hedden P, Genschik P (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell 20: 2117-2129

Adem GD, Roy SJ, Zhou M, Bowman JP, Shabala S (2014) Evaluating contribution of ionic, osmotic and oxidative stress components towards salinity tolerance in barley. Bmc Plant Biol 14

Agarwal PK, Jha B (2010) Transcription factors in plants and ABA dependent and independent abiotic stress signalling. Biol Plantarum 54: 201-212

Agboma PC, PeltonenSainio P, Hinkkanen R, Pehu E (1997) Effect of foliar application of glycinebetaine on yield components of drought-stressed tobacco plants. Exp Agr 33: 345-352

Agrawal GK, Thelen JJ (2006) Large scale identification and quantitative profiling of phosphoproteins expressed during seed filling in oilseed rape. Molecular & Cellular Proteomics 5: 2044-2059

Ahuja I, de Vos RCH, Bones AM, Hall RD (2010) Plant molecular stress responses face climate change. Trends Plant Sci 15: 664-674

Albacete A, Ghanem ME, Martinez-Andujar C, Acosta M, Sanchez-Bravo J, Martinez V, Lutts S, Dodd IC, Perez-Alfocea F (2008) Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (*Solanum lycopersicum L.*) plants. J Exp Bot 59: 4119-4131

Allen GJ, Chu SP, Harrington CL, Schumacher K, Hoffman T, Tang YY, Grill E, Schroeder JI (2001) A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature 411: 1053-1057

Allen GJ, Chu SP, Schumacher K, Shimazaki CT, Vafeados D, Kemper A, Hawke SD, Tallman G, Tsien RY, Harper JF, Chory J, Schroeder JI (2000) Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in Arabidopsis *det3* mutant. Science 289: 2338-2342

Alvarez S, Marsh EL, Schroeder SG, Schachtman DP (2008) Metabolomic and proteomic changes in the xylem sap of maize under drought. Plant Cell Environ 31: 325-340

Andreasson E, Ellis B (2010) Convergence and specificity in the Arabidopsis MAPK nexus. Trends Plant Sci 15: 106-113

Anuradha S, Rao SSR (2003) Application of brassinosteroids to rice seeds (*Oryza sativa L.*) reduced the impact of salt stress on growth, prevented photosynthetic pigment loss and increased nitrate reductase activity. Plant Growth Regul 40: 29-32

Apel K, Hirt H (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373-399

Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. Science 285: 1256-1258

Arenas-Huertero F, Arroyo A, Zhou L, Sheen J, Leon P (2000) Analysis of Arabidopsis glucose insensitive mutants, *gin5* and *gin6*, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Gene Dev 14: 2085-2096

Arpat AB, Waugh M, Sullivan JP, Gonzales M, Frisch D, Main D, Wood T, Leslie A, Wing RA, Wilkins TA (2004) Functional genomics of cell elongation in developing cotton fibers. Plant molecular biology 54: 911-929

Arroyo A, Bossi F, Finkelstein RR, Leon P (2003) Three genes that affect sugar sensing (*Abscisic Acid Insensitive 4, Abscisic Acid Insensitive 5, and Constitutive Triple Response 1*) are differentially regulated by glucose in arabidopsis. Plant Physiol 133: 231-242

Asada K (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol 141: 391-396

Ashraf M (1994) Breeding for Salinity Tolerance in Plants. Crit Rev Plant Sci 13: 17-42

Ashraf M, Ali Q (2008) Relative membrane permeability and activities of some antioxidant enzymes as the key determinants of salt tolerance in canola (*Brassica napus L.*). Environ Exp Bot 63: 266-273

Ashraf M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59: 206-216

Baisakh N, RamanaRao MV, Rajasekaran K, Subudhi P, Janda J, Galbraith D, Vanier C, Pereira A (2012) Enhanced salt stress tolerance of rice plants expressing a vacuolar H plus -*ATPase subunit c1 (SaVHAc1)* gene from the halophyte grass *Spartina alterniflora* Loisel. Plant Biotechnol J 10: 453-464

Balasubramanian K, Bevers EM, Willems GM, Schroit AJ (2001) Binding of annexin V to membrane products of lipid peroxidation. Biochemistry-Us 40: 8672-8676

Bandorowicz-Pikula J, Kirilenko A, van Deursen R, Golczak M, Kuhnel M, Lancelin JM, Pikula S, Buchet R (2003) A putative consensus sequence for the nucleotide-binding site of annexin A6. Biochemistry-Us 42: 9137-9146

Bari R, Jones J (2009) Role of plant hormones in plant defence responses. Plant molecular biology 69: 473-488

Barkla BJ, Castellanos-Cervantes T, de Leon JLD, Matros A, Mock HP, Perez-Alfocea F, Salekdeh GH, Witzel K, Zorb C (2013) Elucidation of salt stress defense and tolerance mechanisms of crop plants using proteomics-Current achievements and perspectives. Proteomics 13: 1885-1900

Bartels D, Dinakar C (2013) Balancing salinity stress responses in halophytes and non-halophytes: a comparison between *Thellungiella* and *Arabidopsis thaliana*. Funct Plant Biol 40: 819-831

Bassani M, Neumann PM, Gepstein S (2004) Differential expression profiles of growth-related genes in the elongation zone of maize primary roots. Plant molecular biology 56: 367-380

Batistic O, Kudla J (2004) Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. Planta 219: 915-924

Battey NH, Blackbourn HD (1993) The Control of Exocytosis in Plant-Cells. New Phytol 125: 307-338

Baxter A, Mittler R, Suzuki N (2014) ROS as key players in plant stress signalling. J Exp Bot 65: 1229-1240

Benz J, Hofmann A (1997) Annexins: From structure to function. Biol Chem 378: 177-183

Berthomieu P, Conejero G, Nublat A, Brackenbury WJ, Lambert C, Savio C, Uozumi N, Oiki S, Yamada K, Cellier F, Gosti F, Simonneau T, Essah PA, Tester M, Very AA, Sentenac H, Casse F (2003) Functional analysis of AtHKT1 in Arabidopsis shows that Na^+ recirculation by the phloem is crucial for salt tolerance. Embo J 22: 2004-2014

Bianchi MW, Damerval C, Vartanian N (2002) Identification of proteins regulated by cross-talk between drought and hormone pathways in Arabidopsis wild-type and auxin-insensitive mutants, *axr1* and *axf2*. Funct Plant Biol 29

Blackbourn HD, Barker PJ, Huskisson NS, Battey NH (1992) Properties and Partial Protein-Sequence of Plant Annexins. Plant Physiol 99: 864-871

Blackbourn HD, Walker JH, Battey NH (1991) Calcium-Dependent Phospholipid-Binding Proteins in Plants - Their Characterization and Potential for Regulating Cell-Growth. Planta 184: 67-73

Blumwald E (2000) Sodium transport and salt tolerance in plants. Curr. Opin. Cell Biol. 12: 431-434

Blumwald E, Aharon GS, Apse MP (2000) Sodium transport in plant cells. Bba-Biomembranes 1465: 140-151

Bohnert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. Trends Biotechnol 14: 89-97

Bolouri-Moghaddam MR, Le Roy K, Xiang L, Rolland F, Van den Ende W (2010) Sugar signalling and antioxidant network connections in plant cells. Febs J 277: 2022-2037

Bose J, Rodrigo-Moreno A, Shabala S (2014) ROS homeostasis in halophytes in the context of salinity stress tolerance. J Exp Bot 65: 1241-1257

Boustead CM, Smallwood M, Small H, Bowles DJ, Walker JH (1989) Identification of Calcium-Dependent Phospholipid-Binding Proteins in Higher-Plant Cells. Febs Lett 244: 456-460

Breton G, Vazquez-Tello A, Danyluk J, Sarhan F (2000) Two novel intrinsic annexins accumulate in wheat membranes in response to low temperature. Plant Cell Physiol 41: 177-184

Buitink J, Leger JJ, Guisle I, Vu BL, Wuilleme S, Lamirault G, Le Bars A, Le Meur N, Becker A, Kuester H, Leprince O (2006) Transcriptome profiling uncovers metabolic and regulatory processes occurring during the transition from desiccation-sensitive to desiccation-tolerant stages in *Medicago truncatula* seeds. Plant J 47: 735-750

Byrt CS, Platten JD, Spielmeyer W, James RA, Lagudah ES, Dennis ES, Tester M, Munns R (2007) HKT1;5-like cation transporters linked to Na+ exclusion loci in wheat, *Nax2* and *Kna1*. Plant Physiol 143: 1918-1928

Cabello JV, Lodeyro AF, Zurbriggen MD (2014) Novel perspectives for the engineering of abiotic stress tolerance in plants. Curr Opin Biotech 26: 62-70

Calvert CM, Gant SJ, Bowles DJ (1996) Tomato annexins p34 and p35 bind to F-actin and display nucleotide phosphodiesterase activity inhibited by phospholipid binding. Plant Cell 8: 333-342

Cantero A, Barthakur S, Bushart T, Chou S, Morgan R, Fernandez M, Clark G, Roux S (2006) Expression profiling of the Arabidopsis annexin gene family during germination, de-etiolation and abiotic stress. Plant Physiology and Biochemistry 44: 13-24

Cao WH, Liu J, He XJ, Mu RL, Zhou HL, Chen SY, Zhang JS (2007) Modulation of ethylene responses affects plant salt-stress responses. Plant Physiol 143: 707-719

Carroll AD, Moyen C, Van Kesteren P, Tooke F, Battey NH, Brownlee C (1998) Ca^{2+}, annexins, and GTP modulate exocytosis from maize root cap protoplasts. Plant Cell 10: 1267-1276

Chang HS, Jones ML, Banowetz GM, Clark DG (2003) Overproduction of cytokinins in petunia flowers transformed with P(SAG12)-IPT delays corolla senescence and decreases sensitivity to ethylene. Plant Physiol 132: 2174-2183

Chang L, Ramireddy E, Schmulling T (2013) Lateral root formation and growth of Arabidopsis is redundantly regulated by cytokinin metabolism and signalling genes. J Exp Bot 64: 5021-5032

Chefdor F, Benedetti H, Depierreux C, Delmotte F, Morabito D, Carpin S (2006) Osmotic stress sensing in Populus: Components identification of a phosphorelay system. Febs Lett 580: 77-81

Chen JX, Mao LC, Mi HB, Lu WJ, Ying TJ, Luo ZS (2016) Involvement of three annexin genes in the ripening of strawberry fruit regulated by phytohormone and calcium signal transduction. Plant Cell Rep 35: 733-743

Chen THH, Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr Opin Plant Biol 5: 250-257

Chen THH, Murata N (2011) Glycinebetaine protects plants against abiotic stress: mechanisms and biotechnological applications. Plant Cell Environ 34: 1-20

Chen Y, Ji FF, Xie H, Liang JS, Zhang JH (2006) The regulator of G-protein signaling proteins involved in sugar and abscisic acid signaling in Arabidopsis seed germination. Plant Physiol 140: 302-310

Chen YS, Lo SF, Sun PK, Lu CA, Ho THD, Yu SM (2015) A late embryogenesis abundant protein HVA1 regulated by an inducible promoter enhances root growth and abiotic stress tolerance in rice without yield penalty. Plant Biotechnol J 13: 105-116

Cheng WH, Endo A, Zhou L, Penney J, Chen HC, Arroyo A, Leon P, Nambara E, Asami T, Seo M, Koshiba T, Sheen J (2002) A unique short-chain dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions. Plant Cell 14: 2723-2743

Chernyad'ev II (2009) The protective action of cytokinins on the photosynthetic machinery and productivity of plants under stress (review). Applied Biochemistry and Microbiology 45: 351-362

Chin K, Moeder W, Yoshioka K (2009) Biological roles of cyclic-nucleotide-gated ion channels in plants: What we know and don't know about this 20 member ion channel family. Botany-Botanique 87: 668-677

Choi HI, Hong JH, Ha JO, Kang JY, Kim SY (2000) ABFs, a family of ABA-responsive element binding factors. J Biol Chem 275: 1723-1730

Chu P, Chen H, Zhou Y, Li Y, Ding Y, Jiang L, Tsang EW, Wu K, Huang S (2012) Proteomic and functional analyses of *Nelumbo nucifera* annexins involved in seed thermotolerance and germination vigor. Planta 235: 1271-1288

Claeys H, De Bodt S, Inze D (2014) Gibberellins and DELLAs: central nodes in growth regulatory networks. Trends Plant Sci 19: 231-239

Clark G, Cantero-Garcia A, Butterfield T, Dauwalder M, Roux SJ (2005) Secretion as a key component of gravitropic growth: implications for annexin involvement in differential growth. Gravitational and space biology bulletin : publication of the American Society for Gravitational and Space Biology 18: 113-114

Clark GB, Dauwalder M, Roux SJ (1992) Purification and Immunolocalization of an Annexin-Like Protein in Pea-Seedlings. Planta 187: 1-9

Clark GB, Dauwalder M, Roux SJ (1994) Immunolocalization of an Annexin-Like Protein in Corn. Adv Space Res 14: 341-346

Clark GB, Morgan RO, Fernandez MP, Roux SJ (2012) Evolutionary adaptation of plant annexins has diversified their molecular structures, interactions and functional roles. New Phytol 196: 695-712

Clark GB, Sessions A, Eastburn DJ, Roux SJ (2001) Differential expression of members of the annexin multigene family in Arabidopsis. Plant Physiol 126: 1072-1084

Clouse SD, Sasse JM (1998) Brassinosteroids: Essential regulators of plant growth and development. Annu Rev Plant Phys 49: 427-451

Colebrook EH, Thomas SG, Phillips AL, Hedden P (2014) The role of gibberellin signalling in plant responses to abiotic stress. J Exp Biol 217: 67-75

Coolen S, Proietti S, Hickman R, Davila Olivas NH, Huang PP, Van Verk MC, Van Pelt JA, Wittenberg AHJ, De Vos M, Prins M, Van Loon JJA, Aarts MGM, Dicke M, Pieterse CMJ, Van Wees SCM (2016) Transcriptome dynamics of Arabidopsis during sequential biotic and abiotic stresses. Plant J 86: 249-267

Cortina C, Culianez-Macia FA (2005) Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. Plant Sci 169: 75-82

Cowan AK, Cairns ALP, Bartels-Rahm B (1999) Regulation of abscisic acid metabolism: towards a metabolic basis for abscisic acid-cytokinin antagonism. J Exp Bot 50: 595-603

Cramer GR, Läuchli A, Polito VS (1985) Displacement of Ca2+ by Na+ from the Plasmalemma of Root Cells A Primary Response to Salt Stress? Plant Physiol. 79: 207-211

Creutz CE, Pazoles CJ, Pollard HB. Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of isolated chromaffin granules. Journal of Biological Chemistry. 1978 Apr 25;253(8):2858-66

Creutz CE, Dowling LG, Sando JJ, Villar-Palasi C, Whipple JH, Zaks WJ. Characterization of the chromobindins. Soluble proteins that bind to the chromaffin granule membrane in the presence of Ca^{2+}. Journal of Biological Chemistry. 1983 Dec 10;258(23):14664-74

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic Acid: Emergence of a Core Signaling Network. Annual Review of Plant Biology, Vol 61 61: 651-679

Dabitz N, Hu NJ, Yusof AM, Tranter N, Winter A, Daley M, Zschornig O, Brisson A, Hofmann A (2005) Structural determinants for plant annexin-membrane interactions. Biochemistry-Us 44: 16292-16300

Dalal A, Kumar A, Yadav D, Gudla T, Viehhauser A, Dietz K-J, Kirti PB (2014a) Alleviation of methyl viologen-mediated oxidative stress by *Brassica juncea* annexin-3 in transgenic Arabidopsis. Plant Sci. 219: 9-18

Dalal A, Vishwakarma A, Singh NK, Gudla T, Bhattacharyya MK, Padmasree K, Viehhauser A, Dietz K-J, Kirti PB (2014b) Attenuation of hydrogen peroxide-mediated oxidative stress by *Brassica juncea* annexin-3 counteracts thiol-specific antioxidant *(TSA1)* deficiency in *Saccharomyces cerevisiae.* FEBS Lett. 588: 584-593

Danquah A, de Zelicourt A, Colcombet J, Hirt H (2014) The role of ABA and MAPK signaling pathways in plant abiotic stress responses. Biotechnol Adv 32: 40-52

Davenport R, James RA, Zakrisson-Plogander A, Tester M, Munns R (2005) Control of sodium transport in durum wheat. Plant Physiol 137: 807-818

Daviere JM, Achard P (2013) Gibberellin signaling in plants. Development 140: 1147-1151

Deinlein U, Stephan AB, Horie T, Luo W, Xu G, Schroeder JI (2014) Plant salt-tolerance mechanisms. Trends Plant Sci 19: 371-379

Delmer DP, Potikha TS (1997) Structures and functions of annexins in plants. Cell Mol Life Sci 53: 546-553

Demidchik V, Shabala SN, Davies JM (2007) Spatial variation in H_2O_2 response of *Arabidopsis thaliana* root epidermal Ca^{2+} flux and plasma membrane Ca^{2+} channels. Plant J 49: 377-386

Dhaubhadel S, Chaudhary S, Dobinson KF, Krishna P (1999) Treatment with 24-epibrassinolide, a brassinosteroid, increases the basic thermotolerance of *Brassica napus* and tomato seedlings. Plant molecular biology 40: 333-342

Diaz-Zorita M, Fernandez-Canigia MV, Grosso GA (2001) Applications of foliar fertilizers containing glycinebetaine improve wheat yields. J Agron Crop Sci 186: 209-215

Dietrich P, Anschutz U, Kugler A, Becker D (2010) Physiology and biophysics of plant ligand-gated ion channels. Plant Biology 12: 80-93

Dill A, Thomas SG, Hu JH, Steber CM, Sun TP (2004) The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. Plant Cell 16: 1392-1405

Ditengou FA, Tealea WD, Kochersperger P, Flittner KA, Kneuper I, van der Graaff E, Nziengui H, Pinosa F, Li XG, Nitschke R, Laux T, Palme K (2008) Mechanical induction of lateral root initiation in *Arabidopsis thaliana*. P Natl Acad Sci USA 105: 18818-18823

Divi UK, Krishna P (2009) Brassinosteroid: a biotechnological target for enhancing crop yield and stress tolerance. New Biotechnol 26: 131-136

Divi UK, Rahman T, Krishna P (2010) Brassinosteroid-mediated stress tolerance in Arabidopsis shows interactions with abscisic acid, ethylene and salicylic acid pathways. Bmc Plant Biol 10

Divya K, Jami S, Kirti P (2010) Constitutive expression of mustard annexin, AnnBj1 enhances abiotic stress tolerance and fiber quality in cotton under stress. Plant Mol. Biol. 73: 293-308

Dodd AN, Kudla J, Sanders D (2010) The Language of Calcium Signaling. Annual Review of Plant Biology, Vol 61 61: 593-620

Dong H, Zhen ZQ, Peng JY, Chang L, Gong QQ, Wang NN (2011) Loss of *ACS7* confers abiotic stress tolerance by modulating ABA sensitivity and accumulation in Arabidopsis. J Exp Bot 62: 4875-4887

Dubiella U, Seybold H, Durian G, Komander E, Lassig R, Witte CP, Schulze WX, Romeis T (2013) Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. P Natl Acad Sci USA 110: 8744-8749

El-Showk S, Ruonala R, Helariutta Y (2013) Crossing paths: cytokinin signalling and crosstalk. Development 140: 1373-1383

Essah PA, Davenport R, Tester M (2003) Sodium influx and accumulation in Arabidopsis. Plant Physiol 133: 307-318

Fahad S, Hussain S, Matloob A, Khan FA, Khaliq A, Saud S, Hassan S, Shan D, Khan F, Ullah N, Faiq M, Khan MR, Tareen AK, Khan A, Ullah A, Ullah N, Huang JL (2015) Phytohormones and plant responses to salinity stress: a review. Plant Growth Regul 75: 391-404

Feng YM, Wei XK, Liao WX, Huang LH, Zhang H, Liang SC, Peng H (2013) Molecular analysis of the annexin gene family in soybean. Biol Plantarum 57: 655-662

Finkelstein R, Lynch T, Reeves W, Petitfils M, Mostachetti M (2011) Accumulation of the transcription factor ABA-insensitive (ABI)4 is tightly regulated post-transcriptionally. J Exp Bot 62: 3971-3979

Finkelstein RR (1994) Mutations at 2 New Arabidopsis Aba Response Loci Are Similar to the Abi3 Mutations. Plant J. 5: 765-771

Finkelstein RR, Gampala SS, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell 14 Suppl: S15-45

Flowers TJ, Colmer TD (2008) Salinity tolerance in halophytes. New Phytol. 179: 945-963

Flowers TJ, Garcia A, Koyama M, Yeo AR (1997) Breeding for salt tolerance in crop plants - the role of molecular biology. Acta Physiol Plant 19: 427-433

Foster R, Izawa T, Chua NH (1994) Plant Bzip Proteins Gather at Acgt Elements. Faseb J 8: 192-200

Foyer CH, Noctor G (2003) Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plantarum 119: 355-364

Fujita Y, Fujita M, Satoh R, Maruyama K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki K (2005) AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. Plant Cell 17: 3470-3488

Gan SS, Amasino RM (1995) Inhibition of Leaf Senescence by Autoregulated Production of Cytokinin. Science 270: 1986-1988

Gao AG, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang JH, Rommens CMT (2000) Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat Biotechnol 18: 1307-1310

Gao SQ, Chen M, Xu ZS, Zhao CP, Li LC, Xu HJ, Tang YM, Zhao X, Ma YZ (2011) The soybean *GmbZIP1* transcription factor enhances multiple abiotic stress tolerances in transgenic plants. Plant molecular biology 75: 537-553

Garg AK, Kim JK, Owens TG, Ranwala AP, Do Choi Y, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. P Natl Acad Sci USA 99: 15898-15903

Gerke V, Creutz CE, Moss SE (2005) Annexins: Linking Ca*2+* signalling to membrane dynamics. Nat Rev Mol Cell Bio 6: 449-461

Gerke V, Moss SE (2002) Annexins: From structure to function. Physiol Rev 82: 331-371

Ghanem ME, Albacete A, Martinez-Andujar C, Acosta M, Romero-Aranda R, Dodd IC, Lutts S, Perez-Alfocea F (2008) Hormonal changes during salinity-induced leaf senescence in tomato (*Solanum lycopersicum L.*). J Exp Bot 59: 3039-3050

Gidrol X, Sabelli PA, Fern YS, Kush AK (1996) Annexin-like protein from Arabidopsis thaliana rescues *Delta oxyR* mutant of *Escherichia coli* from H_2O_2 stress. P Natl Acad Sci USA 93: 11268-11273

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry 48: 909-930

Gilroy S, Bialasek M, Suzuki N, Gorecka M, Devireddy AR, Karpinski S, Mittler R (2016) ROS, Calcium, and Electric Signals: Key Mediators of Rapid Systemic Signaling in Plants. Plant Physiol 171: 1606-1615

Gilroy S, Suzuki N, Miller G, Choi WG, Toyota M, Devireddy AR, Mittler R (2014) A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signaling. Trends Plant Sci 19: 623-630

Gomez-Porras JL, Riano-Pachon DM, Dreyer I, Mayer JE, Mueller-Roeber B (2007) Genome-wide analysis of ABA-responsive elements ABRE and CE3 reveals divergent patterns in Arabidopsis and rice. BMC genomics 8

Gonzalez-Guzman M, Apostolova N, Belles JM, Barrero JM, Piqueras P, Ponce MR, Micol JL, Serrano R, Rodriguez PL (2002) The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. Plant Cell 14: 1833-1846

Gorantla M, Babu PR, Lachagari VBR, Feltus FA, Paterson AH, Reddy AR (2005) Functional genomics of drought stress response in rice: Transcript mapping of annotated unigenes of an *indica* rice (*Oryza sativa L.* cv. Nagina 22). Curr Sci India 89: 496-514

Gorecka KM, Konopka-Postupolska D, Hennig J, Buchet R, Pikula S (2005) Peroxidase activity of annexin 1 from *Arabidopsis thaliana*. Biochem Bioph Res Co 336: 868-875

Gosti F, Beaudoin N, Serizet C, Webb AAR, Vartanian N, Giraudat J (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. Plant Cell 11: 1897-1909

Grieve CM, Shannon MC (1999) Ion accumulation and distribution in shoot components of salt-stressed eucalyptus clones. J Am Soc Hortic Sci 124: 559-563

Griffiths J, Murase K, Rieu I, Zentella R, Zhang ZL, Powers SJ, Gong F, Phillips AL, Hedden P, Sun TP, Thomas SG (2007) Genetic characterization and functional analysis of the GID1 gibberellin receptors in Arabidopsis (vol 18, pg 3399, 2006). Plant Cell 19: 726-726

Guan B, Hu YZ, Zeng YL, Wang Y, Zhang FC (2011) Molecular characterization and functional analysis of a vacuolar Na^+/H^+ antiporter gene (*HcNHX1*) from *Halostachys caspica*. Mol Biol Rep 38: 1889-1899

Halfter U, Ishitani M, Zhu JK (2000) The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. P Natl Acad Sci USA 97: 3735-3740

Hamada A, Shono M, Xia T, Ohta M, Hayashi Y, Tanaka A, Hayakawa T (2001) Isolation and characterization of a Na^+/H^+ antiporter gene from the halophyte *Atriplex gmelini*. Plant molecular biology 46: 35-42

Hamamoto S, Horie T, Hauser F, Deinlein U, Schroeder JI, Uozumi N (2015) HKT transporters mediate salt stress resistance in plants: from structure and function to the field. Curr Opin Biotech 32: 113-120

Hamayun M, Khan SA, Khan AL, Shin JH, Ahmad B, Shin DH, Lee IJ (2010) Exogenous Gibberellic Acid Reprograms Soybean to Higher Growth and Salt Stress Tolerance. J Agr Food Chem 58: 7226-7232

Harinasut P, Tsutsui K, Takabe T, Nomura M, Takabe T, Kishitani S (1996) Exogenous glycinebetaine accumulation and increased salt-tolerance in rice seedlings. Biosci Biotech Bioch 60: 366-368

Harrington GN, Bush DR (2003) The bifunctional role of hexokinase in metabolism and glucose signaling. Plant Cell 15: 2493-2496

Hashimoto M, Toorchi M, Matsushita K, Iwasaki Y, Komatsu S (2009) Proteome Analysis of Rice Root Plasma Membrane and Detection of Cold Stress Responsive Proteins. Protein Peptide Lett 16: 685-697

Hauser F, Horie T (2010) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K^+/Na^+ ratio in leaves during salinity stress. Plant Cell Environ 33: 552-565

Havlova M, Dobrev PI, Motyka V, Storchova H, Libus J, Dobra J, Malbeck J, Gaudinova A, Vankova R (2008) The role of cytokinins in responses to water deficit in tobacco plants over-expressing trans-zeatin *O-glucosyltransferase* gene under 35S or SAG12 promoters. Plant Cell Environ 31: 341-353

Hayashi H, Alia, Mustardy L, Deshnium P, Ida M, Murata N (1997) Transformation of Arabidopsis thaliana with the codA gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant J 12: 133-142

Hayat S, Hasan SA, Yusuf M, Hayat Q, Ahmad A (2010) Effect of 28-homobrassinolide on photosynthesis, fluorescence and antioxidant system in the presence or absence of salinity and temperature in Vigna radiata. Environ Exp Bot 69: 105-112

Hedrich R (2012) Ion Channels in Plants. Physiol Rev 92: 1777-1811

Himabindu Y, Chakradhar T, Reddy MC, Kanygin A, Redding KE, Chandrasekhar T (2016) Salt-tolerant genes from halophytes are potential key players of salt tolerance in glycophytes. Environ Exp Bot 124: 39-63

Hirayama T, Shinozaki K (2010) Research on plant abiotic stress responses in the post-genome era: past, present and future. Plant J 61: 1041-1052

Hochholdinger F (2016) Untapping root system architecture for crop improvement. J Exp Bot 67: 4431-4433

Hofmann A, Delmer DP, Wlodawer A (2003) The crystal structure of annexin Gh1 from *Gossypium hirsutum* reveals an unusual S-3 cluster - Implications for cellulose synthase complex formation and oxidative stress response. Eur J Biochem 270: 2557-2564

Hofmann A, Proust J, Dorowski A, Schantz R, Huber R (2000) Annexin 24 from Capsicum annuum - X-ray structure and biochemical characterization. J Biol Chem 275: 8072-8082

Holmstrom KO, Somersalo S, Mandal A, Palva TE, Welin B (2000) Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. J Exp Bot 51: 177-185

Horie T, Costa A, Kim TH, Han MJ, Horie R, Leung HY, Miyao A, Hirochika H, An G, Schroeder JI (2007) Rice *OsHKT2;1* transporter mediates large Na^+ influx component into K^+-starved roots for growth. Embo J 26: 3003-3014

Horie T, Hauser F, Schroeder JI (2009) HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. Trends Plant Sci 14: 660-668

Hossain MA, Lee Y, Cho JI, Ahn CH, Lee SK, Jeon JS, Kang H, Lee CH, An G, Park PB (2010) The bZIP transcription factor OsABF1 is an ABA responsive element binding factor that enhances abiotic stress signaling in rice. Plant molecular biology 72: 557-566

Hoth S, Morgante M, Sanchez JP, Hanafey MK, Tingey SV, Chua NH (2002) Genome-wide gene expression profiling in *Arabidopsis thaliana* reveals new targets of abscisic acid and largely impaired gene regulation in the *abi1-1* mutant. J Cell Sci 115: 4891-4900

Hu NJ, Yusof AM, Winter A, Osman A, Reeve AK, Hofmann A (2008) The crystal structure of calcium-bound annexin Gh1 from *Gossypium hirsutum* and its implications for membrane binding mechanisms of plant annexins. J Biol Chem 283: 18314-18322

Hu SQ, Brady SR, Kovar DR, Staiger CJ, Clark GB, Roux SJ, Muday GK (2000) Identification of plant actin-binding proteins by F-actin affinity chromatography. Plant J 24: 127-137

Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell 94: 261-271

Huang SB, Spielmeyer W, Lagudah ES, James RA, Platten JD, Dennis ES, Munns R (2006) A sodium transporter (*HKT7*) is a candidate for *Nax1*, a gene for salt tolerance in durum wheat. Plant Physiol 142: 1718-1727

Huang YQ, Wang J, Zhang LD, Zuo KJ (2013) A Cotton Annexin Protein AnxGb6 Regulates Fiber Elongation through Its Interaction with Actin 1. PloS one 8

Hubbard KE, Nishimura N, Hitomi K, Getzoff ED, Schroeder JI (2010) Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. Gene Dev 24: 1695-1708

Huber R, Schneider M, Mayr I, Romisch J, Paques EP (1990) The Calcium-Binding Sites in Human Annexin-V by Crystal-Structure Analysis at 2.0 a Resolution - Implications for Membrane-Binding and Calcium-Channel Activity. Febs Lett 275: 15-21

Huh SM, Noh EK, Kim HG, Jeon BW, Bae K, Hu HC, Kwak JM, Park OK (2010) Arabidopsis annexins AnnAt1 and AnnAt4 interact with each other and regulate drought and salt stress responses. Plant Cell Physiol 51: 1499-1514

Huijser C, Kortstee A, Pego J, Weisbeek P, Wisman E, Smeekens S (2000) The Arabidopsis SUCROSE UNCOUPLED-6 gene is identical to ABSCISIC ACID INSENSITIVE-4: involvement of abscisic acid in sugar responses. Plant J 23: 577-585

Iqbal M, Ashraf M (2007) Seed treatment with auxins modulates growth and ion partitioning in salt-stressed wheat plants. J Integr Plant Biol 49: 1003-1015

Ishitani M, Xiong L, Stevenson B, Zhu JK (1997) Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. Plant Cell 9: 1935-1949

Ismail A, Takeda S, Nick P (2014) Life and death under salt stress: same players, different timing? J Exp Bot 65: 2963-2979

Iwabuchi M, Itoh K, Shimamoto K (1991) Molecular and Cytological Characterization of Repetitive DNA-Sequences in *Brassica*. Theor Appl Genet 81: 349-355

Izawa T, Foster R, Chua NH (1993) Plant Bzip Protein-DNA Binding-Specificity. J Mol Biol 230: 1131-1144

James RA, Davenport RJ, Munns R (2006) Physiological characterization of two genes for Na+ exclusion in durum wheat, *Nax1* and *Nax2*. Plant Physiol 142: 1537-1547

Jami SK, Clark GB, Ayele BT, Roux SJ, Kirti PB (2012) Identification and characterization of annexin gene family in rice. Plant Cell Rep 31: 813-825

Jami SK, Clark GB, Turlapati SA, Handley C, Roux SJ, Kirti PB (2008) Ectopic expression of an annexin from *Brassica juncea* confers tolerance to abiotic and biotic stress treatments in transgenic tobacco. Plant Physiology and Biochemistry 46: 1019-1030

Jami SK, Dalal A, Divya K, Kird PB (2009) Molecular cloning and characterization of five annexin genes from Indian mustard (*Brassica juncea* L. Czern and Coss). Plant Physiology and Biochemistry 47: 977-990

Jami SK, Hill RD, Kirti PB (2010) Transcriptional regulation of annexins in Indian Mustard, *Brassica juncea* and detoxification of ROS in transgenic tobacco plants constitutively expressing *AnnBj1*. Plant signaling & behavior 5: 618-621

Jang IC, Oh SJ, Seo JS, Choi WB, Song SI, Kim CH, Kim YS, Seo HS, Do Choi Y, Nahm BH, Kim JK (2003) Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. Plant Physiol 131: 516-524

Jang JC, Leon P, Zhou L, Sheen J (1997) Hexokinase as a sugar sensor in higher plants. Plant Cell 9: 5-19

Ji YS, Guo HW (2013) From Endoplasmic Reticulum (ER) to Nucleus: EIN2 Bridges the Gap in Ethylene Signaling. Mol Plant 6: 11-14

Jia F, Wang C, Huang J, Yang G, Wu C, Zheng C (2015) SCF E3 ligase PP2-B11 plays a positive role in response to salt stress in Arabidopsis. J. Exp. Bot.: erv245

Johnson CH, Knight MR, Kondo T, Masson P, Sedbrook J, Haley A, Trewavas A (1995) Circadian Oscillations of Cytosolic and Chloroplastic Free Calcium in Plants. Science 269: 1863-1865

Ju CL, Van de Poel B, Cooper ED, Thierer JH, Gibbons TR, Delwiche CF, Chang CR (2015) Conservation of ethylene as a plant hormone over 450 million years of evolution. Nat Plants 1

Kagale S, Divi UK, Krochko JE, Keller WA, Krishna P (2007) Brassinosteroid confers tolerance in Arabidopsis thaliana and *Brassica napus* to a range of abiotic stresses. Planta 225: 353-364

Kang JY, Choi HI, Im MY, Kim SY (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell 14: 343-357

Karakas B, OziasAkins P, Stushnoff C, Suefferheld M, Rieger M (1997) Salinity and drought tolerance of mannitol-accumulating transgenic tobacco. Plant Cell Environ 20: 609-616

Kasuga M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2004) A combination of the Arabidopsis *DREB1A* gene and stress-inducible *rd29A* promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. Plant Cell Physiol 45: 346-350

Kaur S, Gupta AK, Kaur N (1998) Gibberellic acid and kinetin partially reverse the effect of water stress on germination and seedling growth in chickpea. Plant Growth Regul 25: 29-33

Kazan K (2015) Diverse roles of jasmonates and ethylene in abiotic stress tolerance. Trends Plant Sci 20: 219-229

Kazan K, Manners JM (2008) Jasmonate signaling: Toward an integrated view. Plant Physiol 146: 1459-1468

Khripach V, Zhabinskii V, De Groot A (2000) Twenty years of brassinosteroids: Steroidal plant hormones warrant better crops for the XXI century. Ann Bot-London 86: 441-447

Kilian J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, D'Angelo C, Bornberg-Bauer E, Kudla J, Harter K (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. Plant J 50: 347-363

Kim S, Kang JY, Cho DI, Park JH, Kim SY (2004) ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. Plant J 40: 75-87

Kishor PK, Hong Z, Miao G-H, Hu C-AA, Verma DPS (1995) Overexpression of [delta]-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol. 108: 1387-1394

Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, Doke N, Yoshioka H (2007) Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell 19: 1065-1080

Kobayashi S, Abe N, Yoshida KT, Liu SK, Takano T (2012) Molecular cloning and characterization of plasma membrane- and vacuolar-type Na^+/H^+ antiporters of an alkaline-salt-tolerant monocot, *Puccinellia tenuiflora*. J Plant Res 125: 587-594

Koh S, Lee SC, Kim MK, Koh JH, Lee S, An G, Choe S, Kim SR (2007) T-DNA tagged knockout mutation of rice *OsGSK1*, an orthologue of Arabidopsis *BIN2*, with enhanced tolerance to various abiotic stresses. Plant molecular biology 65: 453-466

Konopka-Postupolska D, Clark G, Goch G, Debski J, Floras K, Cantero A, Fijolek B, Roux S, Hennig J (2009) The role of annexin 1 in drought stress in Arabidopsis. Plant Physiol. 150: 1394-1410

Kourie JI, Wood HB (2000) Biophysical and molecular properties of annexin-formed channels. Prog Biophys Mol Bio 73: 91-134

Kronzucker HJ, Britto DT (2011) Sodium transport in plants: a critical review. New Phytol 189: 54-81

Kudla J, Batistic O, Hashimoto K (2010) Calcium Signals: The Lead Currency of Plant Information Processing. Plant Cell 22: 541-563

Kudoyarova GR, Vysotskaya LB, Cherkozyanova A, Dodd IC (2007) Effect of partial rootzone drying on the concentration of zeatin-type cytokinins in tomato (S*olanum lycopersicum* L.) xylem sap and leaves. J Exp Bot 58: 161-168

Kush A, Sabapathy K (2001) Oxy5, a novel protein from Arabidopsis thaliana, protects mammalian cells from oxidative stress. Int J Biochem Cell B 33: 591-602

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E (2004) The Arabidopsis cytochrome P450 CYP707A encodes ABA 8′-hydroxylases: key enzymes in ABA catabolism. The EMBO journal 23: 1647-1656

Laby RJ, Kincaid MS, Kim DG, Gibson SI (2000) The Arabidopsis sugar-insensitive mutants *sis4* and *sis5* are defective in abscisic acid synthesis and response. Plant J 23: 587-596

Lagercrantz U, Lydiate DJ (1996) Comparative genome mapping in *Brassica*. Genetics 144: 1903-1910

Laohavisit A, Brown AT, Cicuta P, Davies JM (2010) Annexins: Components of the Calcium and Reactive Oxygen Signaling Network. Plant Physiol 152: 1824-1829

Laohavisit A, Davies JM (2009) Multifunctional annexins. Plant Sci 177: 532-539

Laohavisit A, Davies JM (2011) Annexins. New Phytol 189: 40-53

Laohavisit A, Shang ZL, Rubio L, Cuin TA, Very AA, Wang AH, Mortimer JC, Macpherson N, Coxon KM, Battey NH, Brownlee C, Park OK, Sentenac H, Shabala S, Webb AAR, Davies JM (2012) Arabidopsis Annexin1 Mediates the Radical-Activated Plasma Membrane Ca^{2+}- and K^{+}-Permeable Conductance in Root Cells. Plant Cell 24: 1522-1533

Lee JT, Prasad V, Yang PT, Wu JF, Ho THD, Charng YY, Chan MT (2003) Expression of Arabidopsis *CBF1* regulated by an ABA/stress inducible promoter in transgenic tomato confers stress tolerance without affecting yield. Plant Cell Environ 26: 1181-1190

Lee S, Lee EJ, Yang EJ, Lee JE, Park AR, Song WH, Park OK (2004) Proteomic identification of annexins, calcium-dependent membrane binding proteins that mediate osmotic stress and abscisic acid signal transduction in Arabidopsis. Plant Cell 16: 1378-1391

Lee SH, Ahsan N, Lee KW, Kim DH, Lee DG, Kwak SS, Kwon SY, Kim TH, Lee BH (2007) Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate

peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. J Plant Physiol 164: 1626-1638

Lee SJ, Park JH, Lee MH, Yu JH, Kim SY (2010) Isolation and functional characterization of CE1 binding proteins. Bmc Plant Biol 10

Li X, Hou S, Gao Q, Zhao P, Chen S, Qi D, Lee BH, Cheng L, Liu G (2013) *LcSAIN1*, a novel salt-induced gene from sheepgrass, confers salt stress tolerance in transgenic Arabidopsis and rice. Plant Cell Physiol 54: 1172-1185

Li B, Li DD, Zhang J, Xia H, Wang XL, Li Y, Li XB (2013) Cotton *AnnGh3* Encoding an Annexin Protein is Preferentially Expressed in Fibers and Promotes Initiation and Elongation of Leaf Trichomes in Transgenic Arabidopsis. J Integr Plant Biol 55: 902-916

Li CH, Wang G, Zhao JL, Zhang LQ, Ai LF, Han YF, Sun DY, Zhang SW, Sun Y (2014) The Receptor-Like Kinase SIT1 Mediates Salt Sensitivity by Activating MAPK3/6 and Regulating Ethylene Homeostasis in Rice. Plant Cell 26: 2538-2553

Liao Y, Zou HF, Wei W, Hao YJ, Tian AG, Huang J, Liu YF, Zhang JS, Chen SY (2008) Soybean GmbZIP44, GmbZIP62 and GmbZIP78 genes function as negative regulator of ABA signaling and confer salt and freezing tolerance in transgenic Arabidopsis. Planta 228: 225-240

Lim EK, Roberts MR, Bowles DJ (1998) Biochemical characterization of tomato annexin p35 - Independence of calcium binding and phosphatase activities. J Biol Chem 273: 34920-34925

Lindermayr C, Saalbach G, Durner J (2005) Proteomic identification of S-nitrosylated proteins in Arabidopsis. Plant Physiol 137: 921-930

Liu JP, Ishitani M, Halfter U, Kim CS, Zhu JK (2000) The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. P Natl Acad Sci USA 97: 3730-3734

Liu JP, Zhu JK (1998) A calcium sensor homolog required for plant salt tolerance. Science 280: 1943-1945

Liu AL, Yu Y, Duan XB, Sun XL, Duanmu HZ, Zhu YM (2015) GsSKP21, a Glycine soja S-phase kinase-associated protein, mediates the regulation of plant alkaline tolerance and ABA sensitivity. Plant molecular biology 87: 111-124

Lu L, Chen Y, Lu Y, Li L (2015) Transcriptome analysis reveals dynamic changes in the gene expression of tobacco seedlings under low potassium stress. Journal of genetics 94: 397-406

Lu Y, Ouyang B, Zhang J, Wang T, Lu C, Han Q, Zhao S, Ye Z, Li H (2012) Genomic organization, phylogenetic comparison and expression profiles of annexin gene family in tomato (*Solanum lycopersicum*). Gene 499: 14-24

Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S, Gruissem W (2002) Calmodulins and calcineurin B-like proteins: Calcium sensors for specific signal response coupling in plants. Plant Cell 14: S389-S400

Luo X, Bai X, Sun XL, Zhu D, Liu BH, Ji W, Cai H, Cao L, Wu J, Hu MR, Liu X, Tang LL, Zhu YM (2013) Expression of wild soybean WRKY20 in Arabidopsis enhances drought tolerance and regulates ABA signalling. J Exp Bot 64: 2155-2169

Lynch J, LÄUCHLI A (1985) Salt stress disturbs the calcium nutrition of barley (Hordeum vulgare L.). New Phytol. 99: 345-354

Ma QQ, Wang W, Li YH, Li DQ, Zou Q (2006) Alleviation of photoinhibition in drought-stressed wheat (*Triticum aestivum*) by foliar-applied glycinebetaine. J Plant Physiol 163: 165-175

Ma SS, Bohnert HJ (2007) Integration of Arabidopsis thaliana stress-related transcript profiles, promoter structures, and cell-specific expression. Genome Biol 8

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C Phosphatase Activity Function as Abscisic Acid Sensors. Science 324: 1064-1068

Maathuis FJM (2014) Sodium in plants: perception, signalling, and regulation of sodium fluxes. J Exp Bot 65: 849-858

Maggio A, Barbieri G, Raimondi G, De Pascale S (2010) Contrasting Effects of GA(3) Treatments on Tomato Plants Exposed to Increasing Salinity. J Plant Growth Regul 29: 63-72

Magome H, Yamaguchi S, Hanada A, Kamiya Y, Oda K (2004) *dwarf and delayed-flowering 1*, a novel Arabidopsis mutant deficient in gibberellin biosynthesis because of overexpression of a putative AP2 transcription factor. Plant J 37: 720-729

Makela P, Munns R, Colmer TD, Condon AG, Peltonen-Sainio P (1998) Effect of foliar applications of glycinebetaine on stomatal conductance, abscisic acid and solute concentrations in leaves of salt- or drought-stressed tomato. Aust J Plant Physiol 25: 655-663

Malamy JE (2005) Intrinsic and environmental response pathways that regulate root system architecture. Plant Cell Environ 28: 67-77

Malamy JE, Ryan KS (2001) Environmental regulation of lateral root initiation in Arabidopsis. Plant Physiol 127: 899-909

Mao XG, Zhang HY, Qian XY, Li A, Zhao GY, Jing RL (2012) TaNAC2, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in Arabidopsis. J Exp Bot 63: 2933-2946

Marin E, Nussaume L, Quesada A, Gonneau M, Sotta B, Hugueney P, Frey A, MarionPoll A (1996) Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of Arabidopsis thaliana. Embo J 15: 2331-2342

Marino D, Dunand C, Puppo A, Pauly N (2012) A burst of plant NADPH oxidases. Trends Plant Sci 17: 9-15

Maser P, Eckelman B, Vaidyanathan R, Horie T, Fairbairn DJ, Kubo M, Yamagami M, Yamaguchi K, Nishimura M, Uozumi N, Robertson W, Sussman MR, Schroeder JI (2002) Altered shoot/root Na^+ distribution and bifurcating salt sensitivity in Arabidopsis by genetic disruption of the Na^+ transporter AtHKTI1. Febs Lett 531: 157-161

Mason MG, Jha D, Salt DE, Tester M, Hill K, Kieber JJ, Schaller GE (2010) Type-B response regulators ARR1 and ARR12 regulate expression of *AtHKT1;1* and accumulation of sodium in Arabidopsis shoots. Plant J 64: 753-763

Mazars C, Bourque S, Mithofer A, Pugin A, Ranjeva R (2009) Calcium homeostasis in plant cell nuclei. New Phytol 181: 261-274

Mazars C, Thuleau P, Lamotte O, Bourque S (2010) Cross-Talk between ROS and Calcium in Regulation of Nuclear Activities. Mol Plant 3: 706-718

McAinsh MR, Pittman JK (2009) Shaping the calcium signature. New Phytol 181: 275-294

Mcclung AD, Carroll AD, Battey NH (1994) Identification and Characterization of Atpase Activity Associated with Maize (*Zea-Mays*) Annexins. Biochem J 303: 709-712

McCormack E, Tsai YC, Braam J (2005) Handling calcium signaling: Arabidopsis CaMs and CMLs. Trends Plant Sci 10: 383-389

McKersie BD, Bowley SR, Harjanto E, Leprince O (1996) Water-deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. Plant Physiol 111: 1177-1181

Melcher K, Xu Y, Ng LM, Zhou XE, Soon FF, Chinnusamy V, Suino-Powell KM, Kovach A, Tham FS, Cutler SR, Li J, Yong EL, Zhu JK, Xu HE (2010) Identification and mechanism of ABA receptor antagonism. Nat Struct Mol Biol 17: 1102-U1110

Merchante C, Alonso JM, Stepanova AN (2013) Ethylene signaling: simple ligand, complex regulation. Curr Opin Plant Biol 16: 554-560

Merlot S, Gosti F, Guerrier D, Vavasseur A, Giraudat J (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. Plant J 25: 295-303

Miller G, Schlauch K, Tam R, Cortes D, Torres MA, Shulaev V, Dangl JL, Mittler R (2009) The Plant NADPH Oxidase RBOHD Mediates Rapid Systemic Signaling in Response to Diverse Stimuli. Sci Signal 2

Miller G, Shulaev V, Mittler R (2008) Reactive oxygen signaling and abiotic stress. Physiol Plantarum 133: 481-489

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ 33: 453-467

Mishra S, Shukla A, Upadhyay S, Sanchita, Sharma P, Singh S, Phukan UJ, Meena A, Khan F, Tripathi V, Shukla RK, Shrama A (2014) Identification, occurrence, and validation of DRE and ABRE Cis- regulatory motifs in the promoter regions of genes of *Arabidopsis thaliana*. J Integr Plant Biol 56: 388-399

Mithofer A, Reichelt M, Nakamura Y (2014) Wound and insect-induced jasmonate accumulation in carnivorous Drosera capensis: two sides of the same coin. Plant Biology 16: 982-987

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9: 490-498

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F (2011) ROS signaling: the new wave? Trends Plant Sci 16: 300-309

Miyazono K, Miyakawa T, Sawano Y, Kubota K, Kang HJ, Asano A, Miyauchi Y, Takahashi M, Zhi YH, Fujita Y, Yoshida T, Kodaira KS, Yamaguchi-Shinozaki K, Tanokura M (2009) Structural basis of abscisic acid signalling. Nature 462: 609-U679

Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2012) AP2/ERF family transcription factors in plant abiotic stress responses. Bba-Gene Regul Mech 1819: 86-96

Moller IS, Gilliham M, Jha D, Mayo GM, Roy SJ, Coates JC, Haseloff J, Tester M (2009) Shoot Na^+ Exclusion and Increased Salinity Tolerance Engineered by Cell Type-Specific Alteration of Na^+ Transport in Arabidopsis. Plant Cell 21: 2163-2178

Moore PB, Dedman JR. Calmodulin, a calmodulin acceptor protein, and calcimedins: unique antibody localizations in hamster sperm. Journal of cellular biochemistry. 1984 Jan 1;25(2):99-107.

Mori IC, Schroeder JI (2004) Reactive oxygen species activation of plant Ca^{2+} channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. Plant Physiol 135: 702-708

Mortimer JC, Laohavisit A, Macpherson N, Webb A, Brownlee C, Battey NH, Davies JM (2008) Annexins: multifunctional components of growth and adaptation. J Exp Bot 59: 533-544

Moss SE, Morgan RO (2004) The annexins. Genome Biol 5

Mostek A, Borner A, Badowiec A, Weidner S (2015) Alterations in root proteome of salt-sensitive and tolerant barley lines under salt stress conditions. J Plant Physiol 174: 166-176

Moller IM, Jensen PE, Hansson A (2007) Oxidative modifications to cellular components in plants. Annu Rev Plant Biol 58: 459-481

Muller R, Morant M, Jarmer H, Nilsson L, Nielsen TH (2007) Genome-wide analysis of the Arabidopsis leaf transcriptome reveals interaction of phosphate and sugar metabolism. Plant Physiol 143: 156-171

Munns R (2005) Genes and salt tolerance: bringing them together. The New phytologist 167: 645-663

Munns R, James RA (2003) Screening methods for salinity tolerance: a case study with tetraploid wheat. Plant Soil 253: 201-218

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59: 651-681

Murata Y, Pei ZM, Mori IC, Schroeder J (2001) Abscisic acid activation of plasma membrane Ca2+ channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in *abi1-1* and *abi2-1* protein phosphatase 2C mutants. Plant Cell 13: 2513-2523

Murgia I, Tarantino D, Vannini C, Bracale M, Carravieri S, Soave C (2004) *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death. Plant J 38: 940-953

Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell 14: 3089-3099

Nah G, Pagliarulo CL, Mohr PG, Luo MZ, Sisneros N, Yu Y, Collura K, Currie J, Goicoechea JL, Wing RA, Schumaker KS (2009) Comparative sequence analysis of the SALT OVERLY SENSITIVE1 orthologous region in *Thellungiella halophila* and *Arabidopsis thaliana*. Genomics 94: 196-203

Nakabayashi R, Saito K (2015) Integrated metabolomics for abiotic stress responses in plants. Curr Opin Plant Biol 24: 10-16

Nakajima M, Shimada A, Takashi Y, Kim YC, Park SH, Ueguchi-Tanaka M, Suzuki H, Katoh E, Iuchi S, Kobayashi M, Maeda T, Matsuoka M, Yamaguchi I (2006) Identification and characterization of Arabidopsis gibberellin receptors. Plant J 46: 880-889

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional Regulatory Networks in Response to Abiotic Stresses in Arabidopsis and Grasses. Plant Physiol 149: 88-95

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu. Rev. Plant Biol. 56: 165-185

Nemhauser JL, Hong FX, Chory J (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell 126: 467-475

Nishimura N, Hitomi K, Arvai AS, Rambo RP, Hitomi C, Cutler SR, Schroeder JI, Getzoff ED (2009) Structural Mechanism of Abscisic Acid Binding and Signaling by Dimeric PYR1. Science 326: 1373-1379

Nishiyama R, Le DT, Watanabe Y, Matsui A, Tanaka M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K, Tran LSP (2012) Transcriptome Analyses of a Salt-Tolerant Cytokinin-Deficient Mutant Reveal Differential Regulation of Salt Stress Response by Cytokinin Deficiency. PloS one 7

Nishiyama R, Watanabe Y, Fujita Y, Le DT, Kojima M, Werner T, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Kakimoto T, Sakakibara H, Schmulling T, Tran LSP (2011) Analysis of Cytokinin Mutants and Regulation of Cytokinin Metabolic Genes Reveals Important Regulatory Roles of Cytokinins in Drought, Salt and Abscisic Acid Responses, and Abscisic Acid Biosynthesis. Plant Cell 23: 2169-2183

Nonogaki H (2015) Seed dormancy and germination—emerging mechanisms and new hypotheses. Advances in Seed Biology: 225

Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S (2003) Gibberellin biosynthesis and response during Arabidopsis seed germination. Plant Cell 15: 1591-1604

Oh DH, Leidi E, Zhang Q, Hwang SM, Li YZ, Quintero FJ, Jiang XY, D'Urzo MP, Lee SY, Zhao YX, Bahk JD, Bressan RA, Yun DJ, Pardo JM, Bohnert HJ (2009) Loss of Halophytism by Interference with *SOS1* Expression. Plant Physiol 151: 210-222

Ohta M, Hayashi Y, Nakashima A, Hamada A, Tanaka A, Nakamura T, Hayakawa T (2002) Introduction of a Na^+/H^+ antiporter gene from *Atriplex gmelini* confers salt tolerance to rice. Febs Lett 532: 279-282

Oliveira TM, da Silva FR, Bonatto D, Neves DM, Morillon R, Maserti BE, Coelho MA, Costa MGC, Pirovani CP, Gesteira AS (2015) Comparative study of the protein profiles of Sunki mandarin and Rangpur lime plants in response to water deficit. Bmc Plant Biol 15

Orellana S, Yanez M, Espinoza A, Verdugo I, Gonzalez E, Ruiz-Lara S, Casaretto JA (2010) The transcription factor *SlAREB1* confers drought, salt stress tolerance and regulates biotic and abiotic stress-related genes in tomato. Plant Cell Environ 33: 2191-2208

Osakabe Y, Yamaguchi-Shinozaki K, Shinozaki K, Tran LSP (2013) Sensing the environment: key roles of membrane-localized kinases in plant perception and response to abiotic stress. J Exp Bot 64: 445-458

Ozdemir F, Bor M, Demiral T, Turkan I (2004) Effects of 24-epibrassinolide on seed germination, seedling growth, lipid peroxidation, proline content and antioxidative system of rice (*Oryza sativa* L.) under salinity stress. Plant Growth Regul 42: 203-211

Ozgur R, Uzilday B, Sekmen AH, Turkan I (2013) Reactive oxygen species regulation and antioxidant defence in halophytes. Funct Plant Biol 40: 832-847

Parent B, Hachez C, Redondo E, Simonneau T, Chaumont F, Tardieu F (2009) Drought and Abscisic Acid Effects on Aquaporin Content Translate into Changes in Hydraulic Conductivity and Leaf Growth Rate: A Trans-Scale Approach. Plant Physiol 149: 2000-2012

Park J, Kim YS, Kim SG, Jung JH, Woo JC, Park CM (2011) Integration of Auxin and Salt Signals by the NAC Transcription Factor NTM2 during Seed Germination in Arabidopsis. Plant Physiol 156: 537-549

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TFF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu JK, Schroeder JI, Volkman BF, Cutler SR (2009) Abscisic Acid Inhibits Type 2C Protein Phosphatases via the PYR/PYL Family of START Proteins. Science 324: 1068-1071

Pedranzani H, Racagni G, Alemano S, Miersch O, Ramirez I, Pena-Cortes H, Taleisnik E, Machado-Domenech E, Abdala G (2003) Salt tolerant tomato plants show increased levels of jasmonic acid. Plant Growth Regul 41: 149-158

Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature 406: 731-734

Peleg Z, Blumwald E (2011) Hormone balance and abiotic stress tolerance in crop plants. Curr Opin Plant Biol 14: 290-295

Petersson SV, Johansson AI, Kowalczyk M, Makoveychuk A, Wang JY, Moritz T, Grebe M, Benfey PN, Sandberg G, Ljung K (2009) An Auxin Gradient and Maximum in the Arabidopsis Root Apex Shown by High-Resolution Cell-Specific Analysis of IAA Distribution and Synthesis. Plant Cell 21: 1659-1668

Pilonsmits EAH, Ebskamp MJM, Paul MJ, Jeuken MJW, Weisbeek PJ, Smeekens SCM (1995) Improved Performance of Transgenic Fructan-Accumulating Tobacco under Drought Stress. Plant Physiol 107: 125-130

Pino MT, Skinner JS, Park EJ, Jeknic Z, Hayes PM, Thornashow MF, Chen THH (2007) Use of a stress inducible promoter to drive ectopic *AtCBF* expression improves potato freezing tolerance while minimizing negative effects on tuber yield. Plant Biotechnol J 5: 591-604

Popescu SC, Popescu GV, Bachan S, Zhang ZM, Gerstein M, Snyder M, Dinesh-Kumar SP (2009) MAPK target networks in Arabidopsis thaliana revealed using functional protein microarrays. Gene Dev 23: 80-92

Pospisilova J, Vagner M, Malbeck J, Travniakova A, Batkova P (2005) Interactions between abscisic acid and cytokinins during water stress and subsequent rehydration. Biol Plantarum 49: 533-540

Potocky M, Pejchar P, Gutkowska M, Jimenez-Quesada MJ, Potocka A, Alche JD, Kost B, Zarsky V (2012) NADPH oxidase activity in pollen tubes is affected by calcium ions, signaling phospholipids and Rac/Rop GTPases. J Plant Physiol 169: 1654-1663

Prasad KVSK, Sharmila P, Kumar PA, Saradhi PP (2000) Transformation of Brassica juncea (L.) Czern with bacterial codA gene enhances its tolerance to salt stress. Mol Breeding 6: 489-499

Qadir M, Tubeileh A, Akhtar J, Larbi A, Minhas PS, Khan MA (2008) Productivity enhancement of salt-affected environments through crop diversification. Land Degradation & Development 19: 429-453

Qiao B, Zhang Q, Liu D, Wang H, Yin J, Wang R, He M, Cui M, Shang Z, Wang D (2015) A calcium-binding protein, rice annexin OsANN1, enhances heat stress tolerance by modulating the production of H_2O_2. J. Exp. Bot. 66: 5853-5866

Qin F, Shinozaki K, Yamaguchi-Shinozaki K (2011a) Achievements and Challenges in Understanding Plant Abiotic Stress Responses and Tolerance. Plant Cell Physiol 52: 1569-1582

Qin H, Gu Q, Zhang JL, Sun L, Kuppu S, Zhang YZ, Burow M, Payton P, Blumwald E, Zhang H (2011b) Regulated Expression of an *Isopentenyltransferase Gene (IPT)* in Peanut Significantly Improves Drought Tolerance and Increases Yield Under Field Conditions. Plant Cell Physiol 52: 1904-1914

Qiu QS, Barkla BJ, Vera-Estrella R, Zhu JK, Schumaker KS (2003) Na^+/H^+ exchange activity in the plasma membrane of Arabidopsis. Plant Physiol 132: 1041-1052

Qiu WM, Liu MY, Qiao GR, Jiang J, Xie LH, Zhuo RY (2012) An Isopentyl Transferase Gene Driven by the Stress-Inducible *rd29A* Promoter Improves Salinity Stress Tolerance in Transgenic Tobacco. Plant molecular biology reporter 30: 519-528

Qiu ZB, Guo JL, Zhu AJ, Zhang L, Zhang MM (2014) Exogenous jasmonic acid can enhance tolerance of wheat seedlings to salt stress. Ecotox Environ Safe 104: 202-208

Quesada V, Ponce MR, Micol JL (2000) Genetic analysis of salt-tolerant mutants in Arabidopsis thaliana. Genetics 154: 421-436

Quintero FJ, Ohta M, Shi HZ, Zhu JK, Pardo JM (2002) Reconstitution in yeast of the Arabidopsis SOS signaling pathway for Na^+ homeostasis. P Natl Acad Sci USA 99: 9061-9066

Raghavendra AS, Gonugunta VK, Christmann A, Grill E (2010) ABA perception and signalling. Trends Plant Sci 15: 395-401

Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2006) A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. P Natl Acad Sci USA 103: 11081-11085

Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37: 1141-1146

Repetto O, Bestel-Corre G, Dumas-Gaudot E, Berta G, Gianinazzi-Pearson V, Gianinazzi S (2003) Targeted proteomics to identify cadmium-induced protein modifications in Glomus mosseae-inoculated pea roots. New Phytol 157: 555-567

Richards DE, King KE, Ait-ali T, Harberd NP (2001) How gibberellin regulates plant growth and development: A molecular genetic analysis of gibberellin signaling. Annu Rev Plant Phys 52: 67-88

Richards SL, Laohavisit A, Mortimer JC, Shabala L, Swarbreck SM, Shabala S, Davies JM (2014) Annexin 1 regulates the H2O2-induced calcium signature in *Arabidopsis thaliana* roots. Plant J 77: 136-145

Rivero RM, Gimeno J, Van Deynze A, Walia H, Blumwald E (2010) Enhanced Cytokinin Synthesis in Tobacco Plants Expressing *P-SARK::IPT* Prevents the Degradation of Photosynthetic Protein Complexes During Drought. Plant Cell Physiol 51: 1929-1941

Rodriguez-Serrano M, Romero-Puertas MC, Pazmino DM, Testillano PS, Risueno MC, del Rio LA, Sandalio LM (2009) Cellular Response of Pea Plants to Cadmium Toxicity: Cross Talk between Reactive Oxygen Species, Nitric Oxide, and Calcium. Plant Physiol 150: 229-243

Rogers ED, Benfey PN (2015) Regulation of plant root system architecture: implications for crop advancement. Curr Opin Biotech 32: 93-98

Rohila JS, Chen M, Chen S, Chen J, Cerny R, Dardick C, Canlas P, Xu X, Gribskov M, Kanrar S, Zhu JK, Ronald P, Fromm ME (2006) Protein-protein interactions of tandem affinity purification-tagged protein kinases in rice. Plant J 46: 1-13

Rojas E, Arispe N, Haigler HT, Burns AL, Pollard HB (1992) Identification of Annexins as Calcium Channels in Biological-Membranes. Bone Miner 17: 214-218

Rajjou L, Duval M, Gallardo K, Catusse J, Bally J, Job C, Job D (2012) Seed germination and vigor. Annu. Rev. Plant Biol. 63: 507-533

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: Conserved and novel mechanisms. Annu Rev Plant Biol 57: 675-709

Rook F, Hadingham SA, Li Y, Bevan MW (2006) Sugar and ABA response pathways and the control of gene expression. Plant Cell Environ 29: 426-434

Rus A, Lee BH, Munoz-Mayor A, Sharkhuu A, Miura K, Zhu JK, Bressan RA, Hasegawa PM (2004) AtHKT1 facilitates Na^+ homeostasis and K^+ nutrition in planta. Plant Physiol 136: 2500-2511

Ryu H, Hwang I (2013) Brassinosteroids in plant developmental signaling networks. J Plant Biol 56: 267-273

Sagi M, Fluhr R (2006) Production of reactive oxygen species by plant NADPH oxidases. Plant Physiol 141: 336-340

Sakamoto A, Murata A, Murata N (1998) Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. Plant molecular biology 38: 1011-1019

Sakamoto A, Murata N (2000) Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance. J Exp Bot 51: 81-88

Salehin M, Estelle M (2015) Ethylene Prunes Translation. Cell 163: 543-544

Santiago J, Dupeux F, Round A, Antoni R, Park SY, Jamin M, Cutler SR, Rodriguez PL, Marquez JA (2009) The abscisic acid receptor PYR1 in complex with abscisic acid. Nature 462: 665-U143

Sarowar S, Kim EN, Kim YJ, Ok SH, Kim KD, Hwang BK, Shin JS (2005) Overexpression of a pepper *ascorbate peroxidase-like 1* gene in tobacco plants enhances tolerance to oxidative stress and pathogens. Plant Sci 169: 55-63

Schmidt R, Mieulet D, Hubberten HM, Obata T, Hoefgen R, Fernie AR, Fisahn J, Segundo BS, Guiderdoni E, Schippers JHM, Mueller-Roeber B (2013) *SALT-RESPONSIVE ERF1* Regulates Reactive Oxygen Species-Dependent Signaling during the Initial Response to Salt Stress in Rice. Plant Cell 25: 2115-2131

Schwartz SH, Qin XQ, Zeevaart JAD (2003) Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. Plant Physiol 131: 1591-1601

Schweighofer A, Hirt H, Meskiene L (2004) Plant PP2C phosphatases: emerging functions in stress signaling. Trends Plant Sci 9: 236-243

Sengupta A, Webb RP, Holaday AS, Allen RD (1993) Overexpression of Superoxide-Dismutase Protects Plants from Oxidative Stress - Induction of Ascorbate Peroxidase in Superoxide Dismutase-Overexpressing Plants. Plant Physiol 103: 1067-1073

Seo M, Koshiba T (2002) Complex regulation of ABA biosynthesis in plants. Trends Plant Sci 7: 41-48

Seo M, Peeters AJM, Koiwai H, Oritani T, Marion-Poll A, Zeevaart JAD, Koornneef M, Kamiya Y, Koshiba T (2000) The Arabidopsis *aldehyde oxidase 3 (AA03)* gene product catalyzes the final step in abscisic acid biosynthesis in leaves. P Natl Acad Sci USA 97: 12908-12913

Seo YJ, Park JB, Cho YJ, Jung C, Seo HS, Park SK, Nahm BH, Song JT (2010) Overexpression of the Ethylene-Responsive Factor Gene *BrERF4* from *Brassica rapa* Increases Tolerance to Salt and Drought in Arabidopsis Plants. Mol Cells 30: 271-277

Shabala S (2013) Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. Ann Bot-London 112: 1209-1221

Shabala S, Bose J, Hedrich R (2014) Salt bladders: do they matter? Trends Plant Sci 19: 687-691

Shabala S, Pottosin I (2014) Regulation of potassium transport in plants under hostile conditions: implications for abiotic and biotic stress tolerance. Physiol Plantarum 151: 257-279

Shahid MA, Pervez MA, Balal RM, Mattson NS, Rashid A, Ahmad R, Ayyub CM, Abbas T (2011) Brassinosteroid (24-epibrassinolide) enhances growth and alleviates the deleterious effects induced by salt stress in pea (*Pisum sativum* L.). Aust J Crop Sci 5: 500-510

Shen B, Jensen RG, Bohnert HJ (1997) Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. Plant Physiol 113: 1177-1183

Shi H, Ishitani M, Kim C, Zhu J-K (2000) The Arabidopsis thaliana salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter. Proceedings of the national academy of sciences 97: 6896-6901

Shi HZ, Lee BH, Wu SJ, Zhu JK (2003) Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in Arabidopsis thaliana. Nat Biotechnol 21: 81-85

Shin HS, Brown RM (1999) GTPase activity and biochemical characterization of a recombinant cotton fiber annexin. Plant Physiol 119: 925-934

Shkolnik-Inbar D, Adler G, Bar-Zvi D (2013) ABI4 downregulates expression of the sodium transporter HKT1;1 in Arabidopsis roots and affects salt tolerance. The Plant journal : for cell and molecular biology 73: 993-1005

Shulaev V, Cortes D, Miller G, Mittler R (2008) Metabolomics for plant stress response. Physiol Plantarum 132: 199-208

Singh A, Kanwar P, Yadav AK, Mishra M, Jha SK, Baranwal V, Pandey A, Kapoor S, Tyagi AK, Pandey GK (2014) Genome-wide expressional and functional analysis of calcium transport elements during abiotic stress and development in rice. Febs J 281: 894-915

Smeekens S, Ma JK, Hanson J, Rolland F (2010) Sugar signals and molecular networks controlling plant growth. Curr Opin Plant Biol 13: 274-279

Snowdon RJ, Kohler W, Friedt W, Kohler A (1997) Genomic in situ hybridization in Brassica amphidiploids and interspecific hybrids. Theor Appl Genet 95: 1320-1324

Sobhanian H, Razavizadeh R, Nanjo Y, Ehsanpour AA, Jazii FR, Motamed N, Komatsu S (2010) Proteome analysis of soybean leaves, hypocotyls and roots under salt stress. Proteome Sci 8

Song K, Osborn TC (1992) Polyphyletic Origins of Brassica-Napus - New Evidence Based on Organelle and Nuclear Rflp Analyses. Genome 35: 992-1001

Steber CM, McCourt P (2001) A role for brassinosteroids in germination in Arabidopsis. Plant Physiol 125: 763-769

Steffens B (2014) The role of ethylene and ROS in salinity, heavy metal, and flooding responses in rice. Front Plant Sci 5

Steinhorst L, Kudla J (2013) Calcium and Reactive Oxygen Species Rule the Waves of Signaling. Plant Physiol 163: 471-485

Stewart CN, Halfhill MD, Warwick SI (2004) Transgene introgression from genetically modified crops to their wild relatives (vol 4, pg 806, 2003). Nat Rev Genet 5: 310-310

Sun TP, Gubler F (2004) Molecular mechanism of gibberellin signaling in plants. Annu Rev Plant Biol 55: 197-223

Sunarpi, Horie T, Motoda J, Kubo M, Yang H, Yoda K, Horie R, Chan WY, Leung HY, Hattori K, Konomi M, Osumi M, Yamagami M, Schroeder JI, Uozumi N (2005) Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na^+ unloading from xylem vessels to xylem parenchyma cells. Plant J 44: 928-938

Suzuki N, Koussevitzky S, Mittler R, Miller G (2012) ROS and redox signalling in the response of plants to abiotic stress. Plant Cell Environ 35: 259-270

Suzuki N, Miller G, Morales J, Shulaev V, Torres MA, Mittler R (2011) Respiratory burst oxidases: the engines of ROS signaling. Curr Opin Plant Biol 14: 691-699

Swarbreck SM, Colaco R, Davies JM (2013) Plant Calcium-Permeable Channels. Plant Physiol 163: 514-522

Szalonek M, Sierpien B, Rymaszewski W, Gieczewska K, Garstka M, Lichocka M, Sass L, Paul K, Vass I, Vankova R (2015) Potato Annexin *STANN1* Promotes Drought Tolerance and Mitigates Light Stress in Transgenic Solanum tuberosum L. Plants. PloS one 10: e0132683

Takeda S, Gapper C, Kaya H, Bell E, Kuchitsu K, Dolan L (2008) Local positive feedback regulation determines cell shape in root hair cells. Science 319: 1241-1244

Tan BC, Schwartz SH, Zeevaart JAD, McCarty DR (1997) Genetic control of abscisic acid biosynthesis in maize. P Natl Acad Sci USA 94: 12235-12240

Tang J, Wang F, Hou X-L, Wang Z, Huang Z-N (2014) Genome-wide fractionation and identification of WRKY transcription factors in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) reveals collinearity and their expression patterns under abiotic and biotic stresses. Plant molecular biology reporter 32: 781-795

Tani T, Sobajima H, Okada K, Chujo T, Arimura SI, Tsutsumi N, Nishimura M, Seto H, Nojiri H, Yamane H (2008) Identification of the *OsOPR7* gene encoding 12-oxophytodienoate reductase involved in the biosynthesis of jasmonic acid in rice. Planta 227: 517-526

Taylor IB, Burbidge A, Thompson AJ (2000) Control of abscisic acid synthesis. J Exp Bot 51: 1563-1574

Tester M, Davenport R (2003) Na+ tolerance and Na^+ transport in higher plants. Ann Bot-London 91: 503-527

Tester M, Langridge P (2010) Breeding technologies to increase crop production in a changing world. Science 327: 818-822

Thompson AJ, Jackson AC, Symonds RC, Mulholland BJ, Dadswell AR, Blake PS, Burbidge A, Taylor IB (2000) Ectopic expression of a tomato *9-cis-epoxycarotenoid dioxygenase* gene causes over-production of abscisic acid. Plant J 23: 363-374

Tracy FE, Gilliham M, Dodd AN, Webb AAR, Tester M (2008) NaCl-induced changes in cytosolic free $Ca^{(2+)}$ in *Arabidopsis thaliana* are heterogeneous and modified by external ionic composition. Plant Cell Environ 31: 1063-1073

Tsonev TD, Lazova GN, Stoinova ZG, Popova LP (1998) A possible role for jasmonic acid in adaptation of barley seedlings to salinity stress. J Plant Growth Regul 17: 153-159

Tuna AL, Kaya C, Dikilitas M, Higgs D (2008) The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. Environ Exp Bot 62: 1-9

Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, Yamaguchi-Shinozaki K (2010) Molecular Basis of the Core Regulatory Network in ABA Responses: Sensing, Signaling and Transport. Plant Cell Physiol 51: 1821-1839

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. P Natl Acad Sci USA 106: 17588-17593

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. P Natl Acad Sci USA 97: 11632-11637

Urao T, Yakubov B, Satoh R, Yamaguchi-Shinozaki K, Seki M, Hirayama T, Shinozaki K (1999) A transmembrane hybrid-type histidine kinase in arabidopsis functions as an osmosensor. Plant Cell 11: 1743-1754

Van Breusegem F, Bailey-Serres J, Mittler R (2008) Unraveling the tapestry of networks involving Reactive Oxygen Species in plants. Plant Physiol 147: 978-984

Vaseva I, Todorova D, Malbeck J, Travnickova A, Machackova I (2008) Response of cytokinin pool and cytokinin oxidase/dehydrogenase activity to abscisic acid exhibits organ specificity in peas. Acta Physiol Plant 30: 151-155

Vincill ED, Bieck AM, Spalding EP (2012) Ca2+ Conduction by an Amino Acid-Gated Ion Channel Related to Glutamate Receptors. Plant Physiol 159: 40-46

Vriet C, Russinova E, Reuzeau C (2012) Boosting Crop Yields with Plant Steroids. Plant Cell 24: 842-857

Wang J, Zhang H, Allen RD (1999) Overexpression of an Arabidopsis peroxisomal ascorbate peroxidase gene in tobacco increases protection against oxidative stress. Plant Cell Physiol 40: 725-732

Wang KLC, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. Plant Cell 14: S131-S151

Wang LK, Niu XW, Lv YH, Zhang TZ, Guo WZ (2010a) Molecular cloning and localization of a novel cotton annexin gene expressed preferentially during fiber development. Mol Biol Rep 37: 3327-3334

Wang PC, Xue L, Batelli G, Lee S, Hou YJ, Van Oosten MJ, Zhang HM, Tao WA, Zhu JK (2013) Quantitative phosphoproteomics identifies SnRK2 protein kinase substrates and reveals the effectors of abscisic acid action. P Natl Acad Sci USA 110: 11205-11210

Wang X, Ma X, Wang H, Li B, Clark G, Guo Y, Roux S, Sun D, Tang W (2015) Proteomic study of microsomal proteins reveals a key role for Arabidopsis annexin 1 in mediating heat stress-induced increase in intracellular calcium levels. Molecular & Cellular Proteomics 14: 686-694

Wang YN, Li KX, Li X (2009) Auxin redistribution modulates plastic development of root system architecture under salt stress in Arabidopsis thaliana. J Plant Physiol 166: 1637-1645

Wang YY, Mopper S, Hasenstein KH (2001) Effects of salinity on endogenous ABA, IAA, JA, and SA in Iris hexagona. J Chem Ecol 27: 327-342

Wang ZF, Wang ZH, Shi L, Wang LJ, Xu FS (2010b) Proteomic alterations of Brassica napus root in response to boron deficiency. Plant Mol. Biol. 74: 265-278

Wasternack C, Hause B (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann Bot-London 111: 1021-1058

Weber M, Trampczynska A, Clemens S (2006) Comparative transcriptome analysis of toxic metal responses in Arabidopsis thaliana and the Cd^{2+}-hypertolerant facultative metallophyte *Arabidopsis halleri*. Plant Cell Environ 29: 950-963

Weston DJ, Gunter LE, Rogers A, Wullschleger SD (2008) Connecting genes, coexpression modules, and molecular signatures to environmental stress phenotypes in plants. Bmc Syst Biol 2

Wilkinson JQ, Lanahan MB, Conner TW, Klee HJ (1995) Identification of Messenger-Rnas with Enhanced Expression in Ripening Strawberry Fruit Using Polymerase Chain-Reaction Differential Display. Plant molecular biology 27: 1097-1108

Wilkinson S, Kudoyarova GR, Veselov DS, Arkhipova TN, Davies WJ (2012) Plant hormone interactions: innovative targets for crop breeding and management. J Exp Bot 63: 3499-3509

Wind JJ, Peviani A, Snel B, Hanson J, Smeekens SC (2013) ABI4: versatile activator and repressor. Trends Plant Sci 18: 125-132

Xia XJ, Zhou YH, Shi K, Zhou J, Foyer CH, Yu JQ (2015) Interplay between reactive oxygen species and hormones in the control of plant development and stress tolerance. J Exp Bot 66: 2839-2856

Xiao WY, Sheen J, Jang JC (2000) The role of hexokinase in plant sugar signal transduction and growth and development. Plant molecular biology 44: 451-461

Xie YJ, Xu S, Han B, Wu MZ, Yuan XX, Han Y, Gu QA, Xu DK, Yang Q, Shen WB (2011) Evidence of Arabidopsis salt acclimation induced by up-regulation of HY1 and the regulatory role of RbohD-derived reactive oxygen species synthesis. Plant J 66: 280-292

Xiong LM, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. Plant Cell 14: S165-S183

Xiong LM, Zhu JK (2003) Regulation of abscisic acid biosynthesis. Plant Physiol 133: 29-36

Xu L, Tang Y, Gao S, Su S, Hong L, Wang W, Fang Z, Li X, Ma J, Quan W, Sun H, Wang Y, Liao X, Gao J, Zhang F, Li L, Zhao C (2016) Comprehensive analyses of the annexin gene family in wheat. BMC Genomics 17: 415

Yadav D, Ahmed I, Kirti P (2015) Genome-wide identification and expression profiling of annexins in Brassica rapa and their phylogenetic sequence comparison with *B. juncea* and *A. thaliana* annexins. Plant Gene 4: 109-124

Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci 10: 88-94

Yang O, Popova OV, Suthoff U, Luking I, Dietz KJ, Golldack D (2009a) The Arabidopsis basic leucine zipper transcription factor *AtbZIP24* regulates complex transcriptional networks involved in abiotic stress resistance. Gene 436: 45-55

Yang Q, Chen ZZ, Zhou XF, Yin HB, Li X, Xin XF, Hong XH, Zhu JK, Gong ZZ (2009b) Overexpression of *SOS (Salt Overly Sensitive)* Genes Increases Salt Tolerance in Transgenic Arabidopsis. Mol Plant 2: 22-31

Yang RL, Jarvis DE, Chen H, Beilstein MA, Grimwood J, Jenkins J, Shu SQ, Prochnik S, Xin MM, Ma C, Schmutz J, Wing RA, Mitchell-Olds T, Schumaker KS, Wang XF (2013) The reference genome of the halophytic plant *Eutrema salsugineum*. Front Plant Sci 4

Yang XH, Liang Z, Wen XG, Lu CM (2008) Genetic engineering of the biosynthesis of glycinebetaine leads to increased tolerance of photosynthesis to salt stress in transgenic tobacco plants. Plant molecular biology 66: 73-86

Yoshida T, Fujita Y, Sayama H, Kidokoro S, Maruyama K, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2010) AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. Plant J 61: 672-685

Yoshida T, Mogami J, Yamaguchi-Shinozaki K (2014) ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. Curr Opin Plant Biol 21: 133-139

Yoshida T, Nishimura N, Kitahata N, Kuromori T, Ito T, Asami T, Shinozaki K, Hirayama T (2006) ABA-Hypersensitive germination3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among Arabidopsis protein phosphatase 2Cs. Plant Physiol 140: 115-126

Yoshimura K, Miyao K, Gaber A, Takeda T, Kanaboshi H, Miyasaka H, Shigeoka S (2004) Enhancement of stress tolerance in transgenic tobacco plants overexpressing *Chlamydomonas* glutathione peroxidase in chloroplasts or cytosol. Plant J 37: 21-33

Yu S, Cao L, Zhou CM, Zhang TQ, Lian H, Sun Y, Wu JQ, Huang JR, Wang GD, Wang JW (2013) Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. Elife 2

Yuan K, Wysocka-Diller J (2006) Phytohormone signalling pathways interact with sugars during seed germination and seedling development. J Exp Bot 57: 3359-3367

Zdunek E, Lips SH (2001) Transport and accumulation rates of abscisic acid and aldehyde oxidase activity in *Pisum sativum L.* in response to suboptimal growth conditions. J Exp Bot 52: 1269-1276

Zhang F, Li S, Yang S, Wang L, Guo W (2015) Overexpression of a cotton annexin gene, *GhAnn1*, enhances drought and salt stress tolerance in transgenic cotton. Plant Mol. Biol. 87: 47-67

Zhang GH, Su Q, An LJ, Wu S (2008) Characterization and expression of a vacuolar Na^+/H^+ antiporter gene from the monocot halophyte *Aeluropus littoralis*. Plant Physiology and Biochemistry 46: 117-126

Zhang M, Smith JAC, Harberd NP, Jiang CF (2016) The regulatory roles of ethylene and reactive oxygen species (ROS) in plant salt stress responses. Plant molecular biology 91: 651-659

Zhang SS, Cai ZY, Wang XL (2009) The primary signaling outputs of brassinosteroids are regulated by abscisic acid signaling. P Natl Acad Sci USA 106: 4543-4548

Zhang WX, Ruan JH, Ho TD, You Y, Yu TT, Quatrano RS (2005) Cis-regulatory element based targeted gene finding: genome-wide identification of abscisic acid- and abiotic stress-responsive genes in *Arabidopsis thaliana*. Bioinformatics 21: 3074-3081

Zhang ZJ, Huang RF (2010) Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor *TERF2/LeERF2* is modulated by ethylene biosynthesis. Plant molecular biology 73: 241-249

Zhang ZJ, Wang J, Zhang RX, Huang RF (2012) The ethylene response factor AtERF98 enhances tolerance to salt through the transcriptional activation of ascorbic acid synthesis in Arabidopsis. Plant J 71: 273-287

Zhao FY, Zhang H (2006) Salt and paraquat stress tolerance results from co-expression of the *Suaeda salsa* glutathione S-transferase and catalase in transgenic rice. Plant Cell Tiss Org 86: 349-358

Zhao PM, Wang LL, Han LB, Wang J, Yao Y, Wang HY, Du XM, Luo YM, Xia GX (2010) Proteomic Identification of Differentially Expressed Proteins in the Ligon lintless Mutant of Upland Cotton (*Gossypium hirsutum* L.). J Proteome Res 9: 1076-1087

Zhao Q, Guo HW (2011) Paradigms and Paradox in the Ethylene Signaling Pathway and Interaction Network. Mol Plant 4: 626-634

Zhao Y, Dong W, Zhang NB, Ai XH, Wang MC, Huang ZG, Xiao LT, Xia GM (2014) A Wheat *Allene Oxide Cyclase* Gene Enhances Salinity Tolerance via Jasmonate Signaling. Plant Physiol 164: 1068-1076

Zhao YK, Wang T, Zhang WS, Li X (2011) SOS3 mediates lateral root development under low salt stress through regulation of auxin redistribution and maxima in Arabidopsis. New Phytol 189: 1122-1134

Zhou M-L, Yang X-B, Zhang Q, Zhou M, Zhao E-Z, Tang Y-X, Zhu X-M, Shao J-R, Wu Y-M (2013) Induction of annexin by heavy metals and jasmonic acid in Zea mays. Functional & integrative genomics 13: 241-251

Zhu GH, Ye NH, Zhang JH (2009) Glucose-Induced Delay of Seed Germination in Rice is Mediated by the Suppression of ABA Catabolism Rather Than an Enhancement of ABA Biosynthesis. Plant Cell Physiol 50: 644-651

Zhu J, Wu X, Yuan S, Qian D, Nan Q, An L, Xiang Y (2014a) Annexin5 plays a vital role in Arabidopsis pollen development via Ca^{2+}-dependent membrane trafficking. PloS one 9: e102407

Zhu J, Yuan S, Wei G, Qian D, Wu X, Jia H, Gui M, Liu W, An L, Xiang Y (2014b) Annexin5 is essential for pollen development in Arabidopsis. Molecular plant 7: 751-754

Zhu SY, Yu XC, Wang XJ, Zhao R, Li Y, Fan RC, Shang Y, Du SY, Wang XF, Wu FQ, Xu YH, Zhang XY, Zhang DP (2007) Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in Arabidopsis. Plant Cell 19: 3019-3036

Zwack PJ, Rashotte AM (2015) Interactions between cytokinin signalling and abiotic stress responses. J Exp Bot 66: 4863-4871