Functional characterization of rice annexin *OsAnn5* for abiotic stress tolerance through heterologous expression

Thesis submitted to the University of Hyderabad for the award of the degree of Doctor of Philosophy

By

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"DECLARATION"

I, **Boyidi Prasanna** hereby declare that this thesis entitled "**Functional characterization of rice annexin *OsAnn5* for abiotic stress tolerance through heterologous expression**" 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.

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This is to certify that the thesis entitled **"Functional characterization of rice annexin *OsAnn5* for abiotic stress tolerance through heterologous expression"** Submitted by **Boyidi Prasanna** bearing registration number **12LPPH16** in partial fulfilment of the requirements for award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by her under my supervision and guidance.

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Parts of this thesis have been

1. Published in the following publications:
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Further, the student has passed the following courses towards fulfillment of course work requirement for Ph.D/ was exempted from doing coursework (recommended by Doctoral Committee) on the basis of the following courses passed during her M.Phil program and the M.Phil degree was awarded.

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5	PL806	Seminar	1	Pass

Prof. P. B. Kirti
Supervisor **Head of the department** **Dean**

Dedicated to my beloved

Guru and Parents

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Abbreviations

µg	Microgram
µl	Microliter
µM	Micro Molar
ABA	Abscisic acid
ATP	Adenosine Triphosphate
BAP	Benzyl Amino Purine
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
Bp	base pairs
BSA	Bovine Serum Albumin
CAMV	Cauliflower Mosaic Virus
cDNA	complementary DNA
CIAP	Calf Intestinal Alkaline Phosphatase
Cm	Centimeter
CTAB	Cetyl Trimethyl Ammonium Bromide
D	Day
DEPC	Diethyl pyrocarbonate
DNA	Deoxy ribonucleic acid
EDTA	Ethylene nucleotide triphosphate
G	Grams
H	Hours
IPTG	Isopropyl-D-Thio galactoside
Kb	Kilo bases
KDa	Kilo Dalton
LB	Luria Bertani
M	Molar
MES	2-(N-Morpholino)-EthaneSulfonic acid
min	Minutes
Ml	Milliliter
MS	Murashige and Skoog
Ng	nano gram
O.D	Optical Density
ORF	Open Reading Frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	revolution per minute
SDS	Sodium Dodecyl Sulphate
TE	Tris EDTA
U	Units
WT	Wild type
DDW	Double Distilled Water
V	Volts

Contents

CHAPTER 1

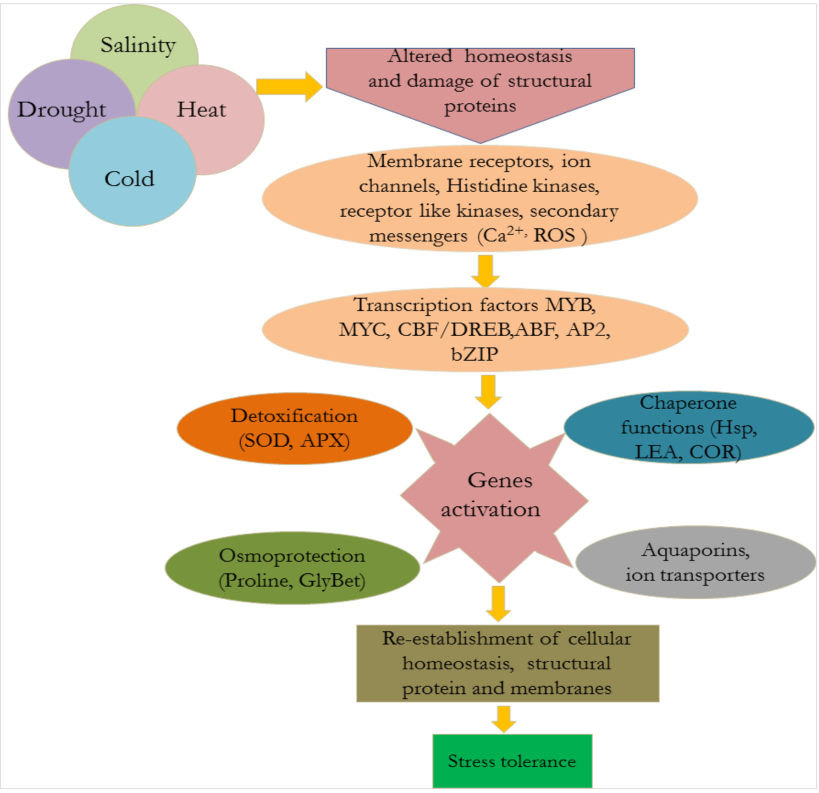
Introduction and Review of Literature

1.1 Stress introduction

Homeostasis is the ability of the organism or a cell to maintain the steady-state levels of the internal biochemical environment when dealing with the external changes. These changes can be achieved by controlled adjustment of the extrinsic and intrinsic mechanisms (Modell et al., 2019). Any alteration in the homeostasis would lead to adverse on the plants called stress. Susceptible plants are those which avoid the stress by completing their lifecycle fast, whereas tolerant plants are those, which are able to tolerate the stress over the time through acclimatization, adaptation and survival processes. Stress can be biotic (due to biological agents) or abiotic (non-biological agents).

Abiotic stress include drought, elevated salinity, cold and heat that drastically decrease food crop yields around the world. Many molecular techniques were used to comprehend the processes by which plants perceive environmental signals and convey them to cellular machinery in order to activate adaptive reactions (Mahajan & Tuteja 2005). During this adaptation/tolerance processes, plants developed different layers of complex mechanisms for stress signal perception, signal transduction, activation of stress-responsive genes or proteins, which can be permanent. Primarily, the extracellular stimulus will be perceived by the cell with help of receptors like ion channels, and receptor-like kinase (RLK), membrane receptors or histidine kinase (HK). These receptors activate a complex intracellular signaling pathways. The stress perception leads to the origin of secondary signaling molecules includes Ca^{+2}, Inositol Phosphates (InsP), Reactive Oxygen Species (ROS) and ABA (Mahajan & Tuteja 2005). The stress signals induce the expression of various stress responsive genes with the help of secondary messengers that transduce the signal into the nucleus. The stress responsive gene products can start the next round of signaling process through generation of signaling molecules like ABA, ethylene and salicylic acid which ultimately leads to plant adaptation or stress tolerance either directly or indirectly (Mahajan et al., 2005). Comprehensively, the stress response is a coordinated action and cross-talk of many genes (Fig.1.1). Small molecules like ABA plays a crucial role in the stress response process (Chinnusamy et al., 2004). This knowledge on mechanism of signal transduction in plants under stress is important for the development of transgenic strategies and stress tolerance crops. Techniques like AFLP, microarray, next-generation sequencing are prominent in identification of differentially expressed genes under stress condition/adaptation.

These genes may be the transcription factors (MYB, MYC, NAC, WRKY, Dof, STAT, and AP2), signaling proteins or stress sensor proteins. Cell perceives the stress through the membrane receptors. Then signal is relayed by the secondary messengers of the cell. Secondary molecules also amplify the signal instead of acting as relaying molecules and leads to the activation of the stress-induced genes. Three such major secondary messengers in the cells are Cyclic nucleotides, Inositol triphosphate (IP_3), Diacylglycerol (DAG) and Ca^{+2} ions (Mahajan et al., 2005).



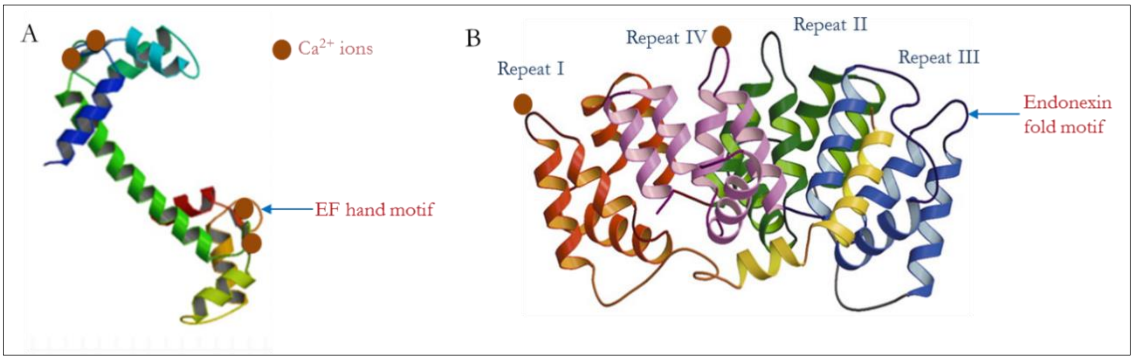
Wang et al., 2003

Fig.1.1 General mechanism of abiotic stress signaling.

Calcium signaling in stress response

Ca^{+2} ions are the most widely used and important secondary messengers in the cell. Many physiological processes like cell growth, cell division, secretion, exocytosis, endocytosis, fertilization, and stress responses are affected by Ca^{+2} ion concentration in a plant cell. Generally, extracellular Ca^{+2} levels are 20,000-100,000 folds higher than the intracellular Ca^{+2} levels (Clapham, 1995). This suggests that a small rise in the intracellular Ca^{+2} levels would still create drastic changes in the cell. Any disturbance in outside of the cell leads to changes in intracellular Ca^{+2} levels. Ca^{+2} amplifies or processes the signal through binding to other proteins, which are called as Ca^{+2} binding proteins. Literature suggests the presence of ~200 different calcium targets in the cells, which in combination with other calcium sensors and signatures generate innumerable signaling pathways (Reddy & Reddy, 2004).

Annexins are one of such important Ca^{+2} targets of the cell. They are different from other Ca^{+2} binding proteins like Calmodulin, Calcineurin B like protein (CBL), Ca^{+2} dependent protein kinase (CDPK). All these proteins need an EF-hand (DxDxDG) motif to bind Ca^{+2} ions. But, C2 domain-containing proteins and annexins do not need EF-hand motif to bind the Ca^{+2} (Clark & Roux, 1995; Laohavisit & Davies, 2011; Reddy et al., 2004). Annexins possess a special kind of motif called Endonexin fold **(G-X-GT (38 residues) D/E)** to bind the Ca^{+2} ions (**Fig. 1. 2**). Comparative to calmodulin, annexins have a lower affinity towards Ca^{+2}.



Rescher et al., 2004

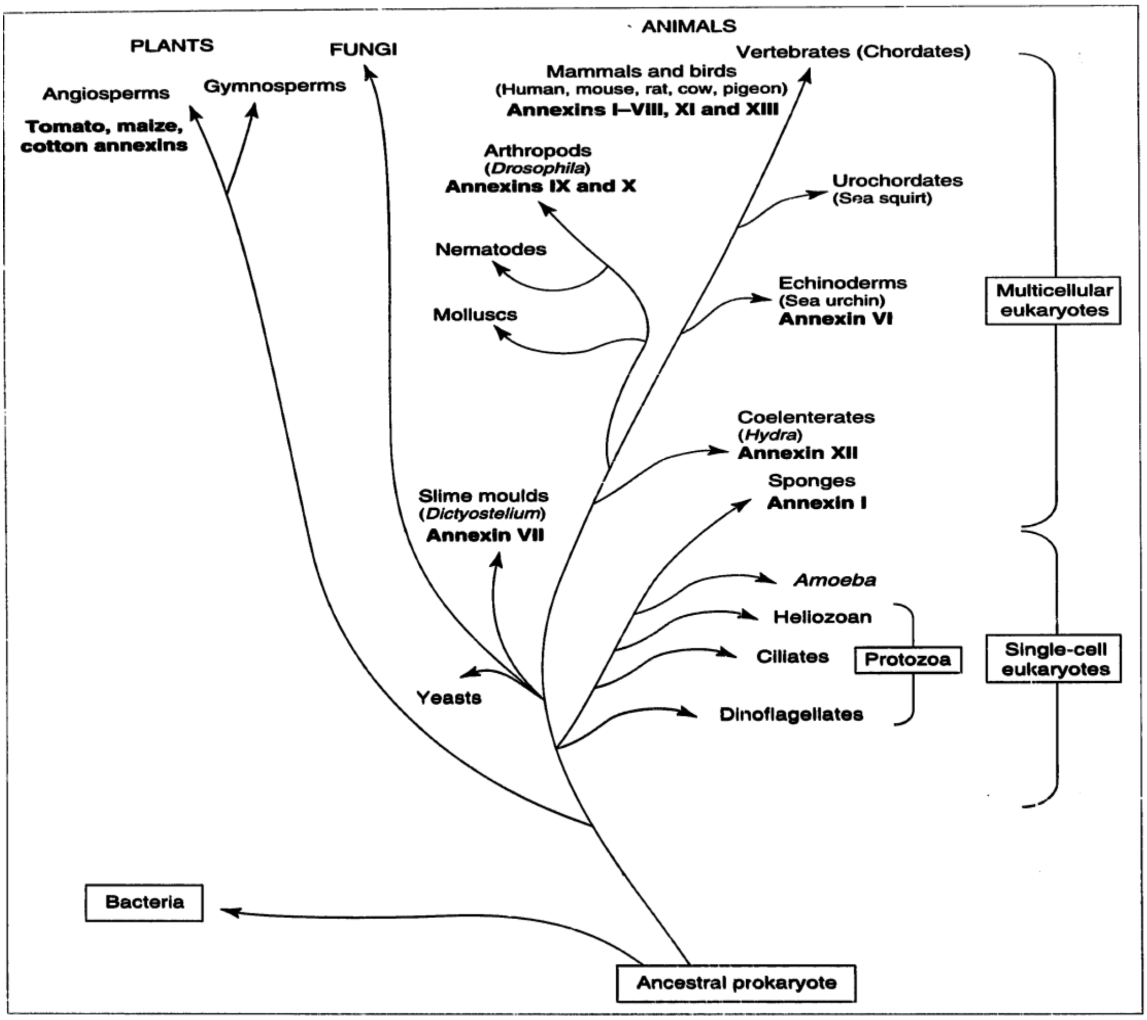
Fig. 1. 2. Difference in the typical structure of calcium-binding proteins. Calmodulin (A), Annexin (B)

1.2 Review of literature

The word annexin was derived from the Greek, *annex* means "bring/hold together". Since they aggregate the membranes at a particular Ca^{+2} ion concentration, they were named as annexins. They exist in animal and plant cells, fungus, protists, bacteria (Clark et al., 2012; Kodavali et al., 2014). However, their identity is not recognized in yeast. This indicates that annexin proteins are almost ubiquitous in nature. Their molecular weight predominantly ranges from 33 kDa-35 kDa. But there are two exceptional cases where the molecular mass is above 35 kDa; one is a celery annexin-like protein, which is of 42 KDa and the second one is a fern annexin, which is of 75 kDa. They are multi-gene, multifunctional proteins. Because of their multi-gene nature, they evolved into a superfamily. The annexin superfamily has been divided into seven sub-families viz., ANXA involves vertebrate annexins and ANXB contains invertebrate annexins, annexins from fungi and unicellular eukaryotic organisms were included in the ANXC. ANXD consists of plant annexins and it comprises 40+ sub families (Clark et al., 2012). The family ANXE comprises protists annexins, ANXF comprises bacterial annexins and ANXG incorporate putative archaeal annexins (Moss & Morgan, 2004; Yadav et al., 2018)).

Phylogeny

They have a complex evolutionary history and shown a large number of gene losses and gene duplications in different taxa (Braun et al., 1998). Mammalian, especially human annexins are well characterized when compared to plant annexins. An accepted general perception is that every mammalian cell type produce a particular subset of annexins that can be seen as a cell unique mark (Gerke & Moss, 2002; Raynal & Pollard, 1994). Molecular phylogeny, chromosomal position, identification and gene structure analysis suggest that annexins are extensively diversified. Plant annexins are distinct from the group of animal annexins (Battey et al.,1996). Though animal annexins were identified first, sequence analysis suggests that the plant annexins evolved and diverged prior to them i.e. 1000 million years ago from the moss, ferns and gymnosperms annexins (Moss & Morgan, 2004). The current data suggests that family of mammalian annexins evolved from four-repeat annexin (**Fig. 1.2.1**) (Smith & Moss, 1994). Clark et al.(2012) proposed that plant annexins descended from green algae.



Smith & Moss, 1994

Fig. 1.2.1 Phylogenetic tree depicting the evolution of the annexins from different kingdoms

Plant annexins discovery

The first search on annexin like proteins in plants has begun in tomato in 1989 (Boustead et al., 1989). During a proteomic study, 33 KDa and 35 KDa Ca^{+2} dependent phospholipid binding proteins were identified in tomato suspension cultures. Later, Smallwood et al. (1990) identified 34 and 35.5 kDa proteins from tomato suspension cultures and confirmed them as annexins through sequencing.

The above two findings strongly suggest that higher plants also have annexin proteins. Soon after this Blackbourn et al. (1991) identified 33 KDa and 35 KDa proteins from maize coleoptiles and confirmed that they also have similar characteristics of annexins by their Ca^{+2} mediated liposomal binding and immunological cross-reactivity. Smallwood et al. (1992) designed primers for a 95 bp fragment of a p34 protein from its genomic DNA. Expression analysis for the same revealed the presence of highest transcript levels in the root tissues of tomato, potato and barley. The transcript levels were also well correlated with the protein abundance levels. Least amount of protein was observed in the mature leaves. Andrawis et al. (1993) identified a similar annexin like protein p34 in cotton fiber, which on association with plasma membrane reduces the activity of (1,3)-β-glucan synthase. This observation gave us a clue that plant annexins also have some enzymatic regulatory role.

Similar observations on isolation and peptide sequencing have also been done on pea, pepper, cotton, celery, rhizoids of ferns and lily pollen tubes (Clark et al., 1995; Clark et al., 2001; Yongan Zhang et al., 2011; Clark&Dauwalder, 1992; Hofmann et al., 1999; Hofmann et al.,2003). There are also evidences for their existence as isoforms. For example, in a 2D analysis, a 35-kDa annexin band from pea plumule proteome resolved into three isoforms at pI's of 6.5, 6.8, and 7.0 and a 34-kD cotton fibers annexin resolved into two isoforms at pI of 6.1 to 6.5 (Clark et al., 1995). This indicates that they might have diverse functions through spatio-temporal regulation.

Structure

Crystallographic studies on annexins disclosed a conserved structural sequence, implicating that they are a family of structurally related proteins (Lim et al., 1998). However, they have conferred different properties and functions while maintaining the moderate level of structural conservation in their primary sequences (Clark & Roux, 1995). Structure of the annexin protein deciphers the involvement of annexins in stress. The fundamental annexin protein has N-terminal head and C-terminal core regions. Generally an N-terminal region of animal annexins is large and variable and exhibit sites required for post-translational modifications, like acetylation with one exception, which shows myristoylation modification (Clark et al., 2012). As it contains the major regulatory motifs this domain is considered as the regulatory region of the protein. Unlike animal annexins, plant annexins have short N-terminal region comprising ten amino acids and may not play an important role in functional diversity (Mortimer et al., 2008). This variation might form the important basis for their functional differences.

Usually animal annexins have four to eight conserved annexin repeats of 70 to 75 amino acids each and all have five helices, named as A-E. The AB, DE loops have the membrane binding sites (Yadav et al., 2018). All the repeats have the ability to bind the Ca^{+2} ions and to the membranes. However, plant annexins have four conserved repeats and only repeat I and IV have characteristic calcium binding Endonexin sequences defined as a (G-X-GTD-{38}-E/D) (Greg B. Clark et al., 2012). It is observed that 3rd repeat in plant annexins is least conserved (Smith & Moss, 1994). Recently an annexin protein having single conserved domain has been reported in *Bacteroidetes/Chlorobi* phylum, *Cytophaga hutchinsonii*. This indicates that they maintain the structural conservation while accommodating different functional properties.

A conserved Histidine, mostly at 39th residue was identified in most of the plant annexins (Mortimer et al., 2008). Some annexins exhibit F-actin binding sites (IRI), GTP-binding regions (GXXXXGKT and DXXG) for phosphodiesterase activity. A cotton annexin has S3 cluster that possibly participate in reduction and oxidation reactions (Hofmann et al., 2003). Comparative to mammalian annexins plant annexins has larger surface area (Clark et al., 2001), perhaps this could be due to extra grooves and clefts, suggesting the occurrence of a broad range of interacting partners and wide range of roles in the cell. The potential role of plant annexins in exocytosis has been identified (Battey & Blackbourn, 1993; Clark & Roux, 1995). They are also involved in DNA replication (Raynal & Pollard, 1994).

Membrane binding activity

As their name suggests, their association with the membrane has been recognized during different physiological processes. Their ability to mediate membrane binding is highly specific for the Calcium ion and showed a decreased order for other ions as follows Cd>Zn>Co>Ba>M for animal annexin AnnexinV. Here, Zn showed a synergistic effect. Ca^{+2} ions acts as a bridge between negatively charged membrane phospholipids and annexins. They can bind to membranes reversibly either in Ca^{+2} dependent or independent manner (Dabitz et al., 2005). Though annexins are calcium-binding proteins, their calcium binding site is distinct from the calcium binding sites of calmodulins.

Literature suggests that membrane binding activity of the annexins depends on the cellular pH and Ca^{+2} concentration. They can adhere to the phospholipids at various Ca^{+2} concentrations i.e. from 10^{-7} – 10^{-4} M. The 33-kD and 35-kD corn annexins exhibited the half of the maximal binding at 150 µM free Ca^{+2} concentration, pH 7.4. The same annexin at pH 6.4 needed 30, 50 µM free Ca^{+2} concentration respectively (Battey & Blackbourn, 1993; Blackbourn et al., 1991). Maize annexin p33 had the requirement of 120 µM free Ca^{+2} for one-half maximal binding to liposomes, whereas p35 required 370 µM (Blackbourn et al., 1991). The sensitivity of annexins to the Ca^{+2} depends on the pH of the surroundings. Maize 33 and 35 kDa proteins had the requirement of 100 µM Ca^{+2} at pH 6.0, and 1 mM Ca^{+2} at higher levels of pH for liposomal binding (Blackbourn et al., 1991). This strongly suggests pH-dependent membrane binding of annexins. Annexin binding to the phospholipids can be specific. Tomato 33 and 35 kDa annexin proteins were able to bind only to the mixture of phosphatidylcholine and phosphatidylserine, but not to phosphatidylcholine lipid molecules (Boustead et al., 1989). However, based on the knowledge on animal annexins, it is likely that the Ca^{+2} concentration required for annexin binding depends on its sub cellular location and kind of tissue (Gruenberg and Emans, 1993; Raynal & Pollard, 1994). This could be one of the reasons for their functional diversity.

Maize root tip annexins p33 and p35 causes the aggregation of the native and artificial vesicles (Battey & Blackbourn, 1993; Blackbourn et al., 1991). Another study by Seigneurin-Berny (2000) revealed that spinach annexin specifically binds to chloroplast sulfo-lipids in the presence of Calcium ions. This is the first study that shows annexins can binds to glycerol lipids other than phospholipids. The 42 kDa celery and tobacco annexin (VCaB42) were found in association with vacuolar membranes (Seals et al., 1994; Seals et al., 1997). Shi et al. (1995) identified plasma membrane bound annexin in soya bean.

Dabitz et al., (2005) highlighted few differences for plant and mammalian annexins on their membrane binding properties. They observed that bell pepper or cotton protein sample ANXD36 bound to PC/PS vesicles (3:1) without the requirement of calcium. This binding percentage is much higher than mammalian annexins. In another study, Hofmann et al. (2002) examined the oligomerization of plant annexins independent of calcium. Breton et al. (2000) identified two groups of proteins in wheat, one group of proteins p36, p34, exist in the cytosolic fractions required calcium ions for phospholipid binding and the other group of annexins p39 and p22.5 were independent of calcium to insert into microsomal membranes.

It is hypothesized that phospholipid membranes helps in the folding of the N-terminal peptides which eventually helps the protein to interact with them. In support to this hypothesis annexinA1 N-terminal domain acting as peripheral membrane anchor during the membrane aggregation (Hu et al., 2008). The repeat IV of tomato p35 plays a crucial role in phospholipid binding (Lim et al.,1998)

Oligomerization

Dimerization has been observed in the animal and plant annexins like synexin, calectin, and the annexin from green pepper (Lim et al., 1998). The first evidence that plant annexins can form homodimers came for Annexin Ca35 (Hoshino et al., 1995). The conserved tryptophan residue in repeat I has been proposed as possible reason for plant annexin oligomerization (Hoshino et al., 1995). Blackbourn et al. (1991) found p68 annexin protein, but they could not find the corresponding cDNA which represents that protein. This observation points to the fact that annexins can form either homodimers or heterodimers. A tomato 70 KDa annexin protein showed the F-actin binding activity, but not the p35 protein. This could imply that p35 shows its catalytic activity in the dimer state only. Gorecka et al.(2007) observed the AtAnn1 structural modification under different pH conditions. When the pH of the buffer was reduced from 7 to 5, they observed the increased hydrophobicity and reversible oligomerization/ aggregation of AtANN1. Further reduction in pH or acidic pH led to irreversible aggregation of the AtAnn1 protein. Hofmann et al. (2002) has done sedimentation equilibrium experiments for few annexins. Where Anx23(Ca38), Anx24(Ca32), and Anx(Gh1) existed in a monomer–trimer equilibrium in solution. AnnGh2 forms dimers and reduced fraction of trimers in the solution. The number of AnnGh2 trimeric species did not changed in the presence of calcium. These experiments proposing the calcium independent self-association of plant annexins.

Exocytosis

Exocytosis activity for plant annexins has been suggested in plants by Battey & Blackbourn, (1993). They suggested that plant annexins may help in the binding of the vesicle to the plasma membrane during the exocytosis or endocytosis process. In an experiment with protoplasts of root cap cells in maize, Carroll et al. (1998) observed that exocytosis was enhanced in the presence of Ca^{+2} and maize annexin p35 and GTP inhibited this process. This evidence implicates the annexins direct role in exocytosis. Generally, actin binding property of a protein may have a regulatory role in exocytosis or endocytosis (Konopka-Postupolska, 2007). Annexins from different species have been reported for their actin binding activity. This leads to an hypothesis that they might have probable regulatory role in the exocytosis or endocytosis (Blackbourn et al., 1992; Delmer & Potikha, 1997; Hoshino et al., 2004; Calvert et al., 1996). In another study, tobacco annexins Ntp32.1 and Ntp32.2 expression was correlated with cell division suggesting their possible role in exocytosis (Proust et a., 1999). A mutant of the Arabidopsis annexin *AtAnn2* shown reduced hypocotyl elongation implicating its role in the exocytosis (Clark et al., 2005a,b).

Channel activity

Plant annexins can also work as unconventional ion channels (Davies, 2014). Unlike conventional channels, they exist as soluble proteins and bound to the membrane upon a stimulus. They appeared to be capable of mediating passive Ca^{+2} ion transport (Mortimer et al., 2008;Gerke & Moss, 2002;Davies, 2014). Pepper annexin (CaANN24) mediated the Ca^{+2} influx into artificial vesicles (Hofmann et al., 2000). A *Zea mays* annexin doublet, ZmANN33 &ZmANN35 promoted Ca^{+2} influx into root epidermal protoplasts and also showed Ca^{+2}-permeable cation conductance in the plasma membrane that resembled planar lipid bilayers (Laohavisit et al., 2009). AtAnn1 was identified as K^{+}- permeable channel at acidic pH in *in vitro*. In support of this observation, acidic pH favored the formation of oligomers and the channel formation is accompanied by the increased amount of β-sheets and also the hydropathic index. Hydroxyl radicle stimulated the conductance of AtAnn1 and was also able to differentiate between Na^{+} and K^{+} (Gorecka et al., 2007) (Table 1).

Table 1: List of annexins which can act as an ion channel

Plant	Gene	Channel property	Reference
Zea mays	*ANN33* *ANN35*	Acts as Ca^{2+} permeable channel	Laohavisit et al., (2009)
Capsicum annuum	*ANN24*	Involved in passive Ca^{2+} transport	Hofmann, et a., (2000a)
Arabidopsis thaliana	*AnnAt1*	Forms K^{+} permeable channels in bilayers, with chanfnel formation, favored at low pH	Gorecka et al., (2007)
Arabidopsis thaliana	*AnnAt1*	Salinity-induced calcium signaling and root adaptation in Arabidopsis require the calcium regulatory protein Annexin1	Laohavisit et al., 2013
Arabidopsis thaliana	*AnnAt1*	Radical-Activated Plasma Membrane Ca^{2+}- and K^{+}-Permeable Conductance in Root Cells	Laohavisit et al., 2012
Arabidopsis thaliana	*AnnAt1*	Regulates the H_2O_2-induced calcium signature in *Arabidopsis thaliana* roots	Richards et al., 2014
Medicago truncatula	*AnnMt1*	Facilitates, the transport of Na^{+}, K^{+}, Ca^{2+} ions across the lipid bilayer and may play an important role in Nod factor signalling	Kodavali et al., 2014

Yadav et al., 2018s

Peroxidase activity

The first report on plant annexins peroxidase activity was given by Gidrol et al. (1996). When the ΔoxyR mutant *E. coli* cells were complemented with AtAnn1, they were able to tolerate the H_2O_2 stress. AtANN1 expressed in *N. benthamiana* showed three times higher peroxidase activity in comparison with prokaryotic expressed AtANN1(Gorecka et al., 2005). This implies that post-translational modifications emphasize the need for the enhanced peroxidase activity. This also suggests that they can play a role in oxidative stress response during the stress. Earlier, a conserved H40 residue was suspected for the peroxidase activity of the annexin due to its structural similarity with the heme-binding motif of other peroxidases (Gidrol et al., 1996). Crystallographic studies on GhAnn1 revealed an S3 cluster, which can act as redox reactive centers during the peroxidase reaction (Hofmann et al., 2000a; Hu et al., 2008). However, the motif responsible for peroxidase activity is clearly not known yet. Overexpression of certain annexins in the transgenics exhibited abiotic stress tolerance through reducing peroxides or lipid peroxidation levels (Ahmed et al., 2017; Divya et al., 2010; Jami et al., 2008; Konopka-Postupolska et al., 2009; Zhang et al., 2011; Zhou et al., 2011). This suggests the annexins can help the plants to tolerate the oxidative stress.

Hydrolysis activity

Plant annexins can bind and hydrolyze purine nucleotides. This activity has been observed in maize (Mcclung et al., 1994), tomato (Calvert et al., 1996; Lim et al., 1998) and cotton (Shin & Brown, 1999). Plant annexins, unlike animal annexins, depend on a Walker A motif (GXXXXGKT/S) and a GTP binding motif (DXXG) for the hydrolysis activity. These kind of motifs has been found in the repeat IV of the AnnGh1, AtAnn2 and AtAnn7 annexins(Clark et al., 2001). Tomato annexin p35 was shown its hydrolysis activity on various substrates like ATP, CTP, GTP, TTP and UTP. Lower activity was found for ADP when compared with NTP's and even less activity was noticed with substrates like AMP, fructose 1-phosphate, UDP-glucose, and inorganic pyrophosphate (Lim et al., 1998). Alignment of cotton annexin, ZmAnn33/35, AnnAt2 revealed that GTP binding motif overlapped with the Calcium binding motif (Mcclung et al., 1994; Shin & Brown, 1999). Tomato annexin upon calcium-mediated phospholipid binding lost its hydrolytic activity (Calvert et al., 1996). Mutation in its calcium binding motif does not affect its GTPase activity (Lim et al., 1998).

Callose synthase activity

Cotton fiber annexins were found to be in association with callose synthase of cotton fibers. This experiment suggested that the association of annexin with callose synthase enzyme has a regulatory role in glucan synthesis (Andrawis et al., 1993).

Post-translational modifications

Andrawis et al. (1993) gave the first report that plant annexins may get phosphorylated. They observed that 34 kDa cotton fiber annexin protein was co-purified with the Mg^{+2}-dependent kinase. They suspected that this kinase could be phosphorylating the annexin *in vitro*. In a proteomic study, AtAnn1 was *S*-nitrosylated (Lindermayr et al., 2005). ABA treatment leads to S-glutathionylation of two Cysteine residues of AtAnn1 and this glutathionylation altered the calcium binding affinity (Konopka-Postupolska et al., 2009). AtAnn1 purified from *E.coli* showed reduced peroxidase activity compared to the protein purified form from *N. benthamiana*. All the above studies strongly suggest that post-translational modifications may play a crucial role in the proper functioning of annexin.

Localization

Mostly annexins are localized in the cytosol and intracellular membrane. But their presence was also found in the nucleus, nucleolus and extracellular matrix (Clark et al., 1998; Kovács et al., 1998). They can also be present in different cellular compartments like chloroplast stroma (Rudella et al., 2006) phloem sap and phloem exudate (Giavalisco et al., 2006). Seals et al. (1994) identified that VCaB42 (42-kD) annexin is specifically localized in the vacuolar membrane. Pea and corn annexins are concentrated in the highly secreted cells like epidermal cells, outer cells of root cap, developing cells of the xylem, phloem (Clark et al.,1994). Immunolocalization studies revealed that high concentrations of annexins get accumulated at polar growing regions like pollen tubes, fern rhizoids, actively involved Golgi mediated secreting cells and plasma membrane (Blackbourn et al., 1992; G B Clark & Roux, 1995). Recent observation suggests that rice annexin OsAnn3 is localized in the peripheral region of the root tip cell (Shen et al., 2017).

Annexins role in abiotic stress

Gene expression study from different species of the plants has found the upregulation of the annexins to the abiotic stresses. The list of identified stress responsive annexins were in the Table 2. The gene expression studies, mutation and overexpression studies reveals the important role of the annexins in abiotic stress tolerance (Yadav et al., 2018).

Table 2: Plant annexins confirmed for their role in plant development and stress tolerance

Plant	Gene	Role in plant	Reference
Arabidopsis thaliana	*AnnAt1*	Drought, salt, heat and osmotic stress tolerance in *A. thaliana*	(Huh et al., 2010; Konopka-Postupolska et al., 2009; Laohavisit et al., 2013; Lee et al., 2004)
Arabidopsis thaliana	*AnnAt2*	Osmotic stress tolerance in *A. thaliana*	(Lee et al., 2004)

Arabidopsis thaliana	*AnnAt4*	Osmotic stress response, Interacts with AnnAt1 and regulates drought and salt stress responses	(Huh et al., 2010; Lee et al., 2004)
Arabidopsis thaliana	*AnnAt5*	Pollen development, pollen germination, pollen tube growth	(Zhu et al., 2014a; Zhu et al., 2014b)
Arabidopsis thaliana	*AnnAt8*	Salt, dehydration and methyl viologen stress tolerance in *A. thaliana* and tobacco	(Yadav et al., 2016)
Brassica juncea	*AnnBj1*	Salt, dehydration and oxidative stress tolerance in Cotton	(Divya et al., 2010)
Brassica juncea	*AnnBj1*	Salt, dehydration, $CdCl_2$ and oxidative stress tolerance to tobacco	(Jami et al., 2008)
Cynanchum komarovii	*CkAnn*	Drought tolerance in cotton	(Zhang et al., 2011)
Brassica juncea	*AnnBj3*	Attenuates methyl viologen-mediated oxidative stress in *A. thaliana*	(Dalal et al., 2014b)
Brassica juncea	*AnnBj3*	Compensate for the thiol-specific antioxidant (TSA1) deficiency in Saccharomyces cerevisiae	(Dalal et al., 2014)
Brassica juncea	*AnnBj2*	Conferred salt tolerance in *Brassica juncea*	(Ahmed et al., 2017)
Brassica juncea	*AnnBj2*	Conferred salt tolerance in tobacco	(Ahmed et al., 2018)
Nelumbo nucifera	*NnAnn1*	Heat stress tolerance during seed germination	(Chu et al., 2012)
Oryza sativa	*OsAnn1*	Heat stress tolerance in rice	(Qiao et al., 2015)
Oryza sativa	*OsAnn3*	Role in cold stress tolerance in rice	(Shen et al., 2017)
Triticum turgidum L. subsp. durum cv.Mahmoudi	*TdAnn6* *TdAnn12*	Heterologous expression of these genes in yeast improved its tolerance to abiotic stresses	(Harabaoui et al., 2018)
Gossypium	*GhAnn1*	Salt and drought stress	(Zhang et al., 2015)

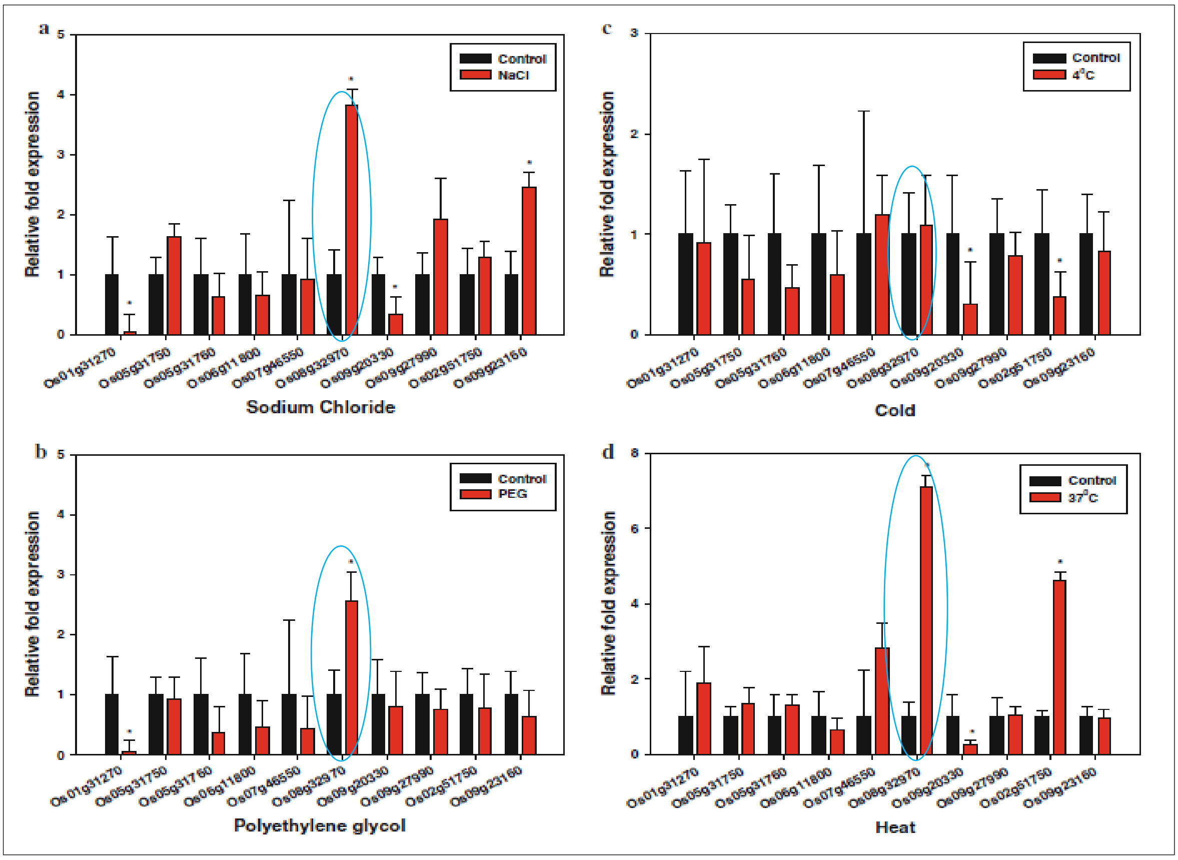
hirsutum		tolerance in cotton	
Medicago sativa	*Ann Ms2*	Osmotic and drought stress responses in *Medicago sativa*	(Kovács et al., 1998)
Gossypium barbadense	*GbAnn6*	Cotton fiber elongation and increased root cell length	(Huang et al., 2013)
Aspergillus fumigatus	*ANXC4*	Anti-stress function	(Khalaj et al., 2011)
Solanum tuberosum	*StAnn1*	Promote drought tolerance and mitigate light stress in potato	(Szalonek et al., 2015)
Solanum pennellii (wild tomato)	*AnnSp2*	Conferred salt and drought stress tolerance in tomato	(Ijaz et al., 2017)
Triticum durum	*TdAnn12*	Its transgenic tobacco shown tolerance to salt, Osmotic and H_2O_2 stresses	(Rania Ben Saad et al., 2019)
Glycine max (L.) Merr	*GmAnn*	Transgenics Arabidopsis shown tolerance to high temperature and humidity stress	(Wei et al., 2019)

Yadav et al., 2018

As annexins are multigenic in nature, genomic sequencing revealed the presence of ten annexin genes in *Oryza sativa* (Jami et al., 2012), eight in *Arabidopsis thaliana* (Yadav et al., 2016), nine in *Solanum tuberosum* (Szalonek et al., 2015), 13 in *Brassica rapa* (Yadav et al., 2015), 23 in *Glycine max* (Feng et al., 2013). They were also identified in tomato, cotton, Alfalfa, Zea maize, capsicum, strawberry plants. Among these, Arabidopsis annexins were well characterized. Functional characterization of annexins from other species is also important in predicting their role in cellular functions and for crop improvement.

Rice is the major food crop throughout the world, especially in China and India. Due to climate change, rice plants experience different types of abiotic stresses, like drought and high salinity. These stress conditions lead to a significant economic loss in rice productivity. To cater to future demands always there is a need for better and improved rice varieties. Crop improvement through conventional breeding is not simple. Genetic engineering can be a promising approach for crop improvement. Diverse functions of the annexins in plant growth and regulation attracting the researchers for their characterization. Since annexins have been proved to have a role in abiotic stress tolerance, the overexpression of annexins might be a promising approach to develop improved stress tolerant crop varieties.

A total of ten annexins have been predicted in the rice genome (Jami et al., 2012). There was an old report on Nagina 22 annexin, which accumulated under the drought stress condition (Gorantla et al., 2005). In a proteomic study a rice, annexin Os05g31750 was shown to interact with various kinases including MAPKK suggesting its involvement in Ca^{2+}-dependent MAPK signaling (Rohila et al., 2006). Later Jami et al. (2012) have performed a gene expression study for all rice annexins under various abiotic stress conditions. All the rice annexins shown differential expression towards the abiotic stress conditions (Fig. 1.2.3).



Jami et al., 2012

Fig. 1.2.3 Relative fold expression of rice annexins under different stress conditions.

In the above study, Os08g32970 has shown significant upregulation within 2 h of abiotic stress treatment suggesting that it is a key player in modulating tolerance

(Jami et al., 2012). Recently another rice annexin *OsAnn3* knock out led to decreased tolerance to the cold stress (Shen et al., 2017) . The function of *OsAnn5* is not characterized till now. As an initial study to elucidate its function in the abiotic stress and to decipher its role in the stress signaling pathway, the following objectives were framed

1) Heterologous expression of *OsAnn5* in tobacco and *E.coli* to assess abiotic stress tolerance.
2) Comparative studies of 35S:*OsAnn5* expressing lines with the mitochondrial and guard cell targeted *OsAnn5* tobacco lines to know their extent of tolerance towards abiotic stress.

CHAPTER 2

Materials and Methods

2.1 Plant materials

All transgenics were developed in *Nicotiana tabacum* cv. Samsun. *OsAnn5* was amplified from the *Oryza sativa* cv. Nipponbare. *Arabidopsis thaliana* var Columbia and *Solanum tuberosum* were used as sources to amplify the DRE (Drought Responsive Elements) and GCSE (Guard Cell Specific Elements) respectively for DGP1 promoter generation.

2.2 Chemicals and enzymes

The chemicals used in the study were purchased from the following company's

Clontech, USA; Sigma St. Louis, MO, USA; Promega Life Science, Madison, WI, USA; Amersham Biosciences, UK; Finnzymes, New England; BioLabs, Ipswich, MA; Fermentas, Germany; Qualigens fine chemicals, Mumbai, India and Himedia Chemicals, Mumbai, India. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, Taq polymerase, RT-enzyme, protein markers were purchased from Thermo Fischer Scientific Inc., USA.

2.3 Plasmids and DNA vectors

2.3.1. pTZ57R/T vector (MBI Fermentas Germany) was used for simple TA cloning which has the ampicillin resistance gene for selection and also suitable for blue-white screening. The amplified CDS of *OsAnn5*, *COXIV*:*OsAnn5* were cloned into this vector.

2.3.2. PET32a (Novagen, USA) is a bacterial expression vector in which T7 promoter is induced by IPTG and was used to overexpress OsANN5 in BL21(DE3) pLysS cells.

2.3.3. pRT100 (Topper et al. 1987). *OsAnn5*, *COXIV*:*OsAnn5*, DGP:*OsAnn5* were cloned into this vector in which MCS is flanked by CaMV promoter and poly-Adenylation signal. This has
β-lactamase gene for ampicillin selection.

2.3.4. PCAMBIA2300 is a binary vector having nptII selection marker and was used to clone *OsAnn5*, COXIV:*OsAnn5*, DGP1:*OsAnn5* expression cassettes, which were further used to develop tobacco transgenic plants through agrobacterium-mediated transformation.

2.3.5. pEGAD vector (Cutler et al., 2000) was used for the confocal microscopy study in which, *OsAnn5* was N-terminally fused with EGFP.

2.4 Bacterial strains

E. Coli strain DH5-α was used for the maintenance of the above-mentioned plasmids

BL-21(DE3) pLysS *E.coli* strain was used for *OSANN5* expression

Agrobacterium tumefaciens strain LBA 4404 was used for transformation of tobacco plants and also for the transient expression studies.

2.5 Total RNA isolation

Total RNA was isolated using TRI reagent as per the manufacturer's protocol (Sigma, USA) from different treatment given plant samples

2.6 Polymerase chain reaction (PCR)

Reagents for PCR were purchased from Sigma-Aldrich (USA) and Invitrogen (USA) and the reactions were performed on Bio-Rad thermal cycler, USA or Eppendorf Personal Thermal cycler, Germany. PCR conditions were optimized according to the template and primer combinations. Quantitative real time PCR was performed with Bio SYBR Premix Ex Taq (Takara, China).

2.7 Agarose gel preparation and electrophoresis

DNA fragments were resolved on 1% Agarose gel prepared by melting 1g of agarose (A-9539, Sigma-Aldrich, St. Louis, USA) in 100ml of 1X TAE buffer [diluted from 50X TAE: 2 M Tris Cl, 1 M glacial acetic acid, 100 mM EDTA (pH 8.0)]. One μl of EtBr was added to it and dispensed into the casting tray, in which a suitable comb was placed. After proper polymerization, the comb was removed and the gel was immersed in an electrophoresis tank, which had 1X TAE buffer. Subsequently, the samples were mixed properly with 6X DNA loading dye (0.03% bromophenol blue, 0.03% Xylene cyanol, 60 mM EDTA, 10 mM tris-HCl, 60% glycerol) to get a final concentration of 1X. Samples were loaded in the wells and the gel was electrophoresed at 70 V until the DNA fragments were properly resolved and checked under UV light to see the band separation.

2.8 DNA fragments purification from the agarose gel

PCR amplifications or confirmed plasmids were electrophoresed on the agarose gel and the gel region where the required DNA fragment was present was cut out to elute the separated DNA fragments according to the protocol given in the kit (Sigma, USA or Nucleopore)

2.9 Ligation

T4 DNA ligase (Fermentas, Germany) was used for ligation purposes. The reaction (20 µl) were prepared, which included 2 µl of 10X ligation buffer, insert, vector (varying quantities) and 1 U enzyme for cohesive end ligations and 3 U for blunt end ligations.

2.10 Dephosphorylation

Single digested plasmid vectors were treated with calf intestine alkaline phosphatase (Fermentas Germany) to remove 5' phosphorylated ends so that self-ligation was avoided. The 50 µl reaction mixture for dephosphorylation included 10 µl of 5X dephosphorylation buffer, 1 µl enzyme, the desired amount of digested plasmid and the mixture was made up to the required volume with water. The reaction mixture was incubated at 37°C for 1 h followed by subjecting it to 65 °C for 15 min to stop the reaction.

2.11 Competent cells preparation

2.11.1 *E.coli* competent cell preparation

The pure DH5α, *E.coli* culture was streaked on the LA media plate and incubated at 37 °C overnight. A single isolated colony from the incubated plate was inoculated in 10 ml LB medium and kept for shaking at 200 rpm, 37 °C overnight. 100 µl inoculum from the overnight culture was added to the 100 ml LB medium, incubated under the above conditions until the culture reaches an OD of 0.2 at 600 nm. This was followed by spinning down the culture at 4000 rpm at 4 °C for 6 min. The supernatant was discarded and the pellet was resuspended in 50 ml of ice cold 0.1 M $CaCl_2$ and incubated on ice for 45 min with interval swirling followed by centrifugation at 4000 rpm for 10 min. The pellet was resuspended in 1.5 ml of 15% glycerol made in 0.1 M $CaCl_2$ solution. 100 µl of this was aliquoted into each 1.5 ml Eppendorf tubes. All these tubes were quick frozen in liquid nitrogen and stored at -70 °C.

2.11.2 Agrobacterium tumefaciens competent cells preparation

The same protocol as *E.coli* competent cell preparation was carried out for the preparation of Agrobacterium competent cells. Except that the culture was grown at 28 °C overnight.

2.12 Transformation of *E. coli* cells

100 ng of plasmids or ligation mix of 1/10th to the volume of the competent cells was added to the *E.coli* competent cells and kept on ice for 5 min. Then, heat shock was given at 42 °C for 90 sec, followed by immediate incubation on ice for 5 min. The volume was made up to 1 ml by adding LB medium and kept on shaking at 37 °C for 1 h. After incubation, the

culture was pelleted and the same was spread on the selection LA media plates, incubated at 37 °C overnight. Next day, the plates were observed for visible colonies and the colonies were further confirmed and used for plasmid isolations.

2.13 Transformation of Agrobacterium tumefaciens cells

2-3 µg of final constructs for plant expression, pCAMIA2300:*OsAnn5*, pCAMIA2300:COXIV:*OsAnn5*, pCAMIA2300:DGP1:*OsAnn5* were added to 100 µl *Agrobacterium tumefaciens* LBA strain competent cells and incubated for 15 min and quick frozen in the liquid nitrogen for 5 min. Then, a heat shock was given at 37 °C for 5 min. After which, the cells were incubated on ice for 5 min and the volume was made up to 1 ml using LB medium. This vial was kept on the rotatory shaker at 28 °C for 3 h. Then the culture was spinned down and spread on the selection media (LA plate supplemented with 25 mg/L rifampicin and 50 mg/L kanamycin) and incubated at 28 °C for 2 days. Later the visible colonies were confirmed through PCR and further used in transformation experiments.

2.14 Plasmid isolation (mini prep) from *E. coli* culture

A single *E.coli* colony grown on selection medium was inoculated in 10 ml LB medium, which is supplemented with specific antibiotic(s) and incubated at 37 °C, overnight. Next day the culture was pelleted down in a 2 ml tube followed by addition of 200 µl of resuspension solution-I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl) to the cell suspension and 400 µl of solution-II (10% SDS, 0.2 N NaOH) to the same tube. The tube was mixed gently until the solution become clear. Then a 350 µl of solution-III [5 M potassium acetate, glacial acetic acid (11.5 ml for 100ml)] was added to the clear lysate, when the mixture turned into a cloudy precipitate. This was centrifuged for 10 min at 12000 rpm and the supernatant was recovered in a fresh tube. To the supernatant, an equal quantity of isopropanol was added, mixed thoroughly and incubated at room temperature for 15 to 20 min. This was followed by centrifugation for 10 min at 12000 rpm at room temperature. The plasmid precipitate was washed with 700 µl wash buffer (70 % ethanol) and dissolved in required volume of Milli Q. This plasmid was used for PCR, digestion confirmation and transformation experiments.

2.15 Transformation of tobacco leaf discs (Horsch et al., 1985)

Second or third leaf of the tobacco plants were collected and rinsed with water, and the same were cut into small pieces. After removing the mid rib and treating with 0.1 % $HgCl_2$ for 5 min, they were washed with autoclaved water 3-4 times. Later, the edges of the leaf pieces were cut off and kept in the *A. tumefaciens* suspension solution for 15 min with gentle shaking at 28°C. These infected tobacco leaf pieces were wet blotted on sterile tissue or blotting paper and kept on the co-cultivation medium (MS media with NAA 0.1 mg/L and BAP 2 mg/L) for 2 days and then shifted to the selection media (co-cultivation+ 500 mg/L cefotaxime, 125 mg/L kanamycin). The leaf pieces started developing callus after 2 weeks after which the same were transferred to fresh selection medium for 20 days on which the shoots were developed after 5 weeks of infection. These shoots were cut off and placed in the rooting media (0.1 mg/L NAA, 250 mg/L cefotaxime, 125 mg/L kanamycin). Properly rooted putative transgenics were shifted to the soil cups for acclimatization and then shifted to the green house for further studies.

2.16 Genomic DNA extraction

Plant genomic DNA was isolated by the CTAB method (Murray et al., 1980). The young leaf was collected from the plant and frozen using liquid nitrogen and then homogenized finely along with a pinch of PVPP powder in the liquid nitrogen. 1 ml of CTAB solution (Sigma, USA) and 0.2 ml of β-mercaptoethanol were added to the fine powder and mixed well to avoid clumps in 2 ml tubes. This mixture was incubated at 65 °C for 1 h and then subjected to centrifugation at 12,000 rpm, 10 min. The supernatant was taken in another tube and an equal amount of chloroform and isopropanol (24:1) was added to this and mixed gently for 20-30 times by inversion and centrifuged for 15 min at 12,000 rpm. The upper aqueous layer was separated carefully and an equal volume of isopropanol was added, mixed and incubated at -20 °C for 20-30 min. Later the samples were centrifuged at 12000 rpm for 10 min. The precipitated genomic DNA was washed with 70 % ethanol, air dried and finally dissolved in an appropriate volume of the Milli Q water according to the pellet size.

2.17 Quantification of DNA and RNA

Concentration and quality of nucleic acids were checked through agarose electrophoresis and Nanodrop (Thermo Scientific NanoDrop® 2000 spectrophotometer).

2.18 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel (12 % acrylamide and N, N'-Methylenebisacrylamide in 30:1 ratio) was used to run the bacterial cell lysate proteins. This was carried out using a standard protocol developed by Laemmli (1970). This was run in 1 x SDS-PAGE buffer (10x SDS-PAGE buffer containing Tris base- 30.0 g, glycine-144.0 g, SDS-10.0 g in 1000 ml of DDW, pH- 8.3). The samples were mixed with sample buffer (2.5 ml 1 M Tris-HCl contains pH 6.8, 1.0 g SDS, 0.8 ml 0.1% Bromophenol Blue, 4 ml 100% glycerol, 2 ml 14.3 M β-mercaptoethanol, 0.5 ml of ddH_2O) and boiled in a water bath for 5 min and then loaded on to the gel. Gels were maintained at 60 V until the tracking dye crossed the stacking gel. Subsequently, the remaining gel was run at 120 V.

2.19 SDS-PAGE staining

Electrophoresed gels were incubated in the Coomassie staining solution (0.1 % Coomassie Brilliant blue-R250, 50% methanol, 10% glacial acetic acid) for 2-3 h and then shifted to the destaining solution (50% methanol, 10% glacial acetic acid) incubated in it until clear bands were visible.

2.20 Cloning and overexpression of *OsAnn5* in *E. coli* cells

The confirmed *OsAnn5* sequence was cloned into the pET32a$^+$ bacterial expression vector (Novagen, USA) at the *Kpn*I and *Bam*HI sites so as to translationally fuse the C-terminal region of OsANN5 protein with 6x Histidine tag. The confirmed recombinant pET32a:*OsAnn5* plasmid was transformed into *E*. *coli* BL21 (DE3) pLysS cells (Takara, China). The transformed BL21 cells were cultured in Luria broth at 37 ˚C till 0.5 O.D growth and the cells were then induced with 0.1 mM IPTG (Sigma, USA) and grown further at 28 ˚C for 3 h. The culture was then centrifuged at 5000 rpm for 10 min at 4 ^oC. The pellet was resuspended in cold lysis buffer (50 mM Tris–HCl, 300 mM NaCl, 10 mM imidazole and 0.1% N-lauroyl sarcosine, pH 7) and the cells were subjected to a 20 s pulse of sonication and a 20 s of pause for 25 cycles on ice. The lysed culture was centrifuged at 12,000 rpm for 40 min at 4 ^oC. The clear supernatant was then gently mixed with 1 ml of Ni^{+2}- NTA agarose beads (Qiagen, Germany) for 30 min and then added to the column with a flow rate of 0.5 ml/min. Later, the column was washed twice with the cold washing buffer (50 mM Tris–HCl, 300 mM NaCl and 20/30 mM imidazole, pH 7.0). The tagged protein was eluted with the cold elution buffer (50 mM Tris–HCl, 300 mM NaCl and 200 mM imidazole pH 7.0). Furthermore, the eluted protein was confirmed by SDS-PAGE (12%) and western blot using anti-His primary antibodies.

Along with the 6x Histidine tag, the thioredoxin (Trx) tag was also fused to OsANN5. Hence to cleave the Trx tag, thrombin enzyme was used. The cleavage reaction consisted of 0.01 units of thrombin enzyme (Novagen, USA), 1 X cleavage buffer and 100 µg of Trx:OsANN5 fusion protein in a final volume of 200 µl and was incubated at 16 °C for 12 h. The tag cleaved OsANN5 was purified and dialyzed against 20 mM Tris-HCl, pH 6.0 and stored for further experiment.

2.21 Western Blotting and Immuno staining

After SDS-PAGE, the gel was equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol). The PVDF membrane was kept on the Whatman filter papers and the gel kept on the membrane while taking care not to trap any air bubbles. This whole set up was placed towards the positive end of Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell apparatus. The transfer was conducted for 1 h at 20 V. The stain was removed by 3-4 washes with TBST buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween-20 (v/v)]. Membrane blocking was carried out using 3% BSA (w/v) in TBS for 1 h at RT followed by 5 washes with TBS for 10 minutes each. The blot was then incubated overnight with the primary antibody diluted in TBS containing 3% BSA at 4 ºC. This was again followed by 5 thorough washes, each of 10 minutes in TBS. Then the blot was incubated in the secondary antibody - Goat anti-rabbit IgG (Bangalore GENEi, India) ALP conjugate. The bands were visualized after staining with BCIP (Bangalore GENEi, India), which was a substrate for ALP conjugate.

To confirm the OsANN5 expression with 6x His tag in, we used anti-His antibodies. The cells were induced with 0.1 mM IPTG and after proper growth, the cells were harvested, re-suspended in the lysis buffer and sonicated. The lysate was run on the gel along with the pellet. Proteins were transferred from the gel to the PVDF membrane using Bio-Rad Trans-blot SD semi-dry transfer cell unit. Anti-His antibodies were used for immunoblotting and the blot was developed with BCIP by keeping the membrane in the dark.

2.22 Ca^{+2} binding activity assay

The *OsAnn5* calcium binding activity assay was performed in *vivo* and in *vitro.* For the in *vivo* analysis, 30 mM $CaCl_2$ was added to the OsANN5 overexpressed BL21 cell lysate to precipitate the calcium bound protein. Through centrifugation (12,000 rpm for 15 min) the supernatant and precipitate were separated. Later, the precipitate was resuspended in the PBS and 30 mM Na_2EDTA was added to chelate the Ca^{2+} ions. This resulted in the clearing of the precipitate. Subsequently, all the samples were analyzed through SDS-PAGE.

For the in *vitro* analysis of *OsAnn5* calcium binding activity, fluorescence spectrophotometer (Horiba Jobin Yvon florimax-3) was used. The 1 ml assay reaction consisted of 20 mM Tris, pH 6.0 with or without 2 mM $CaCl_2$ and 200 µg OsANN5. The fluorescence spectra were taken in the near UV range of 200 nm to 400 nm.

2.23 Stress assays on *E.coli* cells

Spot assay was carried out to check the stress tolerance induced by OsANN5 in the *E. coli* bacterial system. For the spot assay, pET32a:*OsAnn5* and pET32a transformed BL21 PLyS cell cultures were grown to appropriate cell density and induced as per standardized protocols. Different dilutions (10^{-0}, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of both the cultures were spotted and air dried on Luria Agar medium plates (with 100 mg/L ampicillin and 0.1 mM IPTG) containing various concentrations of 0.4 M, 0.5 M, 0.6 M NaCl (high salinity stress), 0.8 M sorbitol (osmotic stress) and 8% and 10% PEG (drought stress). For the high temperature stress treatment after 3 h of IPTG induction, the culture was grown at 50 ^{0}C for 30 min and 1 h and their respective dilutions were spotted on Luria Agar selection plates. The plates were incubated at 37°C overnight and checked for their growth performance.

2.24 Transient expression studies

EGFP fused plasmid were transformed into *Agrobacterium tumefaciens.* The culture was grown at 28°C for overnight under the rifampicin (50 mg/L) and kanamycin (100 mg/L) selection. Later the culture was pelleted down, resuspended in the MS medium (100 µM aceto syringin, 5 % sucrose, 0.02 % silwet- 77) and incubated for 6 h. Then fresh onion scales (1 cm x 1 cm) were placed in the agrobacterium resuspension solution for 6 h as their inner surface immersed in it for infection. Later the infected onion scales were transferred to co-cultivation media (½ MS) for 2 days. After co-cultivation onion scales were rinsed with 0.1 M phosphate buffer and their epidermal peels were peeled off and kept on the glass slide to check under the confocal microscope. Fluorescence images were taken with confocal microscope (Carl Zeiss LSM 710 NLO ConfoCor 3, Germany) under 20X lens at the scale bar of 20 µm.

2.24 Chlorophyll estimation

For chlorophyll isolation, 50 mg of homogenized sample was treated with 80 % acetone until the sample become de-chlorophylyzed. Later the same was centrifuged for 10 min at 12,000 rpm and the supernatant absorbance values were taken at 647 nm and 664 nm. The total chlorophyll content was calculated using below Arnon's equation

Total chlorophyll (µg/ml) = 20.2 (A645) + 8.02 (A663)

2.25 Proline estimation

The protocol developed by Bates et al. (1973) was used for proline estimation. Transgenics and WT seedlings, which were grown on stress media for 15 days were collected and frozen in liquid nitrogen. 50 mg of frozen sample was crushed in 3% sulfosalicylic acid and centrifuged for 10 min at 10,000 rpm. 200 µl of supernatant was treated with 200 µl of acid ninhydrin and 200 µl of glacial acetic acid. The total reaction mixture was subjected to boiling for 1 h and then cooled down on ice to stop the reaction. Later 600 µl of toluene was added to the mixture under vigorous shaking at room temperature to extract the proline from the aqueous phase to the organic phase. Then the organic phase was used to check the absorbance with a spectrophotometer at 520 nm wavelength.

2.26 Lipid peroxidation assay

Lipid peroxidation levels were estimated through the malondialdehyde (MDA) quantification (Heath & Packer, 1968). Each sample (50 mg) was homogenized in 1 ml of 0.1% TCA and the homogenate was centrifuged at 12,000 rpm at 4°C for 10 min. Subsequently, 500 µl of the supernatant was mixed with 1.5 ml of 0.5% (w/v) TBA, which was prepared in 20% TCA (w/v) and incubated at 95°C for 30 min. The reaction was stopped by placing the tubes on ice followed by centrifugation for 5 min at 12,000 rpm (4 °C). The absorbance values for the supernatant were taken at 532 and 600 nm. MDA concentration was calculated using its molar extinction coefficient (155 $mM^{-1}cm^{-1}$). Results were represented as µmol/g FW. The formula used was

MDA (mM) = (A 532 – A 600)/155

2.27 DAB staining and quantification

DAB staining was performed according to the protocol developed by Daudi et al., 2012. In brief, the fourth leaf from one month old transgenic and NS plants were taken for the analysis. Leaf discs were treated with 250 mM NaCl for 48 h. After the treatment, they were incubated in DAB staining solution (1 mg/ml DAB, pH 7.4) overnight. Later, the chlorophyll was removed from the samples by boiling in bleaching solution (ethanol: acetic acid: glycerol,3:1:1) for 20 min. Subsequently, after complete removal of chlorophyll, the leaf discs were stored in 10% glycerol until images were captured.

The DAB stained leaf discs were homogenized in liquid nitrogen and extracted in 1 ml of perchloric acid and centrifuged at 10,000 rpm for 10 min and the absorbance for the supernatant was taken at 450 nm. The amount of DAB accumulation is considered as directly proportional to the amount of H_2O_2 accumulation. The quantified H_2O_2 levels were represented in the graph as µmol/gFW

2.28 Antioxidant enzymatic assays

SOD activity assay was performed according to the protocol developed by Beyer and Fridovich (1987) with minor modifications. The reaction mixture for SOD activity contained 0.66 mM Na_2EDTA, 10 mM L-methionine, 33 µM NBT and 0.0033 mM riboflavin and 100 µg of protein in 50 mM sodium phosphate buffer (pH7.8). The final reaction mixture was kept in light for 15 min. Later, the blue color intensity was measured at 560 nm. One unit of SOD activity is defined as the amount of protein required to inhibit the photo-reduction of NBT by 50 %. The final activity for the all samples were represented in units/mg protein/gFW.

CAT activity was measured using the protocol developed by Çelik and Atak (2012) with minor modifications. The CAT enzymatic reaction mixture included 25 µg of protein, 50 mM phosphate buffer (pH 7.0) and 19.8 mM H_2O_2. The decrease in absorbance for H_2O_2 was taken at 240 nm. Units of activity were calculated using the H_2O_2 molar extinction coefficient (43.6 $M^{-1} cm^{-1}$) and the final activities were expressed as units/mg protein/min.

CHAPTER 3

Heterologous Expression Of *Osann5* In Tobacco And *E.Coli* To Assess Abiotic Stress Tolerance

3.1 Materials and Methods

3.1.1 *In silico* analysis of *OsAnn5*

Ten annexin CDS sequences were obtained from Rice Genome Annotation Project database (RGAP) (http://rice.plantbiology.msu.edu/) using the functional term search ‘annexin’. Out of these, Os08g32970 was selected and when this sequence was subjected to BLAST analysis, it showed high similarity to the *Ann5* of other plants species. Hence, it was named as *OsAnn5*. A 1.5 Kb upstream region of the Os08g32970 sequence was retrieved from RGAP. The putative promoter sequence was analyzed through the online promoter analysis tool PLACE (http://www.dna.affrc.go.jp/PLACE/). The downloaded protein sequence was analyzed for post-translational modifications using various bio-informatics tools (**Table 3.1**).

Table 3.1. Various bio-informatics tools used for the analysis of the OsANN5 sequence.

S.No	Web site	Functional analysis
1.	http://www.cbs.dtu.dk/services/NetPhos/	Phosphorylation prediction
2.	http://bdmpail.biocuckoo.org	Acetylation site
3.	http://sumosp.biocuckoo.org/online.php	Sumoylation
4.	http://bdmpub.biocuckoo.org/	Ubiquitination prediction
5.	http://crdd.osdd.net/raghava/glycoep/index.html	Glycosylation sites prediction
6.	https://string-db.org/version 10.5	Interacting partners

3.1.2 Isolation and amplification of *OsAnn5*:

Two weeks old *Oryza sativa* var *japonica* cv. Nipponbare (kindly provided by Dr. M. Seshu Madhav, Indian Rice Research Institute, Hyderabad) seedlings were subjected to 0.2 M NaCl stress for 24 h and total RNA was isolated by RNAiso plus (*Takara*, China). Subsequently, cDNA was synthesized using M-MLV Reverse Transcriptase (Sigma, USA). Full-length *OsAnn5* CDS was amplified by PCR using primers *OsAnn5* FP, *OsAnn5* RP (**Table 3.2**) with the cDNA as the template. The amplicon was cloned into pTZ57R/T vector (Thermo Fischer Scientific Inc., USA) for the sequence confirmation. The sequence confirmed *OsAnn5* was cloned into pRT100a vector. The *OsAnn5* expression cassette was released from pRT100a and cloned into pCAMBIA2300 binary vector with the help of *HindIII*.

Table 3.2 Primers used for amplification of the *OsAnn5* CDS

Primer	Primer sequence
OsAnn5 FP	5'TAT GGT ACCATG GCG AGC CTG AGC GT'3
OsAnn5 RP	5'ATT GGA TCCTTA GCG GTC GCG GCC'3

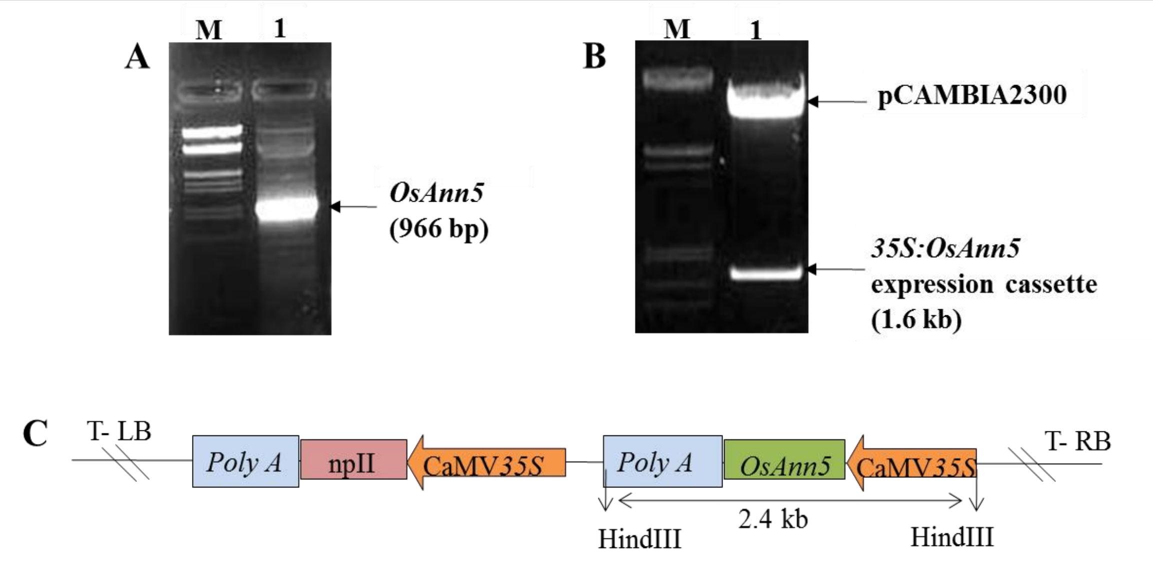


Fig. 3.1.1 Amplification of *OsAnn5* **(A)** and its cloning confirmation from pCAMBIA2300 vector **(B)**. Pictorial representation of T-DAN region of the pCAMBIA2300 having 35S:*OsAnn5* expression cassette **(C)**

3.1.3 Confirmation of 35S:*OsAnn5* tobacco transgenic plants

The putative primary transformants were confirmed by PCR for the presence of *OsAnn5* using specific primers for *OsAnn5* and *nptII* genes. A total of 15 independent 35S:*OsAnn5* transgenics plants were developed. The PCR positive primary transgenic plants were analyzed for low and high expression using a semi-quantitative PCR. The seeds from putative primary transgenic plants were germinated on a half strength MS medium supplemented with 135 mg/l kanamycin to raise the T_1 progeny and to identify the null segregants. The null segregants (NS) were later rescued on MS media without the selection agent and grown to maturity to use as controls in the stress experiments. The homozygous lines were obtained by germinating the self-pollinated seed of T_1 plants on antibiotic selection and identification of 100% germination of the progeny seedlings on the kanamycin selection medium. All the stress assays were conducted with either T_3 seeds or plants.

3.1.4 pEGAD:*OsAnn5* construct preparation:

The *OsAnn5* was amplified using the primers listed out in the **Table 3.2** and cloned into pEGAD vector using EcorI and HindIII restriction enzymes. The *OsAnn5* was translationally fused with Egfp at its c-terminal.

Table 3.2 Primers used for the amplification of *OsAnn5* to clone into pEGAD vector

Primer	Primer sequence
OsAnn5 FP	5'TAT GAA TTC ATG GCG AGC CTG AGC GT'3
OsAnn5 RP	5' GTA AGC TTT TAG CGG TCG CGG C '3

3.1.5 Quantitative Real-time PCR:

To check the role of *OsAnn5* in ABA signaling pathway, 15 d old *Oryza sativa* var *japonica* cv. Nipponbare seedlings were subjected to different treatments viz, 100 µM ABA, 200 mM NaCl, 100 µM fluridone (ABA synthesis inhibitor) and 200 mM NaCl+100 µM fluridone. The samples were collected at different time points i.e. 6, 12, 24 h. Before treating the rice seedlings with 200 mM salt + fluridone, they were pre-treated with 100 µM fluridone for 12 h. After the treatment, all the samples were subjected to RNA isolation followed by cDNA synthesis. Subsequently, real-time PCR was performed to analyze the gene expression levels.

Quantitative real-time PCR was performed with the Bio SYBR Premix Ex Taq (Takara, China) according to the manufacturer's protocol. 100 ng of cDNA was used as a template for the reaction. The reaction was set up in Axygen 96-well PCR microplates with the following reaction conditions: initial denaturation at 95°C for 30 sec, 40 cycles of 95°C for 3 sec, 58°C for 30 sec. Raw data was analyzed through the $\Delta\Delta C_T$ method (Livak et al., 2001). Sigma Plot 11 was used for the representation of the data.

3.2. Results

3.2.1 *In silico* analysis of putative promoter and protein sequences of *OsAnn5* reveals the presence of potential *cis*-elements and functional motifs.

In Silico analysis of the promoter sequence showed the presence of various putative *cis*-acting elements known to be involved in abiotic stress responses like dehydration, low temperature and salt stresses. Important motifs for hormonal response for auxin, gibberellic acid, ABA, ethylene and cytokinin, and light response were observed in the amino acid sequences. Tissue-specific elements for mesophyll, pollen, root hair-specific expression were also observed in the putative promoter region. The binding sites for various transcription factors such as MYB, MYC, WRKY and DOF were recorded. Several light responsive elements were also found in it (**Table 3.3**). This data suggests that OsANN5 is highly regulated during growth and plays a major role during stress responses. This also indicates that *OsAnn5* expression is regulated during different environmental and abiotic stress conditions and is consistent with the *cis*-acting elements predicted in its putative promoter sequence

Table 3.3 A list of different cis-elements and their probable functions identified for the putative promoter region of the *OsAnn5.*

S.No	Cis-elements Name	Sequence	No	Function
Cis-elements involves in Abiotic stress				
1	DRECRTCOREAT	RCCGAC	2	Dehydration responsive elements
2	ACGTATERD1	ACGT	18	
3	CBFHV	RYCGAC	2	
4	LTRECOREATCOR15	CCGAC	3	Low temperature, Drought response elements
5	CCAATBOX	CCAAT	1	Responds to Heat
6	GT1GMSCAM4	GAAAAA	2	Salt inducible
7	SURECOREATSULTR11	GAGAC	2	Sulfur responsive elements
8	CURECORECR	GTAC	4	Copper response elements
9	ARE1	RGTGACNNNGC	1	Antioxidant response element
10	CGCGBOXAT	VCGCGB	20	Ca^{2+}/Calmodulin binding
11	WBOXATNPR1	TTGAC	1	WRKY transcription factor binding site
12	WBOXHVISO1	TGACT	3	
13	WBOXNTERF3	TGACY	4	
14	WRKY71OS	TGAC	15	
	Hormone response			

16	ASF1MOTIFCAMV	TGACG	7	Auxin response elements
17	AUXRETGA2GMGH3	TGACGTGGC	2	
18	SEBFCONSSTPR10A	YTGTCWC	1	Similar to auxin response elements
19	ABREA2HVA1	CCTACGTGG C	1	ABA response elements
20	ABREATCONSENSUS	YACGTGGC	1	
21	ACGTABREMOTIFA2OSEM	ACGTGKC	3	
22	ABRELATERD1	ACGTG	7	
23	ABRERATCAL	MACGYGB	1	
24	GARE1OSREP1	TAACAGA	1	Gibberellin response elements
25	TATCCACHVAL21	TATCCAC	1	
26	ARR1AT	NGATT	10	Induced by cytokine response genes
27	GCCCORE	GCCGCC	6	Ethylene responsive elements
	Transcription factor binding sites			
28	MYBST1	GGATA	4	MYB binding elements
29	MYBCOREATCYCB1	AACGG	1	
30	MYBCORE	CNGTTR	6	
31	TATCCAOSAMY	TATCCA	1	
32	MYB1AT	WAACCA	2	
33	MYB2CONSENSUSAT	YAACKG	1	
34	MYCATERD1	CATGTG	1	MYC binding elements
35	MYCCONSENSUSAT	CANNTG	12	
36	MYCATRD22	CACATG	1	
37	DOFCOREZM	AAAG	7	Dof transcription factor binding sites
	Tissue specific			
39	CACTFTPPCA1	YACT	12	Mesophyll cells specific expression
40	POLLEN1LELAT52	AGAAA	3	Pollen specific expression
41	DPBCOREDCDC3	ACACNNG	2	Embryo specific and induced by ABA
42	OSE1ROOTNODULE	AAAGAT	2	Organ specific elements usually present in the promoters of infected root nodules
43	OSE2ROOTNODULE	CTCTT	5	
44	RHERPATEXPA7	KCACGW	4	Root hair specific elements
45	ROOTMOTIFTAPOX1	ATATT	1	Root specific elements
46	RAV1AAT	CAACA	3	
47	IBOXCORE	GATAA	11	Light responsive elements
48	SORLIP2AT	GGGCC	7	
49	GT1CONSENSUS	GRWAAW	5	
50	ASF1MOTIFCAMV	TGACG	7	
51	SORLIP1AT	GCCAC	13	

Post translational modification (PTM) is another kind of regulatory mechanism, which expands the functional range of the proteins. Various bioinformatics tools were used to identify the possible PTMs sites in OsANN5 protein. This analysis resulted in the identification of some potential phosphorylation sites and disordered regions in the protein. Three disordered regions were identified in OsANN5, which spanned the amino acids at the positions, 2 – 15, 128 – 140, 314 – 319. Probable phosphorylated amino acids and the related kinases were identified as 44 S-Unspecified (Unsp) and PKC, 146 S- Unsp, PKA, 200 S- Unsp, 275 T- Unsp, PKC, 296 S- Unsp. Only those having prediction score of ≥ 0.9 were chosen for presentation. Several Lysine residues in the protein were possible residues that can be ubiquitinated and were also predicted to have equal probability of being acetylated, viz., K 25, K 74, K 227, K 250, K 254, K 261, K 294. We observed one potential site for N-Glycosylation with highest score at N 266. However, no sites for O-Glycosylation were observed in the amino acid sequence. Two consensus sequences for SUMOylation (K 227, K 286) and one SUMO interaction site (IRVVTT) were also identified. One calcium binding motif (GXGT(38 residues)D/E) was identified in the protein. Interestingly, a stretch of six glycine residues (ARF**GGGGGGG**LEH) was observed in the protein sequence, which is not found in other rice annexins. As in other annexins, a conserved histidine residue at the 40^{th} position and a motif for the salt bridge (Laohavisit et al., 2011), F-actin binding site have also been observed in the amino acid sequence (**Fig. 3.2**). Possible interacting partners for OsANN5 were identified as a hypothetical protein (LOC_Os06g34710.1) and a MYB family transcription factor (LOC_Os02g34630.1).

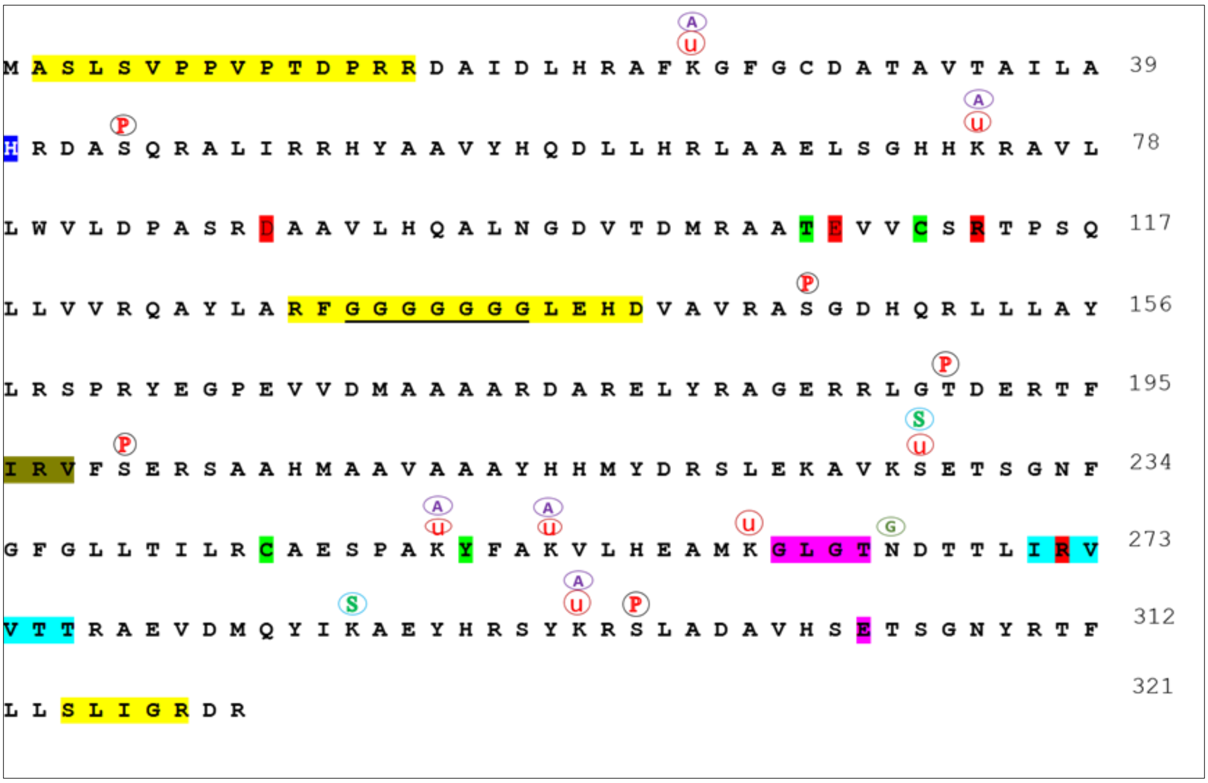


Fig. 3.2 Mapping of putative Phosphorylation (P), N-Glycosylation (G), Sumoylation (S), Ubiquitination (U) and acetylation sites (A) in OsANN5 sequence. Residues involved in salt bridges are in red, conserved histidine residue is highlighted in blue and disordered regions are highlighted in yellow, sumo interaction site is indicated in teal color. Underlined region is the glycine stretch, neon green highlighted amino acid residues are of S3 cluster, olive green highlighted region is the F-actin binding motif, pink highlighted region is the calcium binding motif (Endonexin fold).

3.2.2 OsANN5 overexpression helps *E. coli* cells to combat abiotic stress conditions.

To check whether the overexpression of OsANN5 improves abiotic stress tolerance in *E. coli,* 10 µl of different dilutions of both pET32a transformed and pET32a:OsANN5 transformed BL21 pLysS cultures were spotted on Luria Agar media, which induce high salinity, osmotic, drought stresses along with the ampicillin selection. Their growth performance was observed after 16 h of incubation at 37 °C. Under all the abiotic stress conditions, *E. coli* cells overexpressing OsANN5 were grown well compared to the cells with vector transformation. High salt and heat stress shown major effect on the growth of the cells. Under the salt stress the OsANN5 overexpressing *E.coli* cells took less time to adopt and grow. Similar tolerance was observed to the heat treatment (**Fig. 3.3**). After one hour heat stress (50 °C) treatment, vector cells did not recovered, but OsANN5 overexpressing cells were able to resume normal growth. This clearly suggests that the expression of OsANN5 helps the bacterial cells in combat the the abiotic stresses, especially to the salt and heat stresses.

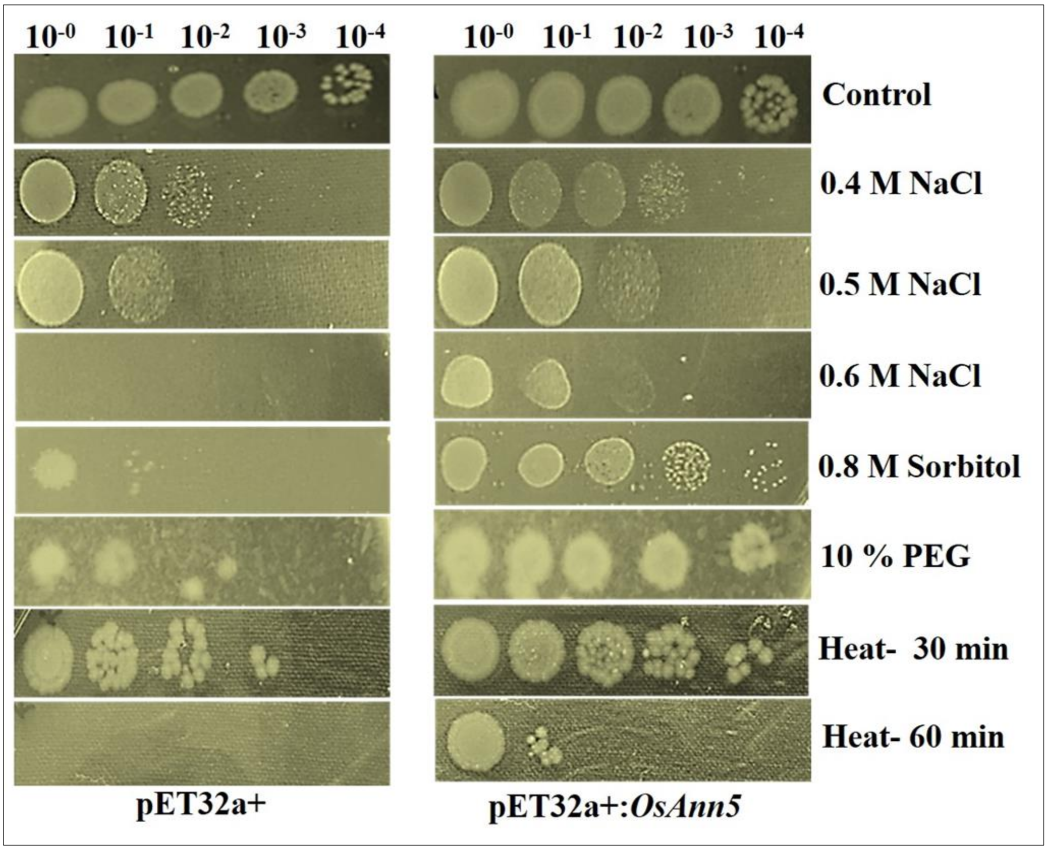


Fig. 3.3 The pET32a+ and pET32a+:*OsAnn5* transformed culture were induced with 0.1 mM IPTG for 3 h at 28 °C. A 10 µl sample from different dilutions (10^{-0} , 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) of the both induced cultures were spotted on different LA plates (Ampicillin + IPTG) which contains 400 mM NaCl, 500 mM NaCl, 600 mM NaCl, 0.8 M sorbitol, 10 % PEG to induce the abiotic stress. The heat stress (50° C for 30 min and 1 h) given cultures were spotted on normal LA medium (100 mg/L Ampicillin + 0.1 mM IPTG). The spotted plates were incubated at 37 °C for minimum of 12-16 h and their growth was observed.

3.2.3 OsANN5 shows Calcium binding activity

Most of the annexins are Ca^{+2} binding proteins but, not all of them (Dabitz et al., 2005). To check the OsANN5 calcium binding activity an assay was performed as described in the material and methods. The overexpressed Trx tag fused OsANN5 (50

KDa) was found in the $CaCl_2$ mediated precipitated proteins. As the concentration of the $CaCl_2$ was increased from the 10 mM to 20 mM the amount of the OsANN5 precipitation was increased and less OsANN5 was observed in the supernatant of 20 mM $CaCl_2$ treated samples compared to 10 mM $CaCl_2$ treated sample. 20 mM and 30 mM $CaCl_2$ treatment lead to the same amount of OsANN5 precipitation. These results suggest that OsANN5 is a calcium binding protein (**Fig. 3.4 A**).

A Fluorescence spectroscopy study was also conducted to assess the calcium binding activity of the OsANN5. Fluorescence emission for purified OsANN5 was detected at 320 nm wavelength. Upon addition of 2 mM $CaCl_2$, a decrease in the fluorescence peak intensity was observed (**Fig. 3.4 B**), suggesting that OsANN5 binds to Ca^{+2} ions, which led to fluorescence quenching and ultimately resulted in a shift in the fluorescence emission intensity. Insignificant or very less fluorescence emission was observed when an uncleaved OsANN5 (Trx:OsANN5) was used for the assay (data not shown).

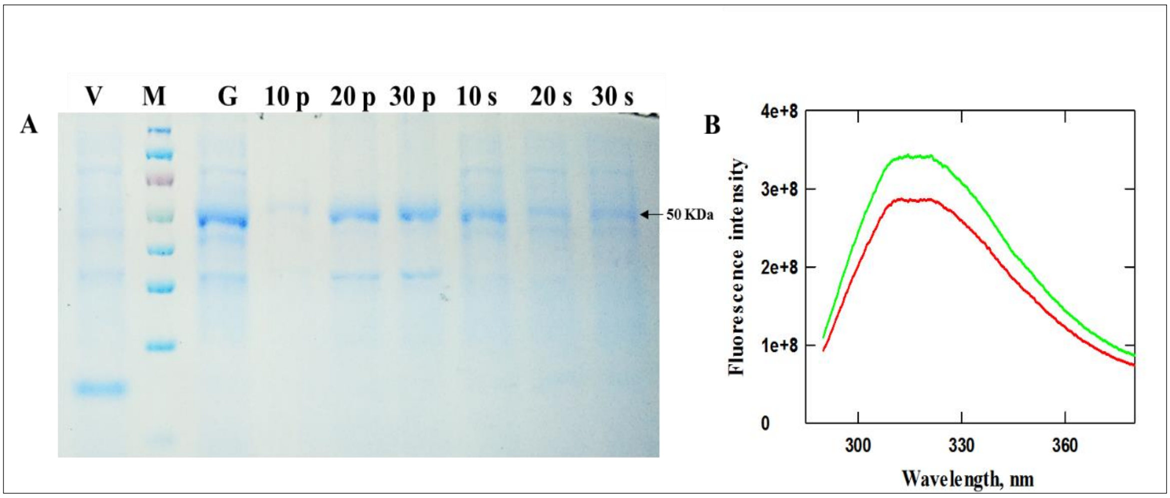


Fig. 3.4 Ca^{+2} binding activity assay for OsANN5 through $CaCl_2$ precipitation and fluorescence spectroscopy method. The IPTG induced pET32a+:*OsAnn5* transformed cell lysate was treated with different concentration of $CaCl_2$ **i.e** 10 mM, 20 mM, 30 mM **(A).** This led to the precipitation of the calcium bound proteins (10p, 20p, 30p are precipitate proteins after 10mM, 20 mM, 30 mM $CaCl_2$ treatment respectively) . After centrifugation the precipitated proteins were resuspended in the lysis buffer having the Na_2EDTA and the supernatant having Ca^{+2} unbound proteins (10s, 20s, 30s are Ca^{+2} unbound proteins after 10mM, 20 mM, 30 mM $CaCl_2$ treatment respectively) were loaded on the SDS-PAGE. V= pET32a+ transformed cell lysate, M=Marker, G= OsANN5 overexpressing cell lysate.

Fluorescence emission spectra for OsANN5 with (Red) and without addition of 2 mM $CaCl_2$ (green) **(B)**.

3.2.4 OsANN5 localizes in the peripheral regions.

Transient expression of OsANN5 fused EGFP was carried out in onion epidermal peels to detect its sub-cellular localization. We observed that the fluorescence signals were localized in the corners of the cells under normal condition. But, upon 200 mM NaCl treatment for 10 min, these signals were observed in the peripheral regions of the cells (**Fig. 3.5**). In the vector transformed cells, the signal is diffused throughout the cell including nucleus.

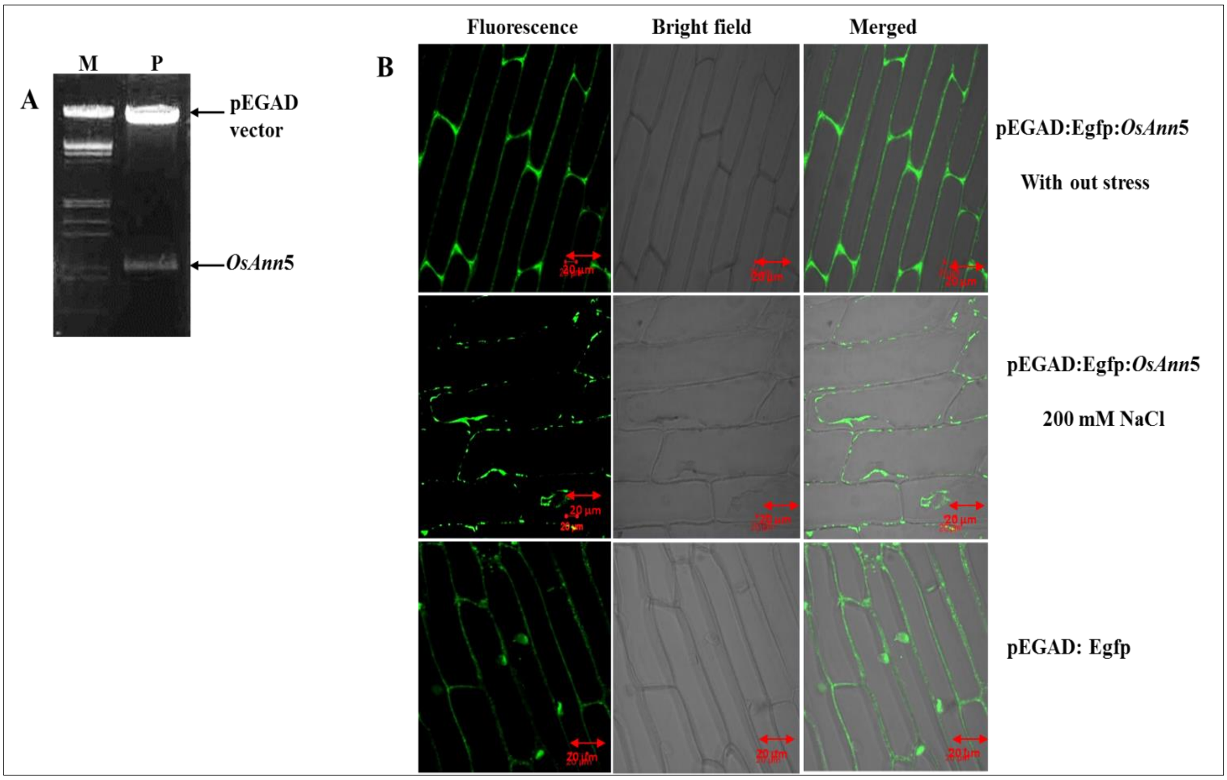


Fig. 3.5 Cloning confirmation of *OsAnn5* from pEGAD vector (**A**)**.** Subcellular localization studies on OsANN5 (**B**). Transient expression of EGFP fused *OsANN5* was done in onion epidermal peels. The fluorescence for Egfp fused *OsAnn5* transformed onion epidermal peels was observed in the corners of the cells. After NaCl treatment the fluorescence was observed in the peripheral region of the cell. In the vector (pEGAD::EGFP) transformed onion epidermal peels, the fluorescence was throughout the cell. Confocal images were taken with the 20X magnification with scale bar of 20 μm.

3.2.5 Confirmation of 35S:OsAnn5 transgenics:

The putative *35S:OsAnn5* tobacco transgenic plants were screened for the presence of *OsAnn5* and *nptII* in their genomic DNA. The confirmed transgenic plants were screened further for low and high expression of the target gene through Semi quantitative PCR analysis using ubiquitin as a reference gene. Among them 35S*:OsAnn5* line I- 2 (A-2) was selected as low expression, while 35S:*OsAnn5* line-A4 (A-4) and 35S:*OsAnn5* line -A7 (A-7) were used as high expression lines for the stress assays (**Fig.3.6**). Various abiotic stress assays were carried out to assess the stress tolerance property of T_3 35S:*OsAnn5* tobacco transgenics.

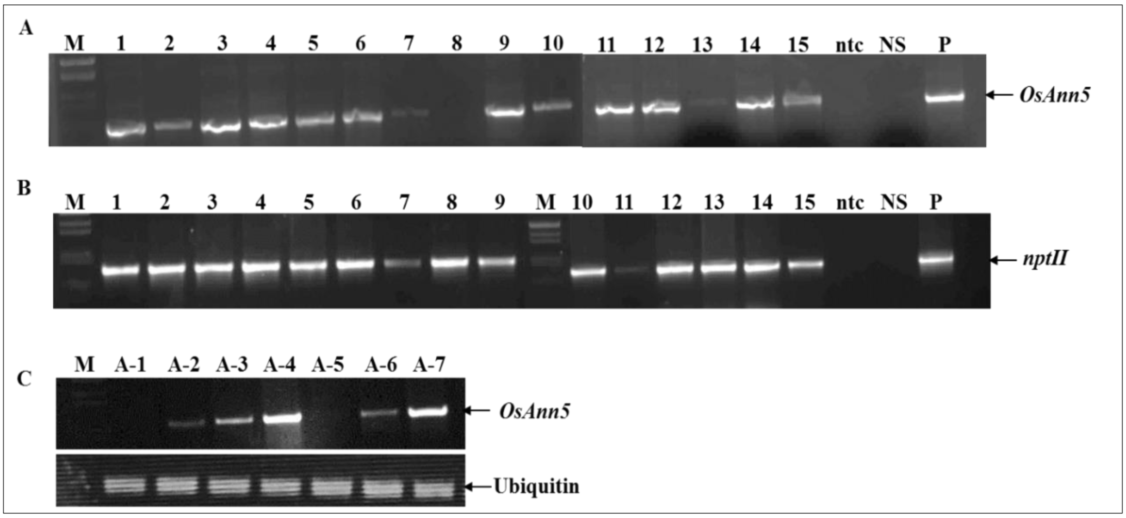


Fig.3.6 PCR confirmation for the 35S:*OsAnn5* transgenics using their genomic DNA for the presence of 966 bp *OsAnn5* (**A**) and 720 bp *nptII* (**B**). Semi-quantitative PCR for the identification of low and high expression lines (**C**) along with the ubiquitin as a reference gene.

3.2.6. Constitutively overexpressing 35S:*OsAnn5* tobacco transgenic shown tolerance to abiotic stress treatments

To assess the abiotic stress tolerance of 35S:*OsAnn5* transgenic plants, various experiments like seed germination percentage, seedling assay and leaf disc assay were performed. Seed germination is an important factor affected by the abiotic stresses. This assay revealed that NS seeds took 10 d for 50 % of germination while the 35S:OsAnn5 transgenic plants showed 50% germination by the 8^{th} day. Without any stress treatment, under control conditions all the seeds germinated by the 3^{rd} day (**Fig.3.7**).

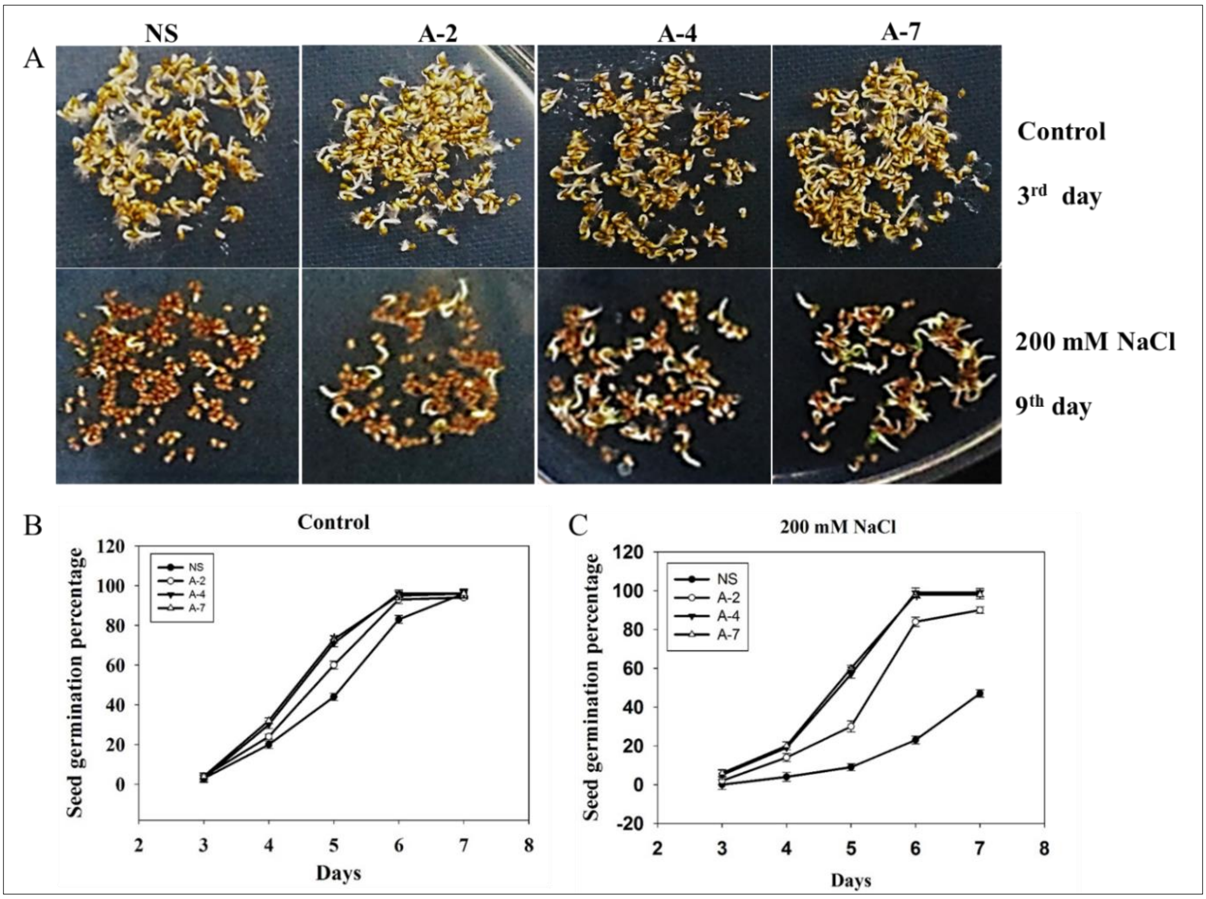


Fig. 3.7 Seed germination assay on null segregants (NS) and 35S:*OsAnn5* transgenic plants. Approximately 100 seeds of NS and transgenics were placed on the ½ MS as a control and on ½ MS+200 mM NaCl media. The germinated seeds were counted on daily basis **(A).** Germination percentage under control **(B)** and NaCl **(C)** conditions were calculated and plotted in a graph. Asterisc represents the significant difference in the germination percentage in comparison with the NS when analyzed through ANOVA. $^*P < 0.05$

For the seedling assay, NS and 35S:*OsAnn5* T_3 transgenic seeds were germinated on ½ MS and ½ MS with kanamycin (125 mg/L) selection media respectively. After germination the 10 d old seedlings were subjected to abiotic stress treatments like high salinity (0.2 M NaCl), drought (10% PEG) and osmotic stresses (0.3 M Sorbitol) and the seedling responses were assessed for their stress tolerance. Compare to NS seedlings, the 35S:*OsAnn5* transgenic seedlings were able to tolerate the given stresses and show significant growth difference in all the treatments. A two fold reduction in the root length was observed for NS under all abiotic stresses while the 35S:*OsAnn5* transgenic seedlings did not shown any significant reduction in their root length (**Fig. 3.8**).

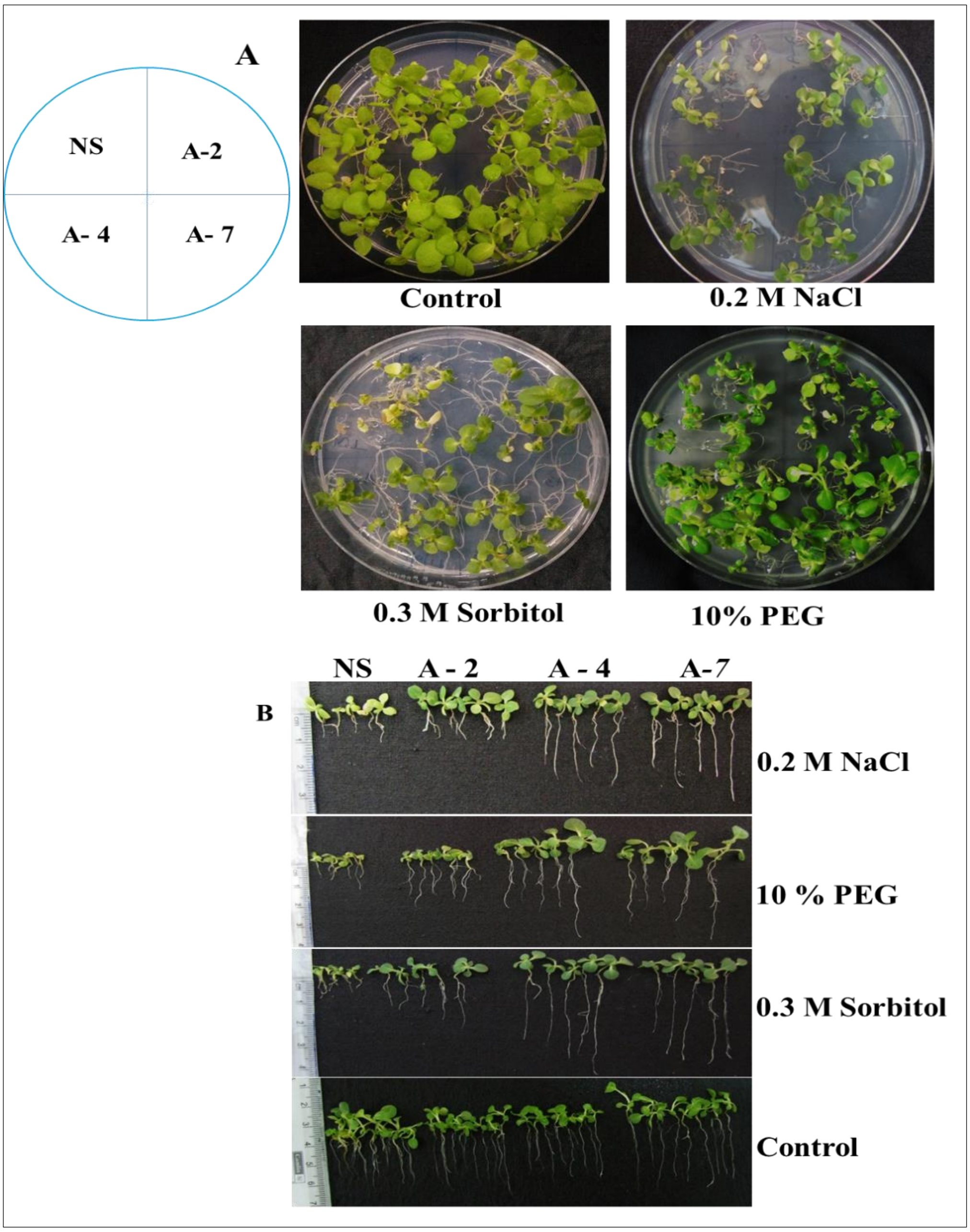


Fig. 3.8 Seedling assay on 10 days old NS and 35S:*OsAnn5* tobacco transgenics. The seedlings were grown on MS media supplemented with 200 mM NaCl, 10 % PEG, 0.8 M sorbitol and on MS media as a control. The difference in the root lengths after the treatment were compared and measured using the scale **(A)**

To check the extent of tolerance to the various abiotic stresses at mature plant stage, a leaf disc assay was performed for all transgenics and NS. The leaf discs were subjected to similar stress as in the seedling assay and their phenotype was assessed (**Fig. 3.9A**). Subsequently, the samples were analyzed for their total chlorophyll content, proline content and the extent of lipid peroxidation under respective treatments.

The NS showed up to 60% reduction in the chlorophyll content under all the stress conditions whereas *35S*:*OsAnn5* transgenic plants showed only 15 - 30% reduction (**Fig. 3.9B**). Proline accumulates in the plants during stress conditions and acts as a compatible solute, which in turns helps in protecting enzymes and proteins during stress (Szabados et al., 2010). The accumulation of free proline by the NS was one fold higher under stress as compared to the control condition. The transgenic tobacco plants accumulated significantly higher amount of free proline under the same stress conditions (**Fig.3.9D**).

The stress on the plant also intensifies lipid peroxidation, which affects the membrane integrity and hence, the viability of cell. MDA is the end product of lipid peroxidation process that represents the oxidation status of the cells during stress conditions. Significantly less MDA levels were present in the *OsAnn5* transgenic plants in comparison with their corresponding NS (**Fig. 3.9C**).

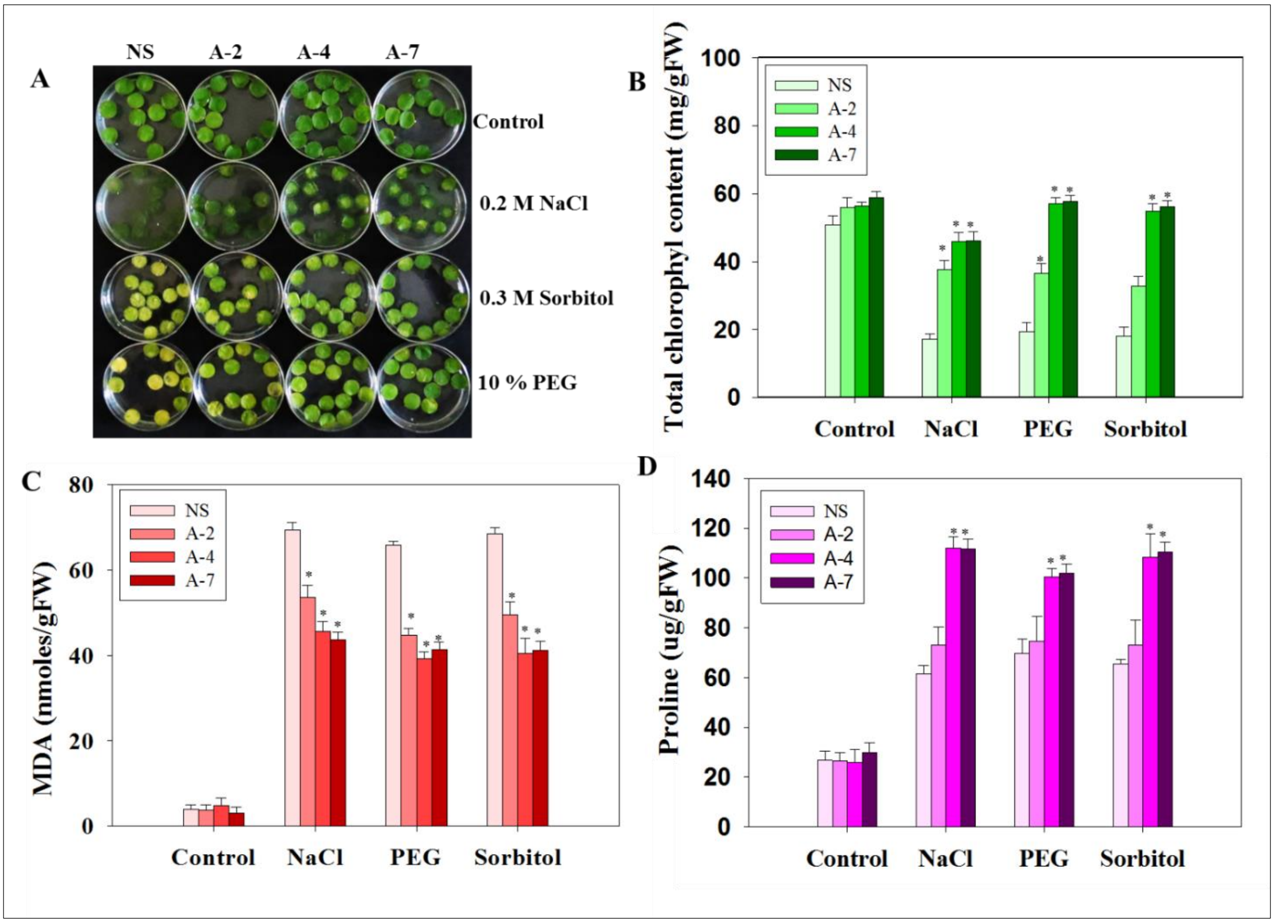


Fig. 3.9 Leaf disc assay for null Segregant and 35S:*OsAnn5* transgenic lines under control and different abiotic stress (200 mM NaCl, 0.3 M Sorbitol, 10% PEG) conditions (A). After 7 d of the treatment the leaf disc samples were analyzed for total chlorophyll content (B), MDA levels (C), proline accumulation (D). Error bars represent the ± SD. Asterisks represent the significant difference in either fold level or percentage level in comparison with stress treated null segregants under respective stress conditions (NS) analyzed through ANOVA. $^{*}P< 0.05$; $^{**}P< 0.01$.

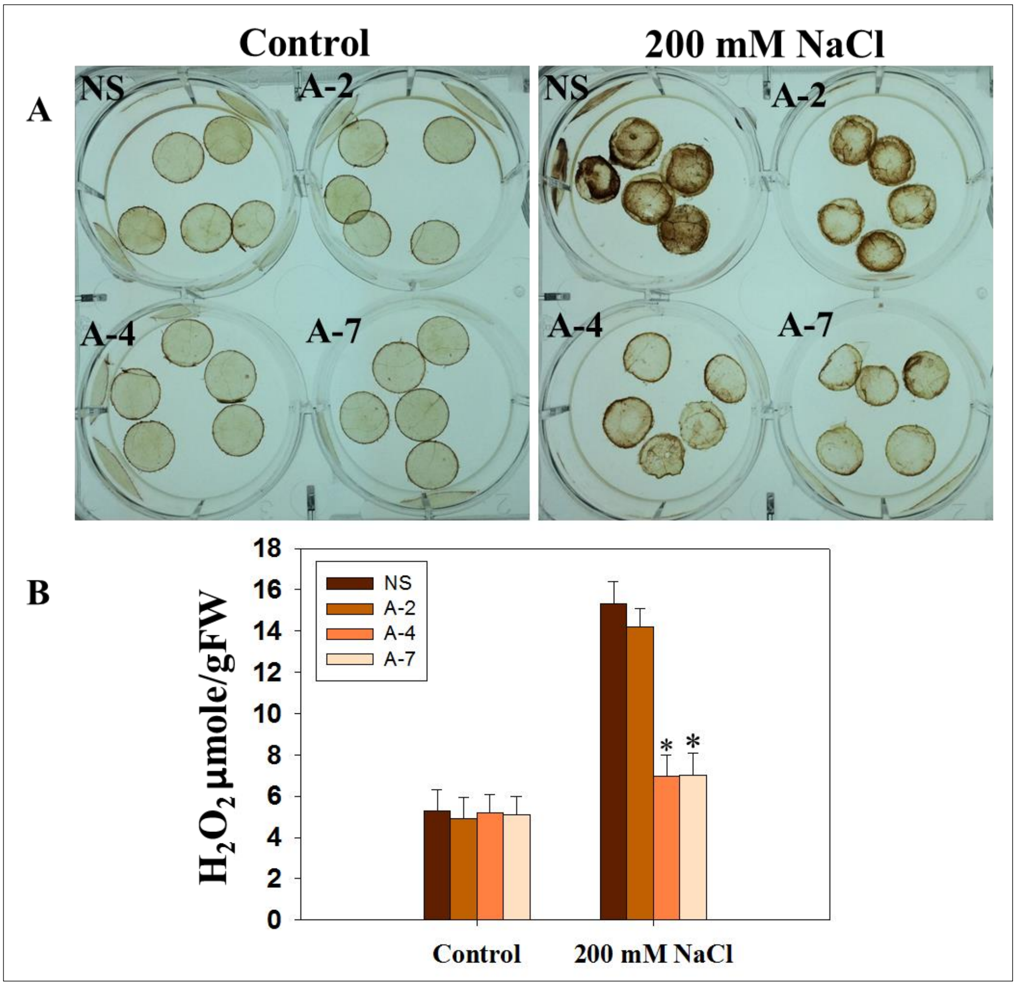


Fig. 3.10 DAB staining assay was performed for the NS and 35S:*OsAnn5* transgenic lines. The brown coloration was directly proportional to the levels of H_2O_2 accumulation. The stress led to more intense staining of the NS leaf disc than transgenic lines (**A**). The DAB stained samples were homogenized in perchloric acid and after centrifugation the absorbance values for the supernatant were represented in the graph (**B**). Error bars represent the ± SD. Asterisks represent the significant difference in either fold level or percentage level in comparison with stress treated null segregants (NS) analyzed through ANOVA. $^{*}P< 0.05$.

The DAB staining assay was performed to observe the extent of H_2O_2 accumulation under high salt stress. The NS and 35S:*OsAnn5* transgenics leaf discs were subjected to 0.2 M NaCl stress for a week and followed by DAB staining. We observed that salt stress treatment lead to strong and intense staining of NS leaf discs when compared to the transgenic plants (**Fig. 3.10 A**). DAB quantification results suggested that *OsAnn5* transgenics shown significantly less H_2O_2 accumulation under the salt stress compared to NS (**Fig. 3.10 B**).

3.2.7 OsANN5 induced Salinity stress tolerance is associated with altered SOD and catalase activity in recombinant *E.coli* and transgenic tobacco

Anti-oxidant enzymatic assay in *E. coli* revealed that the SOD activity was increased in the induced cultures of vector and *OsAnn5* transformed cells (**Fig. 3.11A**). However, heat and high salt treatment lead to decreased SOD activity in the same cultures. But, there was a further decrease in SOD activity in induced *OsAnn5* transformed cells as compared to induced vector transformed cells under stress treatments. In contrast to SOD activity, heat and high salt treatment lead to significantly increased CAT activity levels in the induced *OsAnn5* transformed cells in comparison to induced vector transformed cultures (**Fig. 3.11B**). Un-induced cultures were used as controls.

Interestingly the 35S:*OsAnn5* tobacco transgenics also showed significant reduction in SOD activity **(Fig. 11C)** and increased CAT enzymatic activity **(Fig. 11D)** under the high salt stress compared to the control samples. The above results suggests that OsANN5 mediated oxidative stress tolerance is through the regulation of antioxidant enzymes.

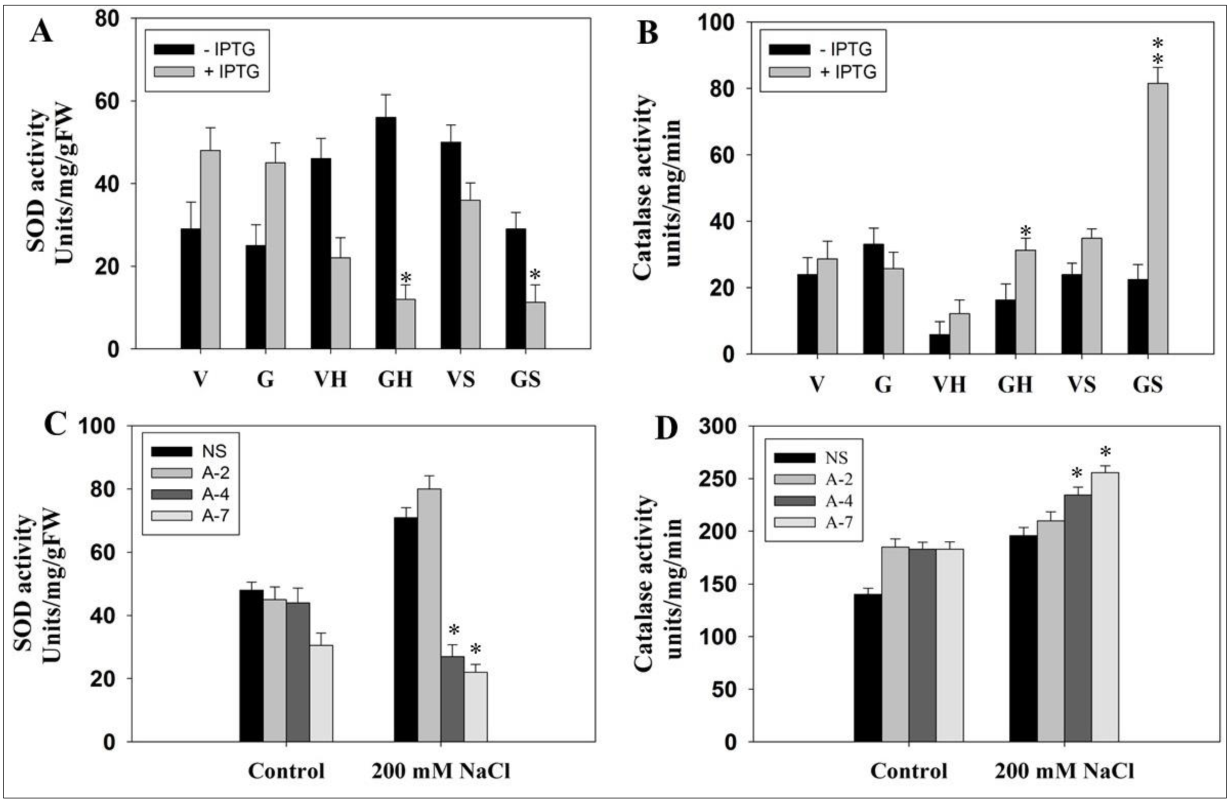


Fig. 3.11 Quantification of anti-oxidant enzyme activity levels in the control and stress treated samples of *E.coli* and transgenics tobacco. In *E.coli* cells, SOD activity **(A)** and CAT activity **(B)** were determined for the pET32a vector (V) and pET32a:*OsAnn5* (G) transformed cells with IPTG (+ IPTG) or without IPTG (- IPTG) induction under heat stress (VH or GH= Heat stress given V or G samples) and high salinity stress (VS or GS = salt stress given V or G sample). In tobacco the SOD activity **(C)** and CAT activity **(D)** levels were measured for null segregant and 35S:*OsAnn5* transgenics samples under control and 200 mM NaCl treatments. Error bars represent the ± SD. Asterisks represent the significant difference in the activity levels of SOD/ CAT in comparison of VH with GH and VS with VH samples in the panel A and B, in comparison to the salt stress treated NS samples of tobacco in panel C & D when analyzed through ANOVA.**P*< 0.05,***P*< 0.01.

3.2.8 ABA-independent expression of *OsAnn5* under salt stress

To check whether the *OsAnn5* expression is independent of or dependent on ABA under salt stress, initially 10 d rice seedlings were treated with ABA and the samples were collected at different points. The samples were analyzed for the *OsAnn5* transcript levels through semi-quantitative PCR (**Fig. 3. 12**). Initially the transcript were reduced at 4 h of the treatment and then increased by 6 h treatment followed by decreasing after 24 h of treatment.

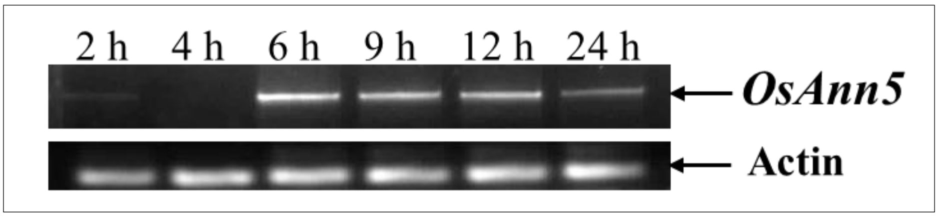


Fig. 3. 12. A semi-quantitative PCR for the ABA (100 µM) treated 10 d rice seed lings with reference to the actin gene expression. The samples were collected at different time points during the treatment (2 h, 4 h, 6 h, 9 h, 12 h, 24 h).

After the semi-quantitative PCR, the 10 d rice seedlings were treated with the ABA, salt, and a combination of salt and fluridone and the transcript levels of *OsANN5* were quantified by qRT-PCR. The *OsAnn5* transcript levels were quantified along with the reference genes. It was observed that *OsAnn5* transcripts were doubled after 6 h of ABA treatment suggesting that *OsAnn5* responded to ABA. A significant eight fold increase was observed after 24 h of 200 mM NaCl salt stress treatment. But, the same salt treatment in combination with fluridone showed an approximately seven folds upregulation of *OsAnn5* transcript levels. This suggests that the fluridone treatment did not significantly affect the *osAnn5* expression under the salt stress. When we compared the basal expression of *OsAnn5* under normal and fluridone treatments, no significant change in the expression levels was observed. This suggests that treatment with fluridone did not affect the *OsAnn5* expression *in vivo* (**Fig. 3.13A**).

To check whether fluridone treatment inhibited the synthesis of ABA, we studied the expression levels of some genes whose expression under ABA has been determined earlier. Expression levels of an ABA-dependent gene *OsDREB1F* (Agarwal et al., 2010) was reduced from 112 folds to 22 folds at 24 h in the fluridone + salt stress treatment (**Fig. 3.13B**). There was no significant change in the expression levels of *OsDREB1A* gene, which is known to act through an ABA-independent pathway (Agarwal et al., 2010), (**Fig. 3.13C**). The transcript levels of *OsABA2* (ZEP) gene, which is known to be regulated by both ABA-dependent and independent pathway (Chen et al., 2014), did not change much during the 6 - 12 h of salt treatment. However, there was 1.8 folds decrease in the transcript levels under the combination treatment of fluridone with salt as compared with salt treatment at 24 h (**Fig. 3.13D**). These observations suggest that the expression of *OsAnn5* is independent of ABA during the initial stress treatment, with some dependence on ABA as the stress progressed.

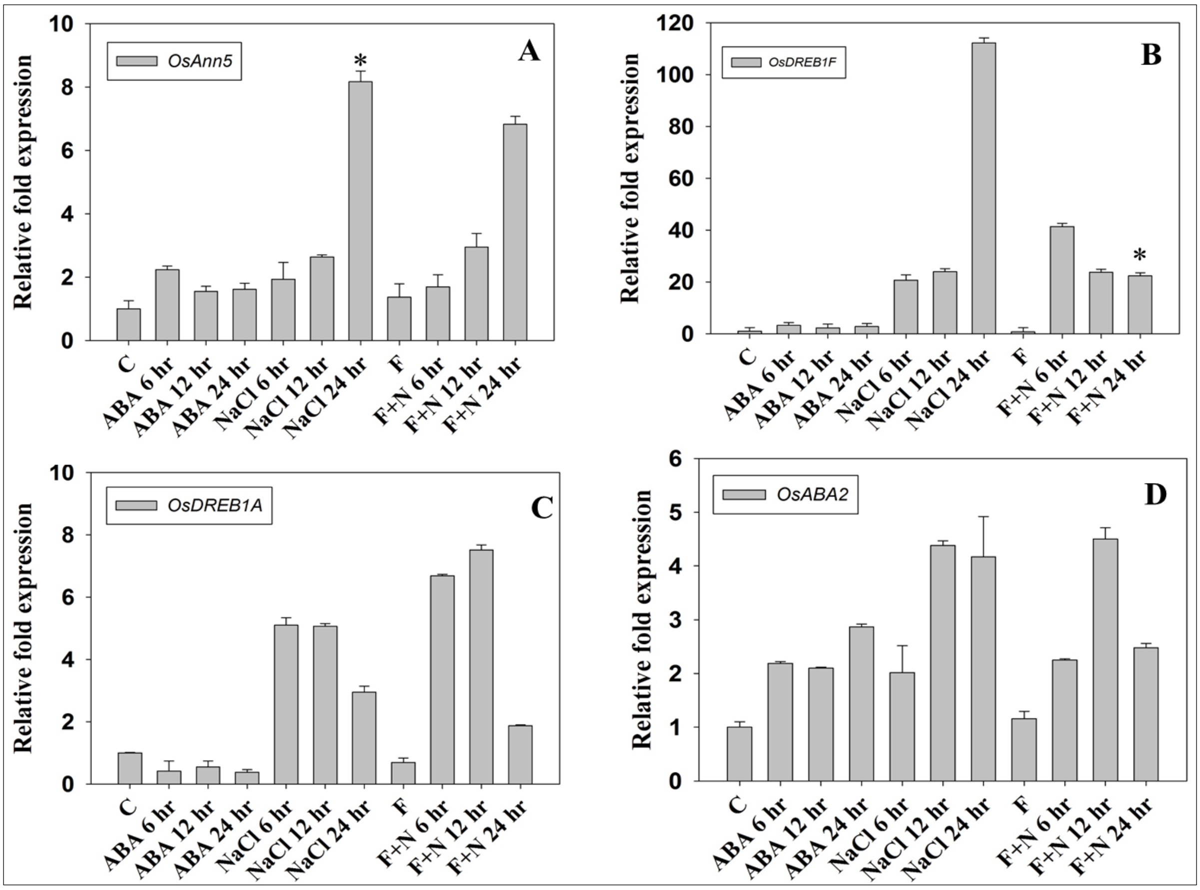


Fig. 3.13. Real-time PCR quantification of *OsAnn5* **(A)**, *OsDREB1F* **(B)**, *OsDREB1A* **(C)**, *OsABA2* (**D**) genes at different time points of the control, ABA (100 µM), NaCl (0.2 M) and fluridone (100 µM) (A) treatments. Error bars represent the ± SD. Asterisks represent the significant difference in either folds level or percentage level in comparison with control samples in panel A and with NaCl 24 h treated samples in panel B when analyzed through ANOVA.$^{*}P< 0.05$.

In continuation with these observations, 10 d old *OsAnn5* transgenic and NS seedlings were grown on MS media supplemented with 4 µM and 8 µM ABA for 15 d. Their phenotype and their root length were observed after two weeks of incubation. Compared to NS, O*sAnn5* transgenic seedlings showed reduced sensitivity to ABA (**Fig. 3.14**).

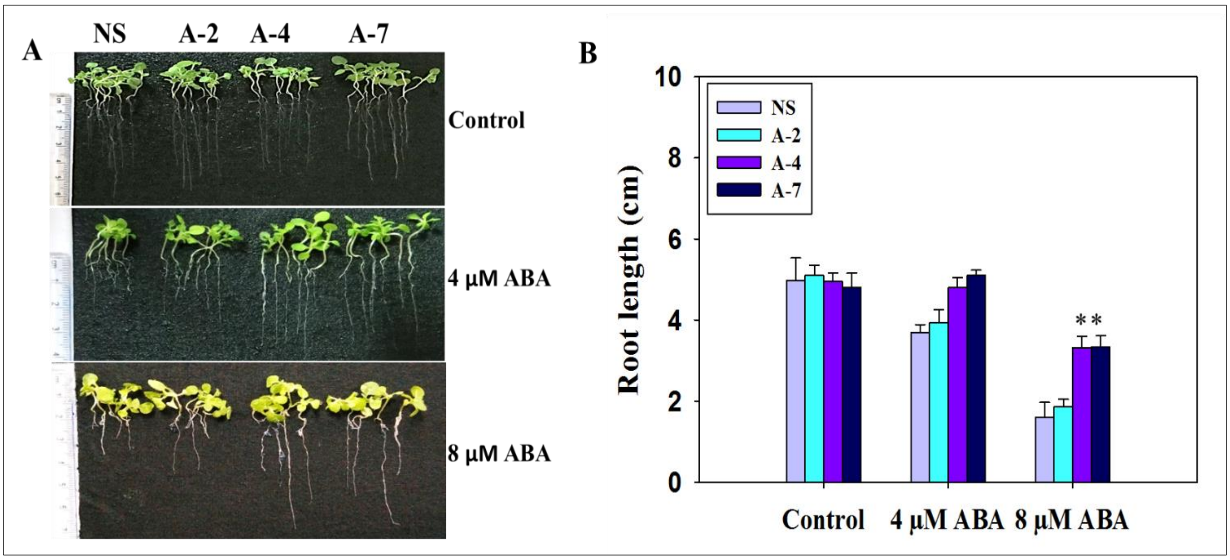


Fig. 3.14. ABA sensitivity assay for control and 35S:*OsAnn5* transgenics at different concentrations of ABA **(A)**. After the treatment, differences in the root length was measured and represented in the graph. Error bars represent the ± SD. Asterisks represent the significant difference in either folds level or percentage level in comparison to null segregants under 8µM ABA treatment when analyzed throuh ANOVA. $^{*}P< 0.05$.

3.3. Discussion

In silico analysis for the upstream region of *OsAnn5* and mapping of important functional sites in OsANN5

A promoter defines when and where the transcription of a gene under its control should occur. Analysis of *cis*-elements in the promoter region generally gives an idea about its functionality that controls corresponding gene action. Various *cis*-elements related to abiotic stress conditions and hormones have been identified in the upstream region of *OsAnn5*. This suggests that these *cis*-elements are responsible for the involvement of OsANN5 in abiotic stresses and physiological responses.

ABRERATCAL is a Ca^{+2} responsive *cis*-element identified in the promoters of the 162 Ca^{+2} responsive genes (Kaplan et al., 2006). It was observed that one motif is enough to induce the overexpression of these genes. We found an ABRERATCAL *cis*-element in the *OsAnn5* putative promoter region. Apart from this a calcium binding motif was identified in the repeat IV of OsANN5 sequence. In support to this, in the calcium binding assays, OsANN5 showed calcium binding activity. Presence of calcium responsive *cis*-elements in the upstream region of *OsAnn5* suggests, Ca^{+2}

ions not only regulates the *OsANN5* function but, also its expression. We also observed the presence of potential N-glycosylation and ubiquitination sites near to the calcium binding motif. This leads to a speculation that the calcium binding activity may be regulated by the PTMs.

The *OsANN5* protein was identified as a probable interacting partner of MYB through the STRING software. In earlier study, Punwani *et al.*, 2007 observed that *Arabidopsis thaliana Annexin8* (*AtANN8*) expression by MYB98 transcription factor in synergid cells. This indicates that a member of the MYB transcription factor family could be a possible upstream regulator of *OsAnn5*, which needs further experimental verification.

The possible occurrence of post-translational modifications in *OsAnn5* can be another level of regulatory mechanism, which can alter protein function by altering cellular and sub-cellular location, protein interactions and the biochemical reaction chains. If a gene is translated into a protein, its functional activity will be majorly directed by the posttranslational modifications. There are few reports on the PTMs of plant annexins (Lindermayr et al., 2005; Gorecka et al., 2005; Konopka-Postupolska et al., 2009). It was observed that posttranslational modification can alter the functional activity of the annexins (Konopka-Postupolska et al., 2009; Gorecka et al., 2005). Phosphorylation, glycosylation, acetylation, ubiquitination and SUMOylation are identified as potential posttranslational modifications that can modify the OsANN5 protein.

We found some highly potential phosphorylation and N-Glycosylation sites in OsANN5, which strongly suggest its functional importance in perceiving or transducing the signals. In our study, we identified the overlapping of SUMO interaction site and salt bridge motifs. This overlapping might have a function related to salt bridge formation in OsANN5. *In silico* prediction of potential PTMs can be explored for further experimental verification. This suggests a need for accelerated research towards post-translational modification of plant annexins for defining and clearly understanding the molecular mechanism of annexins.

Disordered regions in the protein are dynamic in nature and have the tendency to interact with other proteins or are prone to PTMs. The existence of disordered sequence with potential phosphorylation and O-glycosylation sites in the N-terminal region of OsANN5 suggests that it might play a major role in the signaling process. Crystal structure analysis of bell pepper annexin (AnxCa32) revealed that its core region interacts with the short N-terminal region (Hofmann et al., 2000a). Similarly, N-terminal and core disorder regions of OsANN5 possibly interact with each other under stress conditions through their disordered regions.

The alignment of OsANN5 with other plant annexins revealed an unconserved stretch of seven Glycine (Gly) residues. A stretch of Gly residues may decide the localization of the corresponding protein. Poly-glycine stretch in toc75 was necessary for targeting it to chloroplast envelope. Replacement of this glycine stretch with alanine made it miss-targeted to stroma (Inoue et al., 2003). Hence, it can be predicted that Gly stretch of OsANN5 may have a functional role in its localization.

OsANN5 is localized in the peripheral regions after salt treatment:

A given annexin can occupy various positions in cells, which include apoplast, organelles, and in association with membrane (Clark et al., 2012). Tissue-specific and sub-cellular localization of a specific protein may help in the identification of its function. As Ca^{2+} dependent membrane-binding proteins, many annexins possess the ability to dynamically change their cellular localization during certain physiological responses. For example, AtANN1 can be cytosolic, extracellular or can be associated with the plasma membrane, tonoplast and organelle membranes indicate that it can act as a multi-functional protein (Clark et al., 2010). The association of VCaB42 with vacuoles indicates its possible role in early vacuolar biogenesis (Seals et al., 1997). ANNSp2 was localized in the nucleus (Ijaz et al., 2017) predicting that it may have a transcriptional regulatory role. The fluorescence imaging studies reveals that OsANN5 is localized in the corners of the cells under normal condition. After the salinity stress the fluorescence signal was in the peripheral regions of the cell.

***OsAnn5* ameliorate stress tolerance through modulation of the anti-oxidant enzymes**

The *E. coli,* being a simple prokaryotic system, is the first choice for the heterologous expression of genes. There are many studies, which tried to analyse the functional role of the eukaryotic proteins in the *E*. *coli* system (Zhou et al., 2014; Kumari et al., 2009; Hu et al., 2014). Gidrol et al. (1996) observed that an annexin like protein rescued the ΔoxyR mutant of *E. coli* from the H_2O_2 stress. Similarly, the purified AtANN1 from *E. coli* cells exhibited the peroxidase activity, despite the possibility of the lack of posttranslational modification in the prokaryotic system. But, the same *ANNAt1,* purified from the *N. benthamiana* exhibited three times higher peroxidase activity (Gorecka et al., 2005) indicates that PTMs might have enhanced its peroxidase activity. In the present study we also tried to assess the functional activity of the OsANN5 under different abiotic stresses by overexpressing it in *E. coli* cells. OsANN5 overexpressing *E. coli* cells were able to resist the given high salt, heat and drought stress conditions suggesting, that OsANN5 functions through a signaling pathway, which is common to both prokaryotes and eukaryotes in terms of the anti-oxidant systems.

From previous study It is already known that *OsAnn5* shown very quick response to heat stress (Jami, Clark, Ayele, Roux, & Kirti, 2012). Spot assay also revealed that OsANN5 over expressing *E.coli* cells were also able to recovered even after 1 h of heat treatment. This results indicates that *OsAnn5* regulates the thermal stability of cells under stress condition.

To understand the functional role of *OsAnn5* in the abiotic stress, *OsAnn5* tobacco transgenics were developed and various stress assays were conducted. The ectopic expression of *OsAnn5* increased the stress tolerance of tobacco at seed germination stage, seedling stage and at mature plants level to the abiotic stresses in comparison to NS control. Assays with the seedling and leaf discs showed similar results in different abiotic stress treatments. This suggests that *OsAnn5* overexpression provides stress tolerance at different stages during tobacco plant development. At the seedling stage, NS control and A-2 low expression lines exhibited significantly reduced root length and stunted growth under stress conditions compared to the other high expression *OsAnn5* transgenics. Biophysical analysis revealed that the stress tolerance of 35S:*OsAnn5* transgenics are correlated with enhanced osmolyte (proline) accumulation, chlorophyll content and reduced peroxidation in the cells. This kind of correlation was also observed with other annexin transgenics (Ahmed et al., 2017; Huh et al., 2010). DAB staining assay results supported the MDA quantification results for 35S:*OsAnn5* transgenics under salt stress. This suggest that NS were not able to scavenge the H_2O_2 as efficiently as the 35S:*OsAnn5* transgenic plants.

The stress tolerance is generally associated with increased antioxidant defense system of the plant (Wang et al., 2003). It is observed that the anti-oxidant enzyme activity levels vary from plant to plant, kind and intensity of the stress and age of the plants (Mýtinová et al., 2010). In our study, we observed increased CAT activity and decreased SOD activity under the salt or heat stress treatments in both *OsAnn5* transformed *E.coli* and *OsAnn5* tobacco transgenics. These results strongly suggest that *OsAnn5* modulates CAT and SOD levels during the stress conditions and attributes to the stress tolerance of OsANN5. This also suggests that *OsAnn5* could act as a balancing protein by inducing the ROS mediated signaling and scavenging the ROS.

OsAnn5* expression is possibly independent of ABA under salt stress *in vivo

Many of the plant annexins are well known for their crucial role in biotic and abiotic stress tolerance and developmental processes (Yadav et al., 2018). But, the signaling pathways in which they are involved are yet to be clearly identified. Abscisic acid is a key signaling intermediate during abiotic stress, which plays a crucial role in developmental and drought stress tolerance. Many genes would be upregulated or downregulated by this hormone. Some recent reports tried to elucidate the role of annexins in correlation with ABA in stress signaling pathway. For example, Bianchi et al. (2002)suggested that AnnAt1 could be acting downstream of a cross talk between ABA-Auxin. A *Solanum pennellii* annexin, Annsp2 is associated with ABA accumulation during drought stress when over expressed in tomato and showed insensitivity to external ABA application during the seed germination stage (Ijaz et al., 2017). The annexin, *AnnBj2* overexpressing transgenic mustard plants were also insensitive to ABA during the seed germination stage. Interestingly, gene expression studies in the *AnnBj2* transgenic mustard plants revealed increased transcript levels of ABA catabolic gene *CYP707A2* (Ahmed et al., 2017). In line with these results, *OsAnn3* knock down transgenics showed ABA sensitivity at seed germination stage (Shen et al., 2017) suggesting that annexins may enhance seed germination by promoting the degradation of ABA. The above studies strongly suggest the functional role of annexins in abiotic stress tolerance that is associated with ABA. Hence, as a first step towards understanding OsANN5 involvement in ABA signalling pathway, we tried to assess whether any alteration in ABA levels would affect *OsAnn5* expression in stress condition, which eventually gives a clue also whether it is ABA-dependent or independent or both.

Earlier Jami et al. (2012) reported that no rice annexin got responded to the ABA treatment. In our case OsAnn5 shown upregulation at 6 h of ABA treatment. So we tried to know whether OsAnn5 expression dependent of ABA under salt stress. we observed not much significant difference in the expression levels of the *OsAnn5* when compare with salt stress and salt stress in combination with fluridone. In line with this, 35S:*OsAnn5* transgenic seedlings showed reduced sensitivity to external ABA. This clearly suggests that OsAnn5 may acts majorly through an ABA independent pathway under salt stress. The presence of ABRE along with DRE in the *OsAnn5* upstream region suggesting *OsAnn5* possibly act through ABA-dependent and independent pathway under stress conditions.

3.4. Conclusion

Presence of several probable post translational modifications sites in annexins suggesting that it can play a major role in stress tolerance. This was evinced through heterologous overexpression of *OsAnn5* in *E.coli* and tobacco, which resulted in their enhanced tolerance to abiotic stresses. Under salt stress, *OsAnn5* works through an ABA-independent pathway. ROS scavenging activity exhibited by 35S:OsAnn5 transgenics was correlated with the modulation of their antioxidant enzyme levels elucidating the possible involvement of OsAnn5 in stress tolerance. Hence, a new dimension of research related to the identification of the binding partners and post-translational modifications of OsAnn5 would throw more light on the detailed mechanism of its action.

CHAPTER IV

Comparison of over-expressing OsAnn5 lines with mitochondrial and guard cell targeted overexpressing lines for abiotic stress tolerance in tobacco

4.1 Results

4.1.1 Fusion of CoxIV with *OsAnn5* by overlapping PCR

To express the OsANN5 specifically in mitochondria, signal peptide of Cytochrome C oxidase subunit IV (CoxIV) was used (Hurt et al., 1984). For this, 966 bp full length *OsAnn5* CDS was fused with 75 bp of CoxIV by overlapping PCR. This was carried out in two steps. In the first step *CoxIV* sequence was PCR amplified with *CoxIV* FP and *CoxIV* RP_o. The *CoxIV* RP_o is the reverse primer for *CoxIV* with an overhang sequence at its end, which is complementary to 28 nucleotide sequence of the 5' end of *OsAnn5.* This amplified CoxIV template was used in the second step along with purified *OsAnn5* template without adding any primers. 15 cycles of PCR were performed. Later, *CoxIV FP* and *OsAnn5* RP primers were added to the same reaction mixture to develop CoxIV:*OsAnn5* fusion*.* The amplified *CoxIV:OsAnn5* product was confirmed through Sanger sequencing. The corresponding primers were listed in **Table 4.1**

Table 4.1 Primers used for the CoxIV:*OsAnn5* expression cassette development

Gene	Primer sequence
CoxIV FP	5'GGG TAC CAT GCT TTC ACT ACG TCA A'3
CoxIV RP	5'AAG CAG ATA TCT AGA GCT ACA CAA A'3
CoxIV RP_o	5'GCT CAG GCT CGC CAT AAG CAG ATA TCT AGA'3
OsAnn5 RP	5'ATT GGA TCC TTA GCG GTC GCG GCC'3

4.1.2 Construction of pCAMBIA2300:*CoxIV:OsAnn5* vector

CoxIV:OsAnn5 fusion product was amplified and cloned into pRT100a vector at *KpnI* and *BamHI* restriction enzyme sites **(Fig. 4.1)**. The 1.7 Kb final expression cassette, 35S:*CoxIV:OsAnn5*:*polyA* was released from pRT100a and cloned into pCAMBIA2300 binary vector using *PstI* restriction enzyme. The confirmed pCAMBIA2300:*CoxIV:OsAnn5* **(Fig. 4.1)** was transformed into *A. tumefaciens* strain LBA4404 through the freeze-thaw method of transformation.

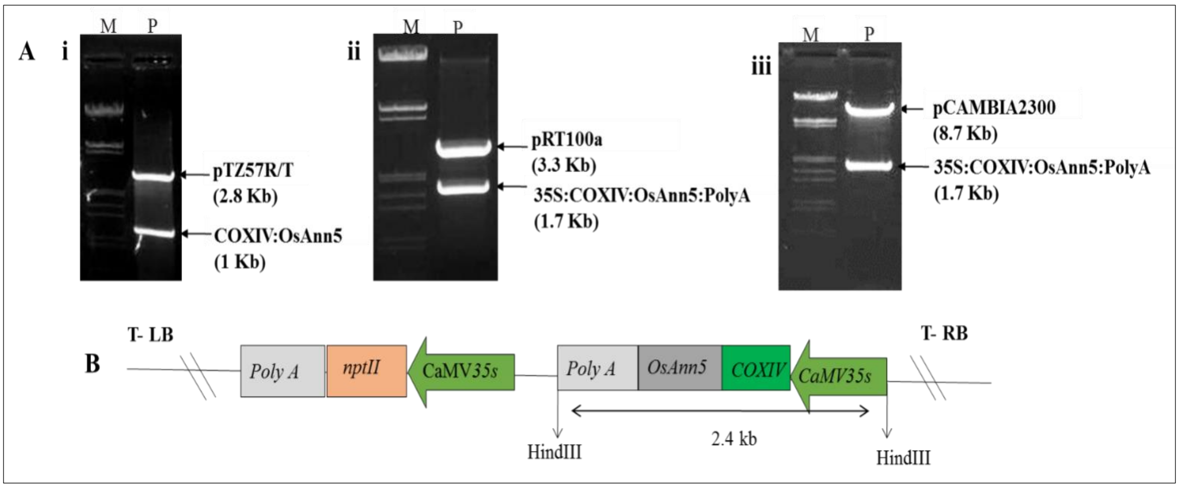


Fig. 4.1 Confirmation of *CoxIV:OsAnn5* from pTZ57R/T vector (**A-i**). The release of 35S:*CoxIV:OsAnn5*:polyA, expression cassette from pRT100a **(A-ii)** and pCAMBIA2300 binary vectors **(A- iii)**. M = λ DNA / *Eco*RI + *Hin*dIII marker, P = plasmid. Pictorial representation of T-DNA region of pCAMBIA2300 having CoxIV:*OsAnn5* expression cassette along with *nptII* gene as the selection marker **(B)**.

4.1.3 Construction of *CoxIV:OsAnn5*:*Egfp* vector

To confirm the mitochondrial targeting of *CoxIV:OsAnn5*, *CoxIV:OsAnn5* was translationally fused to *EGFP* at the N' terminal by cloning it into pRT100 (Kumar & Kirti, 2011) with *Sma*I restriction enzyme. The final 35S:*CoxIV:OsAnn5*:*Egfp* expression cassette was released from pRT100 and cloned into pCAMBIA2300 using *Hind*III restriction enzyme **(Fig. 4.2 A)**.

4.1.4 Confirmation of CoxIV:*OsAnn5* targeting to mitochondria

The final pCAMBIA2300:35S:*CoxIV:OsAnn5*:*Egfp* was used for transient expression in onion epidermal peels (Sun et al., 2007). Transient expression study was done according to the protocol explained in material and methods (2. 24).

The peels were pre-treated with 20nM Mito-Tracker dye CMXRos (Thermo Fischer Scientific Inc., USA) and washed twice with 1x PBS before observing under confocal microscope. The red fluorescence, represents the mitochondrial staining (wavelength range 579-599 nM) and the green fluorescence (wavelength range
489 nM-509 nM), represents the *OsAnn5*:*Egfp* localization. Overlay of these two fluorescence results in yellow fluorescence which suggests the localization of CoxIV:*OsAnn5* to the mitochondria. **(Fig.4.2B)**.

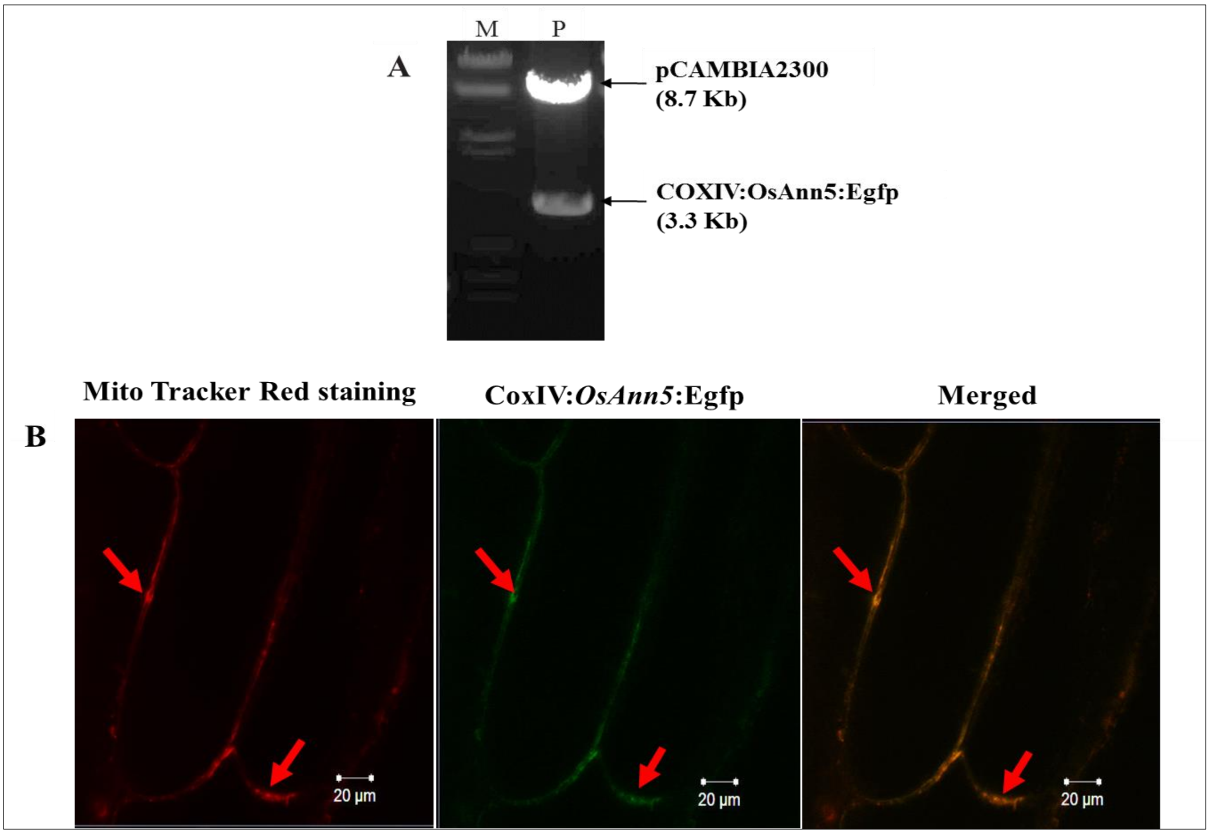


Fig. 4.2 Cloning confirmation and transient expression of CoxIV:*OsAnn5*:*Egfp* in onion epidermal peels. The release of 3.3 Kb *Egfp* fused CoxIV:*OsAnn5* fragment from the pCAMBIA2300 vector **(A)**. Mitochondria stained with mito-tracker red showing red fluorescence. Green fluorescence represents the expression of *CoxIV:OsAnn5*:*Egfp* in the cell. An overlapping image of the both showing the yellow fluorescence confirms the mitochondrial targeting of the fusion protein. Fluorescence images were taken at 20X magnification at the scale bar of 20 µm **(B)**.

4.1.5 Generation and confirmation of 35S:*CoxIV:OsAnn5* transgenics.

The pCAMBIA2300:*CoxIV:OsAnn5* transformed agrobacterium LBA4404 strain was used for developing tobacco transgenic plants using the standard leaf disc transformation protocol developed by Horsch et al., 1985. Putative T_0 transgenic plants were confirmed through PCR amplification for *CoxIV:OsAnn5* and *nptII* using genomic DNA as template (**Fig. 4.3 A,B**). Semi- quantitative PCR was performed to identify high and low expression lines. CoxIV:OsAnn5 transgenic line 2 (C-2) was used as low expression line while lines 4 (C-4) and 6

(C-6) were used as high expression lines (**Fig.4.3C**). The T_3 plants were used for all the assays.

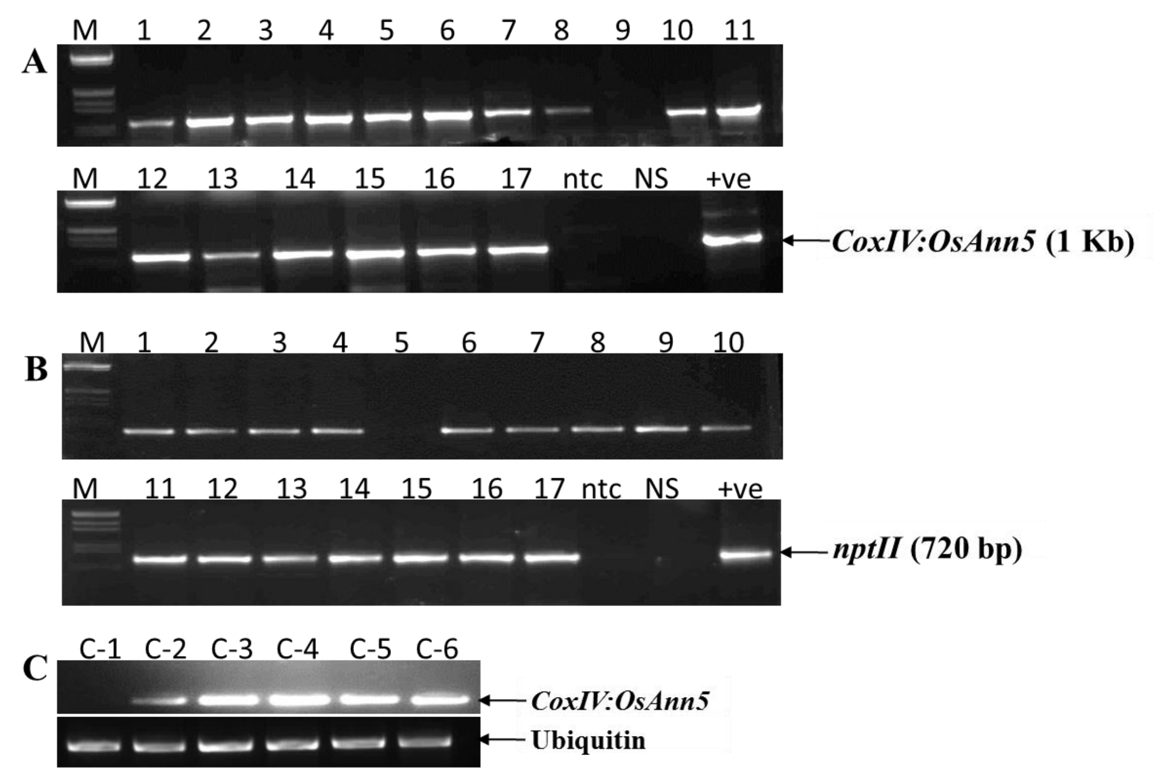


Fig. 4.3 Molecular confirmation of CoxIV:*OsAnn5* tobacco transgenic plants. PCR confirmation for CoxIV:*OsAnn5* **(A)** , *nptII* **(B)** from genomic DNA. Semi quantitative RT-PCR for *CoxIV:OsAnn5* expression with reference to the actin gene **(C)**. M= marker, ntc = Non template control, NS= null segregant, + ve = positive control, 1-17 = CoxIV:*OsAnn5* transgenic lines.

4.1.6 Seedling assay of CoxIV:*OsAnn5* transgenics

The NS seeds were germinated on the ½ MS media and T_3 *CoxIV:OsAnn5* transgenic seeds were germinated on the ½ MS+ kanamycin (135 mg/L) media. The 10 d old seedlings were placed on ½ MS media and ½ MS media supplemented with 0.2 M NaCl, 0.3 M Sorbitol, 10 % PEG and their stress tolerance ability was assessed after two weeks of treatment (**Fig. 4.4**). *CoxIV:OsAnn5* transgenics were susceptible to osmotic stress, but exhibited tolerance towards high salt and shown moderate stress tolerance to drought stresses.

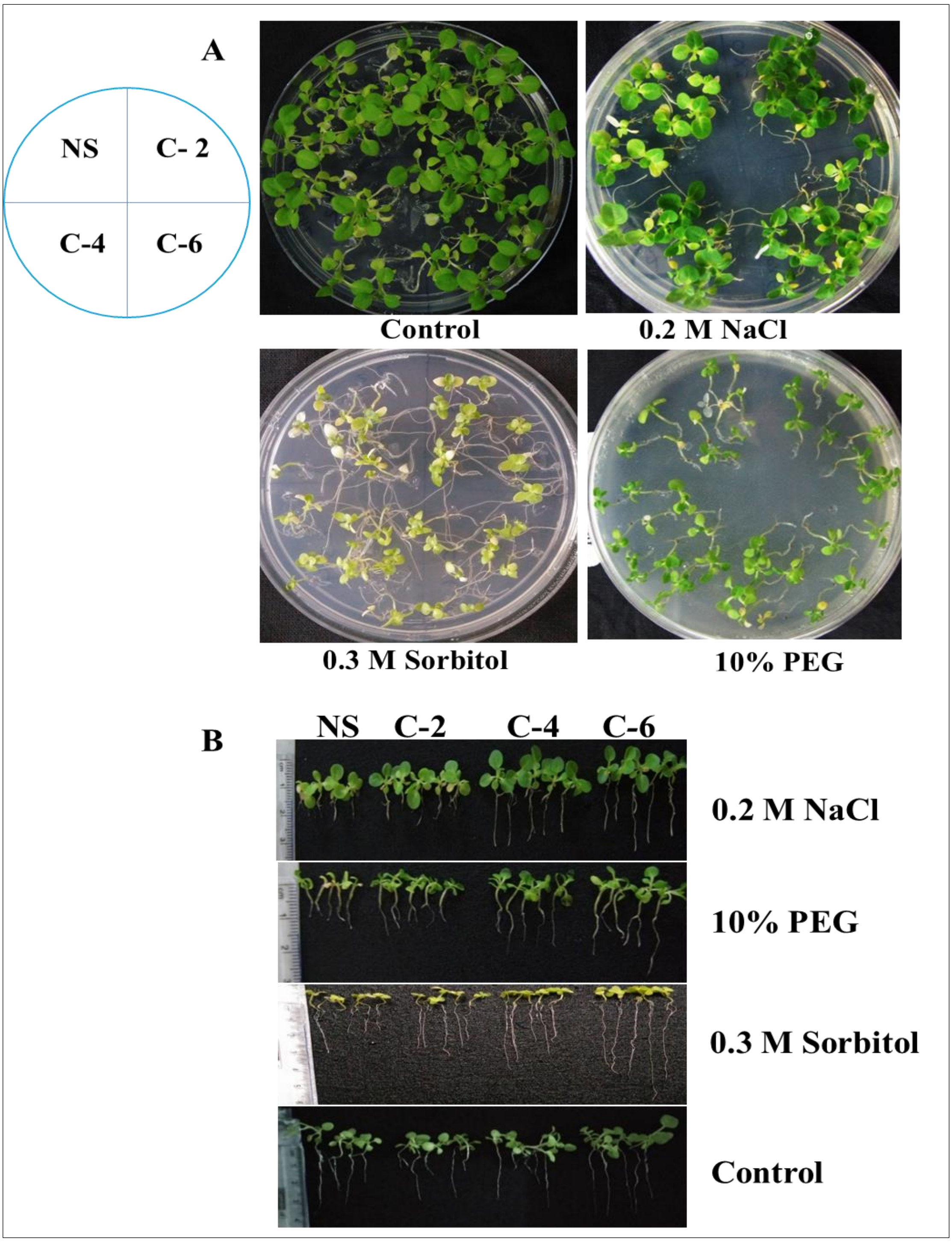


Fig. 4.4 Seedling assay with the CoxIV:*OsAnn5* transgenic lines under various abiotic stress treatments **(A)**. Comparison of root lengths of seedlings after various stress treatments **(B)**.

4.1.7 Leaf disc assay of CoxIV:*OsAnn5* transgenics

For this assay fully grown leaves were collected from NS and transgenic plant and leaf disc of 1 cm diameter were prepared. Then they were subjected to various stresses as in seedling assay for one week and their phenotype was observed (**Fig. 4.5**). Comparative to NS and CoxIV:*OsAnn5* low expression lines, high expression lines were shown less chlorophyll bleaching only under salinity stress.

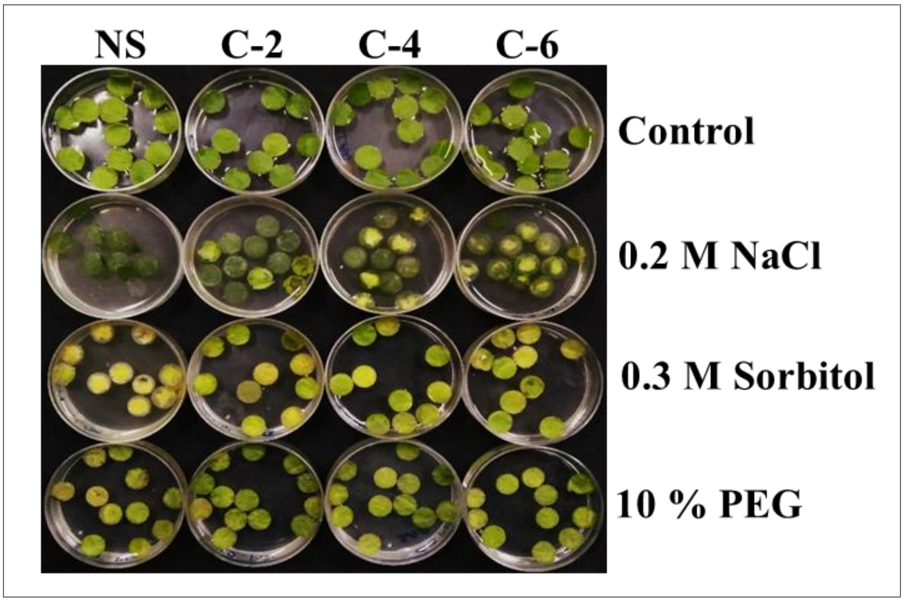


Fig. 4.5 Leaf disc assay for NS and CoxIV:*OsAnn5* transgenics lines. Their leaf discs were subjects to different stresses (0.2 M NaCl, 0.3 M Sorbitol, 10 % PEG) for one week and their phenotype was observed.

4.1.8 DAB staining assay for CoxIV:*OsAnn5:*

The leaf discs of CoxIV:*OsAnn5* transgenics and its NS were prepared and subjected to 0.2 M NaCl for 4 days and then followed by DAB staining. The high expression lines were shown less intensive staining and accumulated less quantity of DAB under high salinity stress (**Fig. 4.6**). NS and C-2 low expression lines were shown intensive staining. The DAB stained samples were quantified for the DAB accumulation and its equivalent values were represented as µmoles of H_2O_2 in gFW. The values were correlated with the staining results.

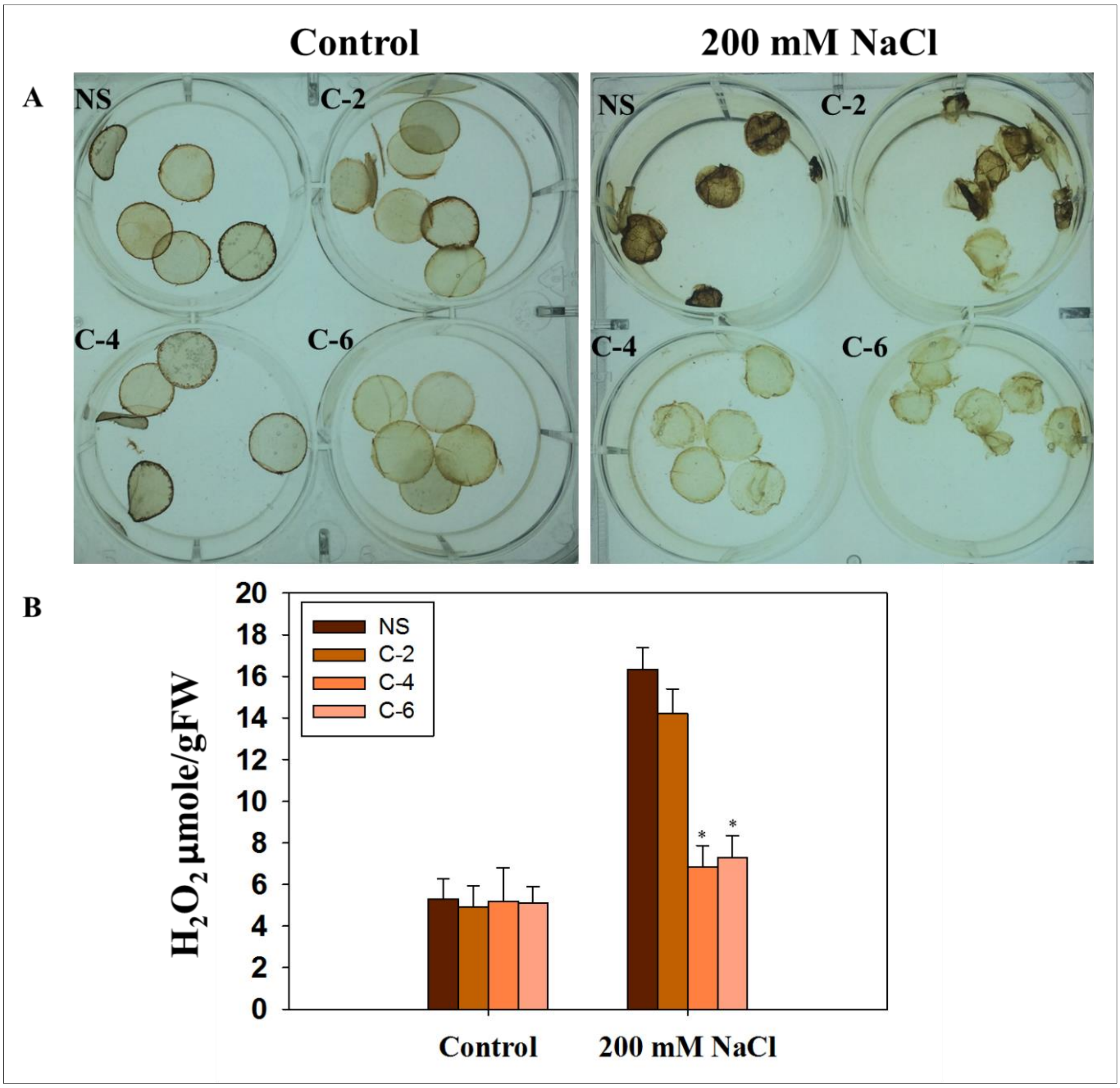


Fig. 4.6. DAB staining assay with the NS and CoxIV:*OsAnn5* transgenics after the high salinity (0.2 M NaCl) stress treatment **(A).** Quantification of DAB from the DAB stained sample of NS and CoxIV:*OsAnn5* transgenics. The amount of DAB accumulation is directly proportional to the H_2O_2 accumulation in the samples **(B)**. Error bars represent the ± SD. Asterisks represent the significant difference in the H_2O_2 accumulation in comparison with the NS under stress conditions when analyzed through ANOVA. * $P<$ 0.05.

4.2.1 Construction of DGP1:*OsAnn5* construct vector

For guard cell specific expression of *OsAnn5,* the DGP1 promoter has been used in the place of 35S enhancer. The DGP1 promoter was prepared as demonstrated by Jun et al. (2005). DGP1 is the fusion of two DNA fragments, one is the Drought Responsive Element (DRE), a 250 bp fragment of *rd29A* gene promoter of *A .thaliana*, which has one 9 bp TACCGACAT motif that is responsible for binding of the DREB factor (Yamaguchi-shinozakiaib & Shinozaki, 1994). Another one is Guard Cell Specific Element (GCSE), which is also a 250 bp fragment amplified from the *kstI* gene promoter of potato which has two TAAAG motifs responsible for guard cell specific expression of the targeted gene (Plesch et al., 2001) .

The 250 bp GCSE was amplified from the potato genomic DNA and 250 bp DRE was amplified from the *A. thaliana* genomic DNA. These two fragments were fused using *XbaI* restriction enzyme and ligation in the order of DRE followed by GCSE. The DGP1 3' end (**D**RE:**G**CSE **P**romoter) was fused to the 5' end of *OsAnn5* gene using the *KpnI* restriction site. The *OsAnn5* 3' end is already fused with 5' end of *PolyA* terminal signal sequence using the *BamHI* restriction enzyme. The whole cassette was amplified using DRE FP having *Hin*dIII site and polyA RP also having a *Hin*dIII restriction site. Primers used for the DGP1:*OsAnn5* construct are listed in the **Table 4.2**. The final DGP1:*OsAnn5* expression cassette was cloned into the pCAMBIA2300 binary vector using *Hin*dIII restriction site (**Fig. 4.7**). It was used for developing tobacco transgenic plants for guard cell specific expression under abiotic stress.

Table 4.2 Primers used in developing DGP1:*OsAnn5* expression cassette

Gene	Primer sequence
DRE FP	5'ATT AAG CTT AGA AGG ATG TGC CGT TTG T'3
DRE RP	5'TCT AGA GAG AGA CTG AGA GAG ATA AA'3
GCSE FP	5'GTCT AGA GCTTTTGGTAACAATCTCT'3
GCSE RP	5'GGTACC CAA TGT GTA ATA TTT AA'3
OsAnn5 RP	5' GTA AGC TTT TAG CGG TCG CGG C '3
PolyA RP	5'GAAGCTTACTGGATTTTGGTTTTAGG'3

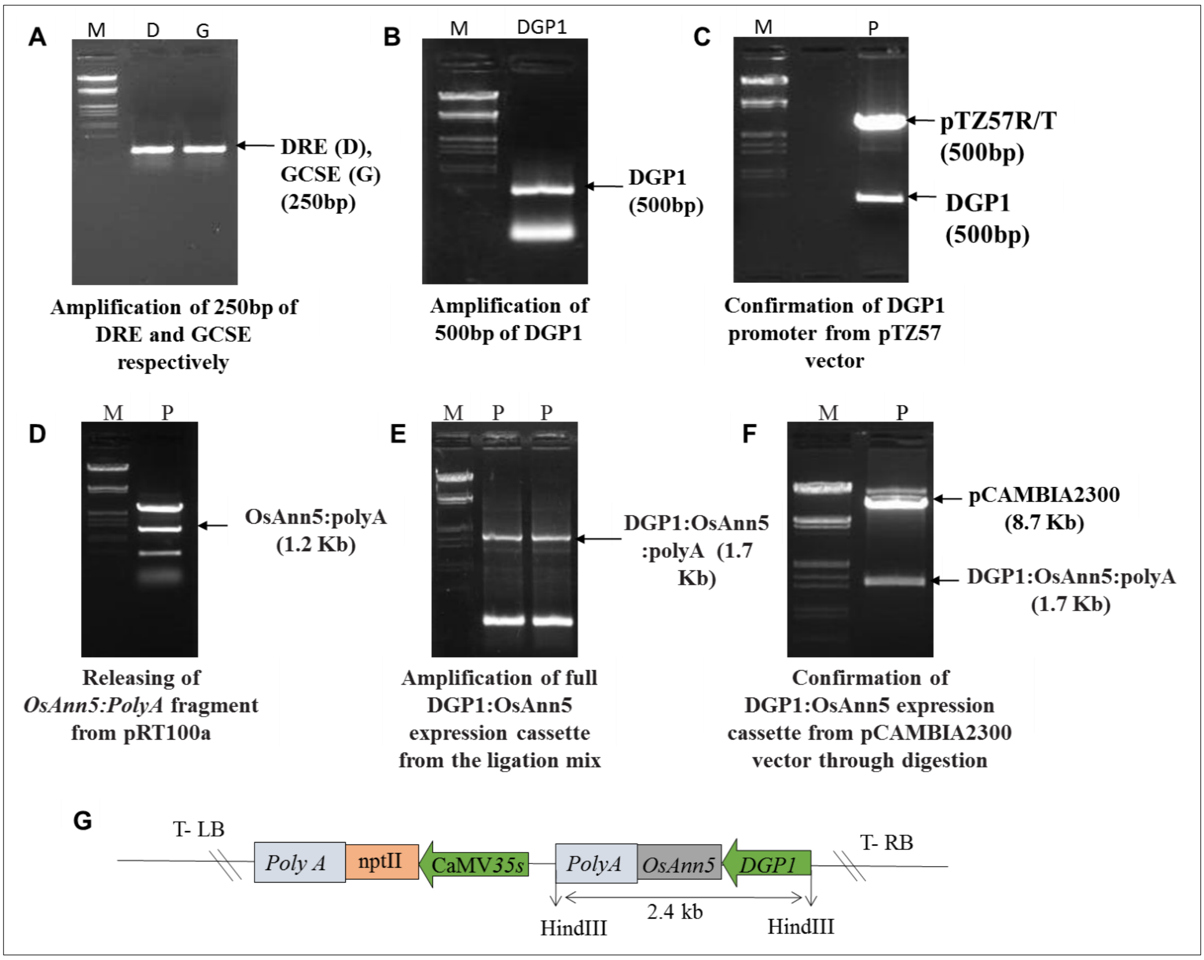


Fig. 4.7 Procedure illustrating the pCAMBIA:DGP:*OsAnn5* construct preparation **(A-F)**. Pictorial representation of T-DNA region of the pCAMBIA2300 which has DGP1:*OsAnn5* expression cassette **(G)**. M = λ DNA / *Eco*RI + *Hin*dIII marker, D= DRE amplification, G= GCSE amplification, DGP1= Fusion of DRE and GCSE, P= plasmids.

4.2.2 Confirmation of DGP1:*OsAnn5* transgenics

Putative T_0 tobacco transgenic plants expressing the DGP1:*OsAnn5* cassette were confirmed through the amplification of 1.46 Kb DGP1:*OsAnn5* and 720 bp *nptII* gene from the genomic DNA **(Fig. 4.8 A, B)**. We also confirmed the expression *OsAnn5* at mRNA level. To identify the high and low expression lines of the DGP1:*OsAnn5* transgenic plants, their epidermal peels were peeled off and total RNA was isolated. cDNA was prepared from the total RNA sample and a semi quantitative PCR was performed. DGP1:*OsAnn5* transgenic line D-4 was used as low expression line, while the lines D-7 and D-17 were used as high expression lines for further studies (**Fig. 4.8 C**).

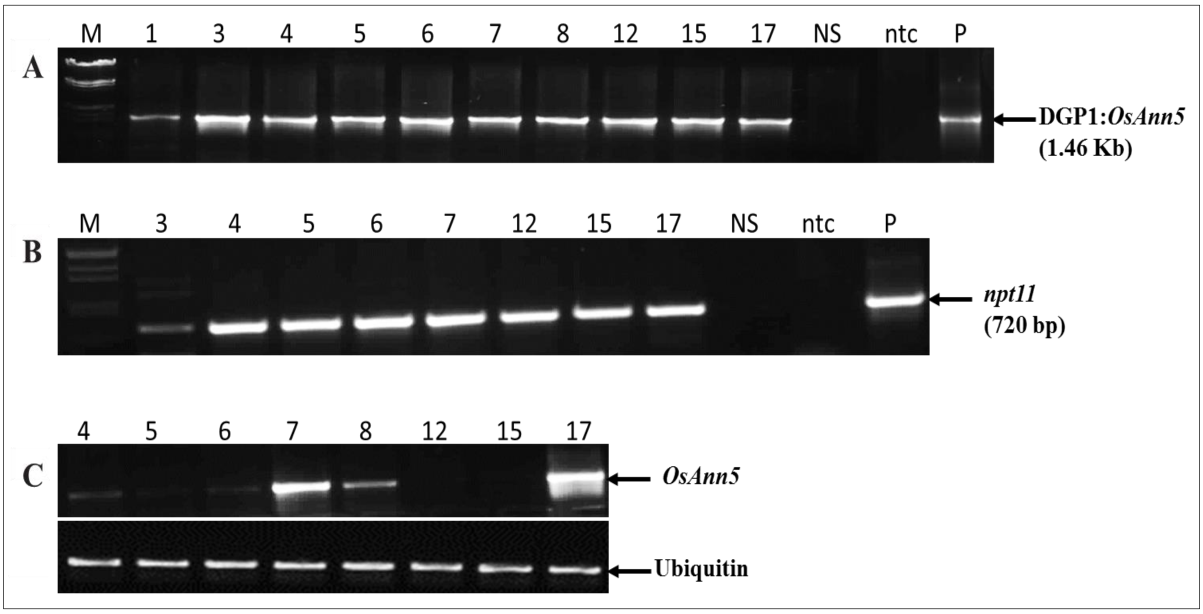


Fig. 4.8. Putative transgenic plat confirmation through amplification of DGP1:*OsAnn5* **(A),** *nptII* **(B)** from genomic DNA. Semi-quantitative PCR for *OsAnn5* confirmation and to identify the low and high expression lines with reference to ubiquitin control as internal **(C)**

4.2.3 Seedling assay of DGP1:*OsAnn5* transgenics

The NS and T_3 DGP1:*OsAnn5* transgenic seeds were germinated on the ½ MS and ½ MS kanamycin (135 mg/L) selection media respectively. The 10 d old seedlings were subjected to different stress assays viz.0.2 M NaCl, 0.3 M Sorbitol, 10% PEG and their stress response was assessed (**Fig. 4.9A**). Compare to NS the high expression lines of DGP1:*OsAnn5* transgenics were tolerant to drought stress and able to maintain good root length but were susceptible to high salt and osmotic stresses and shown less root growth **(Fig 4.9B)**.

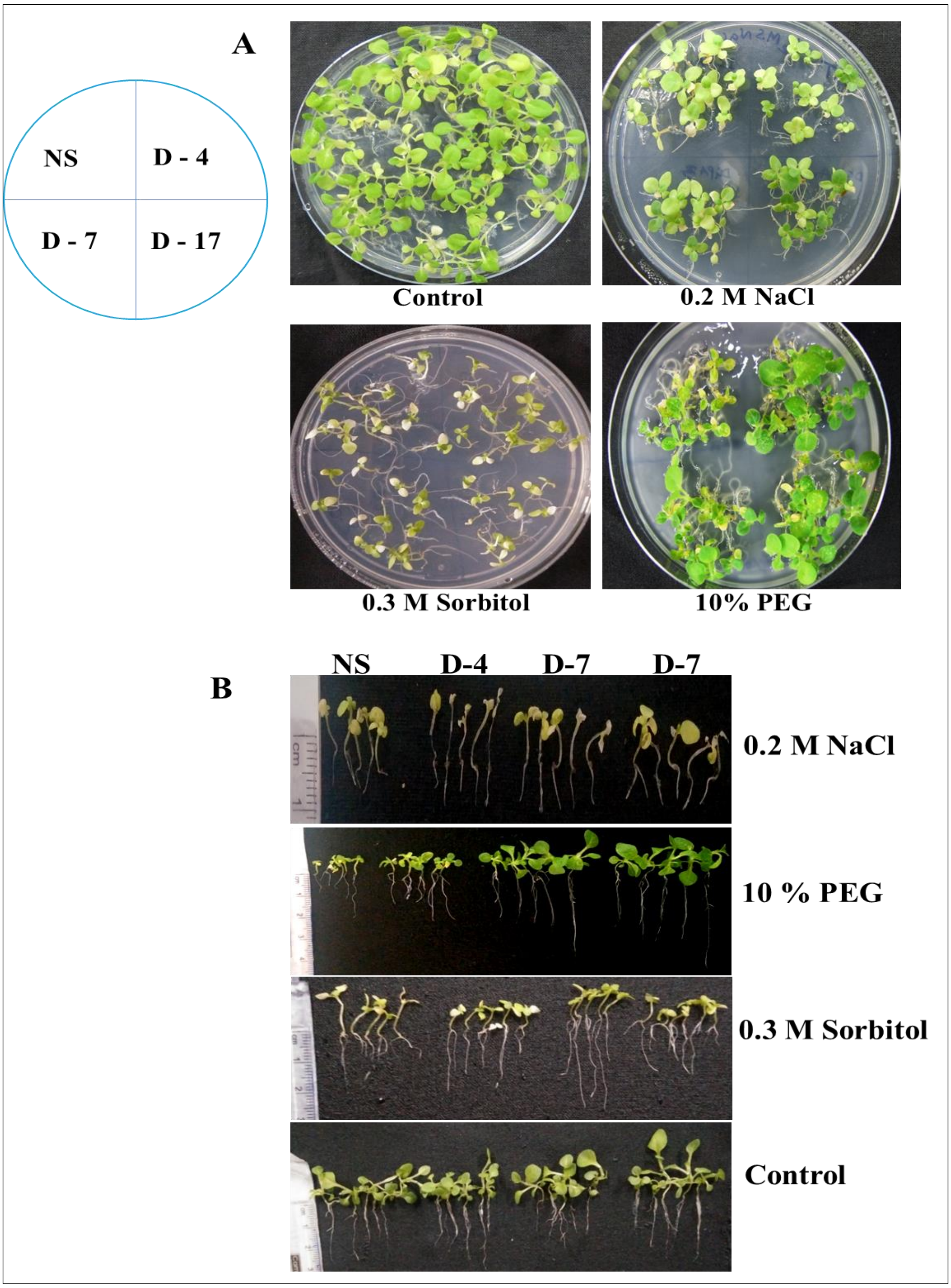


Fig. 4.9 Seedling assay with the NS and DGP1:*OsAnn5* transgenic lines under various stress conditions i.e 0.2 M NaCl, 10 % PEG, 0.3 M Sorbitol along with control **(A).** After the seedling assay the seedlings were compared each other for their root lengths **(B).**

4.2.4 Leaf disc assay for DGP1:*OsAnn5*

For this assay fully grown mature leaf from NS and transgenic plants leaves were collected and leaf disc of 1 cm diameter were prepared. Then they were subjected to various stresses as in seedling assay for one week and their phenotype was observed. DGP1:*OsAnn5* transgenics were shown more chlorophyll bleaching under high salinity and osmotic stress but not under drought stress **(Fig.4.10)**.

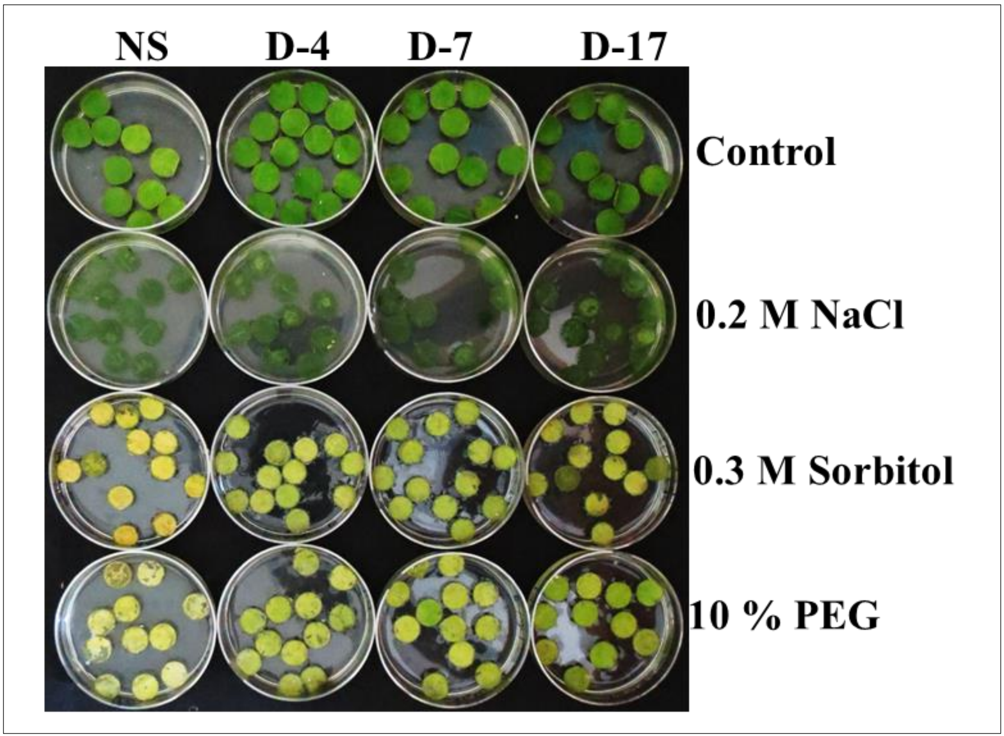
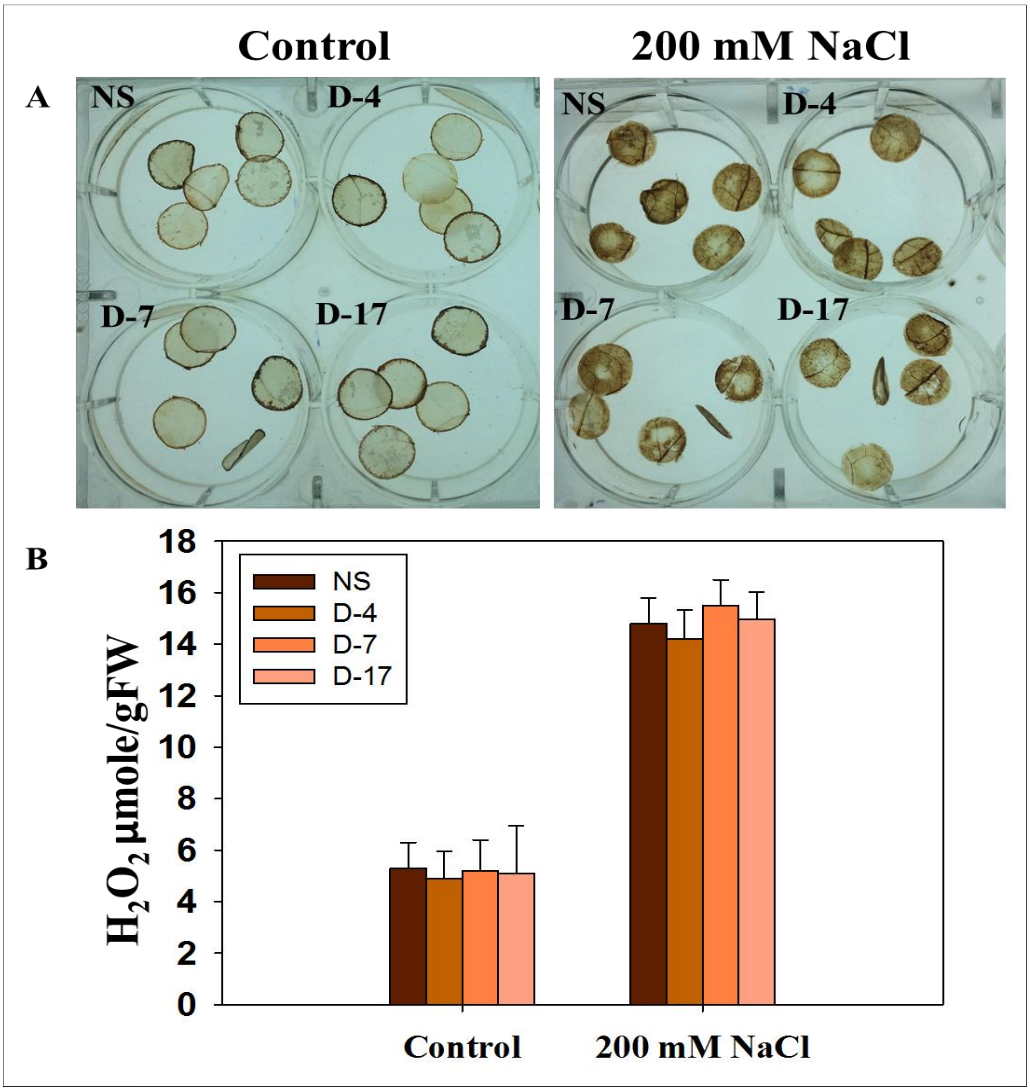


Fig. 4.10 Leaf disc assay for NS and DGP1:*OsAnn5* transgenics with different stresses.

4.2.5 DAB staining assay for DGP1:OsAnn5

The leaf discs of DGP1:*OsAnn5* transgenics and its NS were subjected to 0.2 M NaCl for 4 days and then followed by DAB staining as explained in material and methods. The accumulated DAB extracted with perchloric acid and quantified. Generally, the quantified DAB is directly proportional to the internally accumulated H_2O_2 levels. So, the DAB quantified values are represented as μmoles of H_2O_2 accumulated in gFW. The DGP1:*OsAnn5* null segregatns and it low and high expression lines were stained similarly and accumulated approximately equal amount of DAB under the given salt stress treatment (**Fig. 4.11**).



4.11 DAB staining assay with the DGP1:*OsAnn5* transgenics after the high salinity (0.2 M NaCl) stress treatment **(A).** Quantification of DAB from the DAB stained sample of NS and DGP1:*OsAnn5* transgenics and the values were represented as µmoles of H_2O_2/gFW **(B).**

4.3 Comparative analysis of mitochondrial and guard cell targeted *OsAnn5* transgenics with OsANN5 over-expression transgenics to assess their extent of abiotic stress tolerance.

The high expression lines of all the transgenics were used for the comparison studies. The stress treated samples were examined for their root length, chlorophyll, proline, MDA levels and H_2O_2 were compared.

4.3.1 Root length assay

The average root lengths of the stress given high expression lines of all the transgenics were quantified and represented in a graph. We observed that the 35S:*OsAnn5* transgenics were able to sustain good root length growth almost as in control condition even after the stress treatment. In comparison with 35S:*OsAnn5* transgenics, the CoxIV:*OsAnn5* transgenics showed poor root growth under all the given stress treatments, while the DGP1:*OsAnn5* transgenics showed poor growth to the salinity and osmotic stress treatments **(Fig.4.12**).

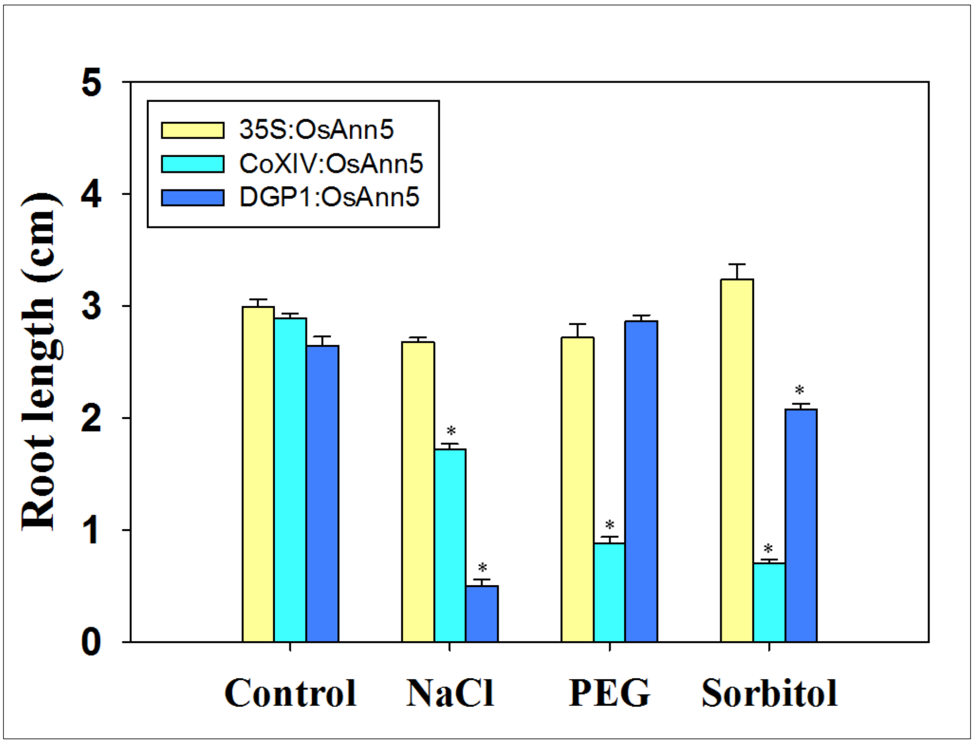


Fig. 4.12 After seedling assay the root lengths of high expression lines of all transgenics were compared, measured and plotted in a graph. Error bars represent the ± SD. Asterisks represent the significant difference in the root length of targeted transgenics in comparison with the 35S:OsAnn5 transgenics under stress conditions when analyzed through ANOVA. * $P< 0.05$.

4.3.2 Comparison of biochemical parameters of the transgenics after the stress treatment

Generally stress leads various biochemical and metabolic changes that eventually effects the crop yields and growth. The major parameters are chlorophyll, osmolyte contents and lipid peroxidation levels. To check the extent of damage in different transgenic plants to the various abiotic stresses at mature plant stage, a leaf disc assay was performed for all the transgenics and their respective NS. The leaf discs were subjected to 200 mM NaCl, 300 mM sorbitol and 10% PEG and the samples were subsequently analyzed for their total chlorophyll and proline contents, and the extent of their membrane lipid peroxidation.

Chlorophyll content

The 35S:*OsAnn5* transgenics shown only 15- 30 % reduction. Interestingly, the *CoxIV:OsAnn5* high expression lines showed 18-35 % reduction, while the DGP1:*OsAnn5* high expression lines exhibited 30-42 % reduction in the chlorophyll content under all the stress treatments (**Fig. 4. 13**).

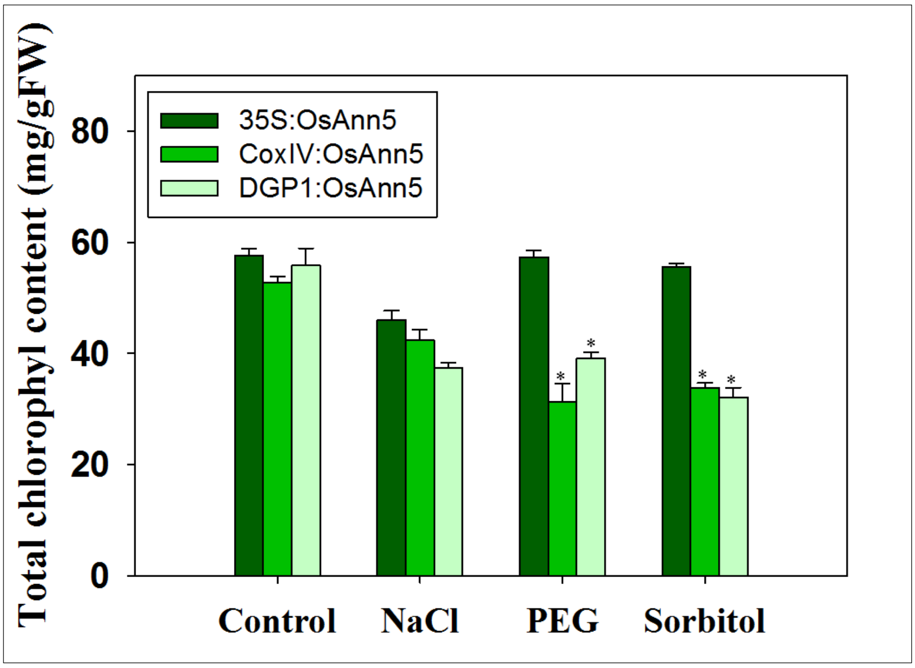


Fig. 4.13 Total chlorophyll estimation for the high expression lines of all the transgenics was done after different stress treatments. Error bars represent the ± SD. Asterisks represent the significant difference in the total chlorophyll content of the targeted transgenics in comparison with the 35S:OsAnn5 transgenics under stress conditions when analyzed through ANOVA. * *P*< 0.05.

Proline content

Proline plays a major role in stress amelioration of the plants and is accumulated during stress conditions. It acts as a compatible solute, which helps in protecting the enzymes and proteins during stress (Szabados et al., 2010). 35S:*OsAnn5* transgenics accumulated two folds higher levels of proline in the stress conditions as compared to controls **(Fig 4. 14)**. The *CoxIV:OsAnn5* transgenics also accumulated two folds more free proline as 35S:*OsAnn5* transgenics to the stress treatments and this accumulation is even more under the drought stress. In comparison with 35S:*OsAnn5* transgenics DGP1:*OsAnn5* transgenics shown significantly less proline accumulation except under drought stress.

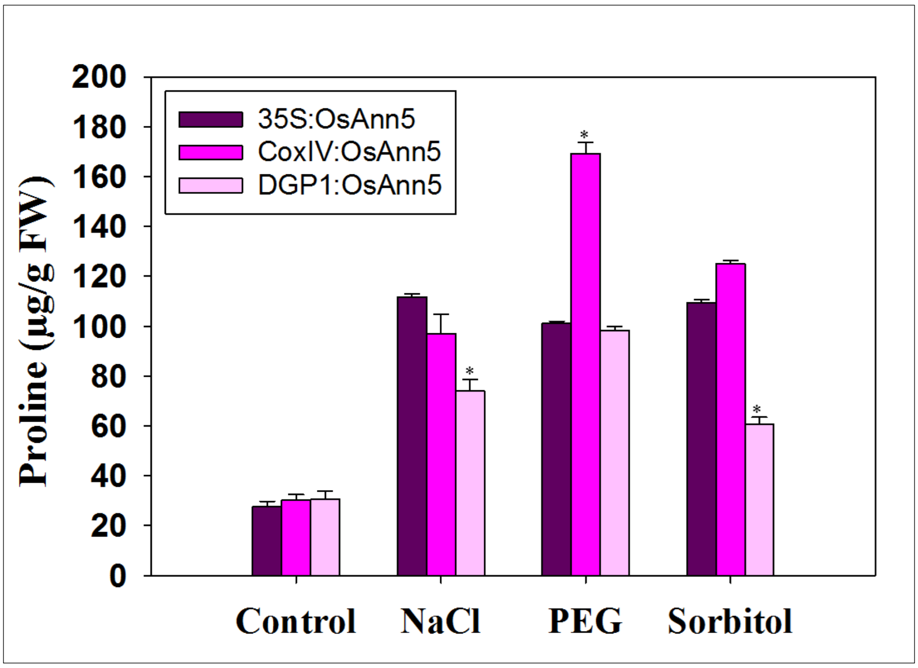


Fig. 4.14. Proline quantification for the high expression lines of all transgenics after different stress (0.2 M NaCl, 10% PEG, 0.3 M Sorbitol) treatments. Error bars represent the ± SD. Asterisks represent the significant difference in the proline accumulation in comparison with the 35S:*OsAnn5* transgenics under stress conditions when analyzed through ANOVA. * *P*< 0.05.

Lipid peroxidation

In general stress intensifies the lipid peroxidation, which affects the viability of cells and indicates membrane damage in cells under stress. Malondialdehyde (MDA) levels represent the level of lipid peroxidation that occur in the cell during stress conditions. Compare to 35S:*OsAnn5*, *CoxIV:OsAnn5* transgenics had increased MDA level in all given stresses while *DGP1:OsAnn5* transgenic samples showed reduced MDA levels only in drought stress treatment but not in other stress treatments **(Fig. 4.15)**

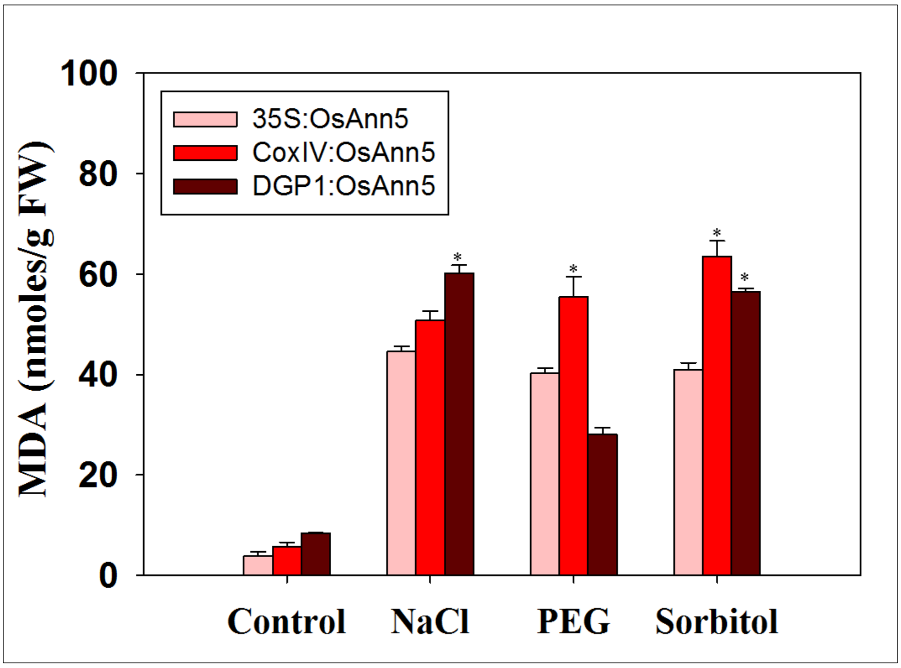


Fig. 4.15 Total MDA level estimation for the high expression lines of all the transgenic plants after various stress treatments (0.2 M NaCl, 10% PEG, 0.3 M sorbitol). Error bars represent the ± SD. Asterisks represent the significant difference in MDA levels in comparison with the 35S:*OsAnn5* transgenics under stress conditions when analyzed through ANOVA. * *P*< 0.05.

H_2O_2 accumulation

DAB staining assay was performed to see the extent of H_2O_2 accumulated in the leaf discs under the high salinity stress (200 mM salt) treatment. The intensity of staining is directly proportional to the H_2O_2 accumulation. We observed strong and intense staining in the leaf discs of DGP1:*OsAnn5* when compare with the 35S:*OsAnn5* and CoxIV:*OsAnn5* transgenic plants. The CoxIV*:OsAnn5* transgenics exhibited less intense staining as 35S:OsAnn5 transgenics but with a certain degree of damage (**Fig. 4.16**).

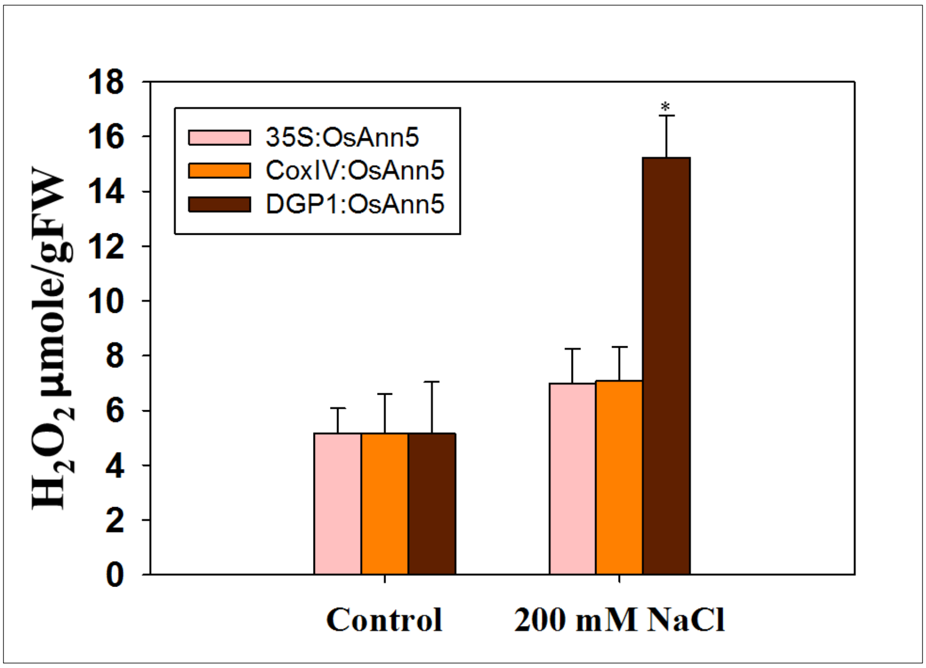


Fig. 4.16 DAB staining quantification for the high expression lines of all transgenics lines after 0.2 M NaCl stress. Error bars represent the ± SD. Asterisks represent the significant difference in MDA levels in comparison with the 35S:*OsAnn5* transgenics under stress conditions when analyzed through ANOVA. * $P< 0.05$.

4.4 Discussion

Constitutive overexpression of the genes in the transgenic plants had been using as a tool to enhance the tolerance of the plants to abiotic stress conditions. Always a homeostatic condition is needed between the nucleus and cellular organelle to develop the abiotic stress tolerance in the plants (Robles & Quesada, 2019). Instead of systematic over expression, targeted expression of genes is also done to understand the functional activity of the genes (Daloso et al., 2015). An animal annexin VI was overexpressed only in the heart tissue to know its function (Gunteski-Hamblin AM et al., 1996). However, no targeted expressions studies has been done so far for plant annexins.

Guard cells and mitochondria are the active sites of the calcium signaling and palys major role under stress response (Medvedev, 2005). Mitochondria is drastically affected by abiotic stress and is also a site for developing stress tolerance (Robles & Quesada, 2019). In our study, to know whether targeted overexpression of *OsAnn5* will enhance the abiotic stress tolerance, different tobacco transgenics were developed. In one kind of transgenics the expressed

OsANN5 was targeted to the mitochondria with the help of its fused mitochondrial matrix targeting signal peptide called CoxIV. Another set of tobacco transgenics were developed in which *OsAnn5* expresses specifically in the guard cells and under drought stress condition with the help of DGP1 promoter (Jun et al., 2005). The 35S:*OsAnn5* tobacco transgenics and mitochondrial targeted 35S:CoxIV:*OsAnn5* tobacco transgenics and guard cell targeted DGP1:*OsAnn5* tobacco transgenics phenotypes and their biochemical parameters after stress treatment were compared each other with respect to their stress tolerance property.

Stress assays suggests that 35S:OsAnn5 transgenics were tolerant to all the given stresses. Unlike 35S:*OsAnn5* tobacco transgenics, targeted tobacco transgenics were not able to tolerate all given stresses. CoxIV:*OsAnn5* transgenics were tolerant only to the high salinity stress and showed moderate tolerance to the drought stress but not to the osmotic stress. Whereas guard cell targeted *OsAnn5* tobacco transgenics were able to tolerate to the only drought stress, but not to the other stresses. Their phenotypes were correlated with root length measurements under abiotic stress treatment.

Table 4.2 Comparison of NS and transgenics how they responded to the given stresses

Stress	**NS**	**35S:OsAnn5**	***CoxIV:OsAnn5***	**DGP1:OsAnn5**
No stress	✓	✓	✓	✓
Salinity	X	✓	✓	X
Osmotic	X	✓	X	X
Drought	X	✓	X	✓

When we compared the biochemical parameters after the stress treatment 35S:*OsAnn5* transgenics were able to retain high chlorophyll content whereas CoxIV:*OsAnn5* and DGP1:*OsAnn5* transgenics did not retain much chlorophyll. This implicates that targeted expression of *OsAnn5* gene doesn't help the plant to retain more chlorophyll under the given stress conditions.

Under all the stress treatments, the *CoxIV:OsAnn5* transgenics accumulated more free proline osmolyte as 35S:*OsAnn5* transgenics. They were unable to maintain low levels of MDA under osmotic and drought stress. But after salt stress treatment, *CoxIV:OsAnn5* transgenics accumulated low MDA levels which is correlating with its salt stress tolerance. The drought stress tolerance of DGP1:OsAnn5 transgenics is also associated with the less MDA levels accumulation. This make us to suspect that OsAnn5 overexpression might be helping the plant in membrane integrity under stress and that lead to less lipid peroxidation, less MDA accumulation under stress.

Usually plant experiences two kind of stress to the salt stress. They are osmotic and ionic stresses. As 35S:*OsAnn5* transgenics, CoxIV:*OsAnn5* transgenics were also able to tolerate the osmotic stress created by the salinity stress, but not to the non-ionic osmotic stress created by the sorbitol. This suggest that *OsAnn5* might be helping the plant to tolerate abiotic stress more by maintaining the ionic misbalance.

Unlike other transgenics, DGP1 transgenics were unable to tolerate the salt stress. This intolerance shown positive correlation with the DAB staining assay where they accumulated more H_2O_2 after the salt stress. This can be due to that *OsAnn5* expression was limited to guard cells but not to other parts of the cell.

4.5. Conclusion

The targeted expression of the *OsAnn5* either to mitochondria or guard cell did not shown any enhanced tolerance. The stress tolerance ability of the targeted transgenics was shown negative correlation with its MDA levels. This study certainly suggest that systemic overexpression of *OsAnn5* is needed to develop stress tolerance in the plant.

CHAPTER 5

Summary of the Work

The current study tried to investigated the role of *OsAnn5* for abiotic stress tolerance through heterologous expression in transgenic tobacco and *E.coli*. *In silico* analysis of the 5' upstream region of *OsAnn5* identified several *cis*-elements that have been shown earlier to be essential for hormonal and stress regulation. *In silico* analysis of the protein sequence of *OsAnn5* revealed the presence of several probable sites for post-translational modifications and also the presence of a glycine-rich stretch.

Since *E.coli* is a simple system we tried to assess the functional role of the OsAnn5 by doing heterologous overexpression in it. The purified OsANN5 shown calcium binding activity. The *E.coli* cells, overexpressing the OsANN5 shown tolerance to high salinity, osmotic, drought and heat stresses. This study is implicating the functional role of the *OsAnn5* in abiotic stress tolerance. The 35S:OsAnn5 tobacco transgenic plants constitutively over-expressing *OsAnn5* (35S:*OsANN5*) showed tolerance to abiotic stresses like high salinity, drought and osmotic stress at the seedling stage and mature plant stage. Fluorescent signals for transiently expressed EGFP:OsANN5 were observed in the peripheral membrane regions of the onion epidermal cells under salt stress treatment.

We have also generated two other tobacco transgenics; in one of them OsANN5 was targeted to the mitochondria (*COXIV*:*OsAnn5)* and in the other, OsANN5 was expressed under a strong guard cell promoter, which expresses *OsAnn5* only in guard cells upon drought stress (DGP1:*OsAnn5)*. We performed a comparative analysis of the extent of tolerance, phenotypic and biochemical parameters under abiotic stresses in these three different tobacco transgenic plants, which revealed that 35S:*OsAnn5* transgenic plants are highly tolerant to all the stresses; However, the *COXIV*:*OsAnn5* transgenics were able to tolerate high salt and shown moderate tolerance to the drought stresses, while the DGP1:*OsAnn5* transgenic plants were tolerant only to drought stress. This comparison study suggests that, instead of targeted *OsAnn5* expression systemic OsAnn5 expression helped the tobacco transgenics to tolerate the given abiotic stress treatments.

It is observed that *OsAnn5* over expression lead to enhanced tolerance to abiotic stress through efficient scavenging of the ROS and balanced expression of SOD and CAT antioxidant enzymes in both the systems under stress treatments. Expression analysis of *OsAnn5* under ABA synthesis inhibitor and salinity stress revealed that it appears to act through an ABA-independent pathway under salt stress. In support to this ABA sensitivity assay suggests that 35S:OsAnn5 transgenics were insensitive to externally supplemented ABA.

Bibliography

Agarwal, P. K., & Jha, B. (2010). Transcription factors in plants and ABA dependent and independent abiotic stress signalling. *Biologia Plantarum*. https://doi.org/10.1007/s10535-010-0038-7

Ahmed, I., Yadav, D., Shukla, P., Vineeth, T., Sharma, P. C., & Kirti, P. B. (2017). Constitutive expression of Brassica juncea annexin, AnnBj2 confers salt tolerance and glucose and ABA insensitivity in transgenic plants. *Plant Science*. https://doi.org/10.1016/j.plantsci.2017.09.010

Andrawis, A., Solomon, M., & Delmer, D. P. (1993). Cotton fiber annexins: a potential role in the regulation of callose synthase. *The Plant Journal*, *3*(6), 763–772. https://doi.org/10.1111/j.1365-313X.1993.00763.x

Battey, N. H., & Blackbourn, H. D. (1993). Tansley Review No. 57. The control of exocytosis in plant cells. *New Phytologist*, *125*(2), 307–338.

BATTEY, N. H., & BLACKBOURN, H. D. (1993). The control of exocytosis in plant cells. *New Phytologist*, *125*(2), 307–338. https://doi.org/10.1111/j.1469-8137.1993.tb03883.x

Battey, N. H., James, N. C., & Greenland, a J. (1996). cDNA isolation and gene expression of the maize annexins p33 and p35. *Plant Physiology*, *112*(3), 1391–6. https://doi.org/112/3/1391 [pii]

Beyer, W. F., & Fridovich, I. (1987). Assaying for superoxide dismutase activity: Some large consequences of minor changes in conditions. *Analytical Biochemistry*. https://doi.org/10.1016/0003-2697(87)90489-1

Blackbourn, H. D., Barker, P. J., Huskisson, N. S., & Battey, N. H. (1992). Properties and partial protein sequence of plant annexins. *Plant Physiology*, *99*(3), 864–71. https://doi.org/10.1104/pp.99.3.864

Blackbourn, H. D., Walker, J. H., & Battey, N. H. (1991). Calcium-dependent phospholipid-binding proteins in plants. Their characterization and potential for regulating cell growth. *Planta*, *184*, 67–73.

Boustead, C. M., Smallwood, M., Small, H., Bowles, D. J., & Walker, J. H. (1989).

Identification of calcium-dependent phospholipid-binding proteins in higher plant cells. *FEBS Letters*, *244*(2), 456–460. https://doi.org/10.1016/0014-5793(89)80582-4

Braun, E. L., Kang, S., Nelson, M. A., & Natvig, D. O. (1998). Identification of the first fungal annexin: Analysis of annexin gene duplications and implications for eukaryotic evolution. *Journal of Molecular Evolution*, *47*(5), 531–543. https://doi.org/10.1007/PL00006409

Breton, G., Vazquez-Tello, A., Danyluk, J., & Sarhan, F. (2000). Two novel intrinsic annexins accumulate in wheat membranes in response to low temperature. *Plant and Cell Physiology*, *41*(2), 177–184. https://doi.org/10.1093/pcp/41.2.177

Calvert, C. M., Gant, S. J., & Bowles, D. J. (1996). Tomato annexins p34 and p35 bind to F-actin and display nucleotide phosphodiesterase activity inhibited by phospholipid binding. *The Plant Cell*, *8*(2), 333–42. https://doi.org/10.1105/tpc.8.2.333

Carroll, A., Moyen, C., Van Kesteren P, Tooke, F., Battey, N., & Brownlee, C. (1998). Ca2+, annexins, and GTP modulate exocytosis from maize root cap protoplasts. *The Plant Cell*, *10*(August), 1267–1276. https://doi.org/10.1105/tpc.10.8.1267

Çelik, Ö., & Atak, Ç. (2012). Th e eff ect of salt stress on antioxidative enzymes and proline content of two Turkish tobacco varieties. *Turk J Biol*. https://doi.org/10.3906/biy-1108-11

Chen, Q. F., Ya, H. Y., Feng, Y. R., & Jiao, Z. (2014). Expression of the key genes involved in ABA biosynthesis in rice implanted by ion beam. *Applied Biochemistry and Biotechnology*, *173*(1), 239–247. https://doi.org/10.1007/s12010-014-0837-y

Chinnusamy, V., Schumaker, K., & Zhu, J. K. (2004). Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. In *Journal of Experimental Botany* (Vol. 55, pp. 225–236). https://doi.org/10.1093/jxb/erh005

Clapham, D. E. (1995). Calcium signaling. *Cell*. https://doi.org/10.1016/0092-8674(95)90408-5

Clark, G. B., Lee, D., Dauwalder, M., & Roux, S. J. (2005). Immunolocalization and histochemical evidence for the association of two different Arabidopsis annexins with secretion during early seedling growth and development. *Planta*, *220*(4), 621–631. https://doi.org/10.1007/s00425-004-1374-7

Clark, G. B., Morgan, R. O., Fernandez, M. P., & Roux, S. J. (2012). Evolutionary adaptation of plant annexins has diversified their molecular structures, interactions and functional roles. *New Phytologist*, *196*(3), 695–712. https://doi.org/10.1111/j.1469-8137.2012.04308.x

Clark, G. B., & Roux, S. J. (1995). Annexins of plant cells. *Plant Physiology*, *109*(4), 1133–9.

Clark, G. B., Sessions, A., Eastburn, D. J., & Roux, S. J. (2001). Differential expression of members of the annexin multigene family in Arabidopsis. *Plant Physiology*, *126*(July), 1072–1084. https://doi.org/10.1104/pp.126.3.1072

Clark, G., Konopka-Postupolska, D., Hennig, J., & Roux, S. (2010). Is annexin 1 a multifunctional protein during stress responses? *Plant Signaling & Behavior*, *5*(3), 303–307. https://doi.org/10.4161/psb.5.3.10835

Cutler, S. R., Ehrhardt, D. W., Griffitts, J. S., & Somerville, C. R. (2000). Random GFP::cDNA fusions enable visualization of subcellular structures in cells of Arabidopsis at a high frequency. *Proceedings of the National Academy of Sciences*, *97*(7), 3718–3723. https://doi.org/10.1073/pnas.97.7.3718

Dabitz, N., Hu, N. J., Yusof, A. M., Tranter, N., Winter, A., Daley, M., … Hofmann, A. (2005). Structural determinants for plant annexin-membrane interactions. *Biochemistry*, *44*(49), 16292–16300. https://doi.org/10.1021/bi0516226

Daloso, D. M., Williams, T. C. R., Antunes, W. C., Pinheiro, D. P., & Caroline, M. (2015). Guard cell-specific upregulation of sucrose synthase 3 reveals that the role of sucrose in stomatal function is primarily energetic.

Daudi, A., & O'Brien, J. A. (2012). Detection of Hydrogen Peroxide by DAB Staining in Arabidopsis Leaves. *Bio-Protocol*, *2*(18), 1–5. https://doi.org/10.1007/BF00139728.5

Davies, J. (2014). Annexin-Mediated Calcium Signalling in Plants. *Plants*, *3*(1), 128–

140. https://doi.org/10.3390/plants3010128

Delmer, D. P., & Potikha, T. S. (1997). Structures and functions of annexins in plants. *Cellular and Molecular Life Sciences*, *53*(6), 546–553. https://doi.org/10.1007/s000180050070

Divya, K., Jami, S. K., & Kirti, P. B. (2010). Constitutive expression of mustard annexin, AnnBj1 enhances abiotic stress tolerance and fiber quality in cotton under stress. *Plant Molecular Biology*, *73*(3), 293–308. https://doi.org/10.1007/s11103-010-9615-6

Feng, Y. M., Wei, X. K., Liao, W. X., Huang, L. H., Zhang, H., Liang, S. C., & Peng, H. (2013). Molecular analysis of the annexin gene family in soybean, *57*(4), 655–662. https://doi.org/10.1007/s10535-013-0334-0

G. B. Clark, M. Dauwalder, and S. J. R. (1992). Purification and immunolocalization of an annexin-like protein in pea seedlings. *Planta*, *187*(3), 1–9. https://doi.org/10.1007/sl0869-007-9037-x

Gene, P., Yadav, D., Ahmed, I., & Kirti, P. B. (2015). Genome-wide identi fi cation and expression pro fi ling of annexins in Brassica rapa and their phylogenetic sequence comparison with B . juncea and A . thaliana annexins. *PLGENE*, *4*, 109–124. https://doi.org/10.1016/j.plgene.2015.10.001

Gerke, V., & Moss, S. E. (2002). [LIDO/COMPLEXO] Annexins : From Structure to Function. *Physiol. Rev*, *82*, 331–371. https://doi.org/10.1152/physrev.00030.2001

Gidrol, X., Sabelli, P. A., Fern, Y. S., & Kush, A. K. (1996). Annexin-like protein from Arabidopsis thaliana rescues delta oxyR mutant of Escherichia coli from H2O2 stress. *Proceedings of the National Academy of Sciences*, *93*(20), 11268–11273. https://doi.org/10.1073/pnas.93.20.11268

Gorantla, M., Babu, P. R., Lachagari, V. B. R., Feltus, F. A., Paterson, A. H., & Reddy, A. R. (2005). Functional genomics of drought stress response in rice: Transcript mapping of annotated unigenes of an indica rice (Oryza sativa L. cv. Nagina 22). *Current Science*, *89*(3), 496–514.

Gorecka, K. M., Konopka-Postupolska, D., Hennig, J., Buchet, R., & Pikula, S. (2005).

Peroxidase activity of annexin 1 from Arabidopsis thaliana. *Biochemical and Biophysical Research Communications*, *336*(3), 868–875. https://doi.org/10.1016/j.bbrc.2005.08.181

Gorecka, K. M., Thouverey, C., Buchet, R., & Pikula, S. (2007). Potential role of annexin AnnAt1 from Arabidopsis thaliana in pH-mediated cellular response to environmental stimuli. *Plant and Cell Physiology*, *48*(6), 792–803. https://doi.org/10.1093/pcp/pcm046

Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*, *125*(1), 189–198. https://doi.org/10.1016/0003-9861(68)90654-1

Hofmann, A., Delmer, D. P., & Wlodawer, A. (2003). The crystal structure of annexin Gh1 from Gossypium hirsutum reveals an unusual S3 cluster: Implications for cellulose synthase complex formation and oxidative stress response. *European Journal of Biochemistry*, *270*(12), 2557–2564. https://doi.org/10.1046/j.1432-1033.2003.03612.x

Hofmann, A., Proust, J., Dorowski, A., Schantz, R., & Huber, R. (2000). Annexin 24 from Capsicum annuum. *Biochemistry*, *275*(11), 8072–8082.

Hofmann, A., Ruvinov, S., Hess, S., Schantz, R., Delmer, D. P., & Wlodawer, A. (2002). Plant annexins form calcium-independent oligomers in solution. *Protein Science : A Publication of the Protein Society*, *11*(8), 2033–40. https://doi.org/10.1110/ps.4770102

Hoshino, D., Hayashi, A., Temmei, Y., Kanzawa, N., & Tsuchiya, T. (2004). Biochemical and immunohistochemical characterization of Mimosa annexin. *Planta*, *219*(5), 867–875. https://doi.org/10.1007/s00425-004-1285-7

Hu, N. J., Yusof, A. M., Winter, A., Osman, A., Reeve, A. K., & Hofmann, A. (2008). The crystal structure of calcium-bound annexin Gh1 from Gossypium hirsutum and its implications for membrane binding mechanisms of plant annexins. *Journal of Biological Chemistry*, *283*(26), 18314–18322. https://doi.org/10.1074/jbc.M801051200

Hu, T., Yang, J., Yang, Y., & Wu, Y. (2014). Molecular characterization, heterologous expression and resistance analysis of OsLEA3-1 from Oryza sativa. *Biologia*, *69*(5). https://doi.org/10.2478/s11756-014-0362-z

Huh, S. M., Noh, E. K., Kim, H. G., Jeon, B. W., Bae, K., Hu, H. C., … Park, O. K. (2010). Arabidopsis annexins AnnAt1 and AnnAt4 interact with each other and regulate drought and salt stress responses. *Plant and Cell Physiology*, *51*(9), 1499–1514. https://doi.org/10.1093/pcp/pcq111

Hurt, E. C., Pesold-hurt, B., & Schatz, G. (1984). The cleavable prepiece of an imported mitochondrial protein is sufficient to direct cytosolic dihydrofolate reductase into the mitochondrial matrixHurt, E. C., Pesold-hurt, B., & Schatz, G. (1984). The cleavable prepiece of an imported mitochondrial prote, *178*(2).

Ijaz, R., Ejaz, J., Gao, S., Liu, T., Imtiaz, M., Ye, Z., & Wang, T. (2017). Overexpression of annexin gene AnnSp2, enhances drought and salt tolerance through modulation of ABA synthesis and scavenging ROS in tomato. *Scientific Reports*, *7*(1), 12087. https://doi.org/10.1038/s41598-017-11168-2

Inoue, K., & Keegstra, K. (2003). A polyglycine stretch is necessary for proper targeting of the protein translocation channel precursor to the outer envelope membrane of chloroplasts. *Plant Journal*, *34*(5), 661–669. https://doi.org/10.1046/j.1365-313X.2003.01755.x

Jami, S. K., Clark, G. B., Ayele, B. T., Roux, S. J., & Kirti, P. B. (2012). Identification and characterization of annexin gene family in rice. *Plant Cell Reports*, *31*(5), 813–825. https://doi.org/10.1007/s00299-011-1201-0

Jami, S. K., Clark, G. B., Turlapati, S. A., Handley, C., Roux, S. J., & Kirti, P. B. (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*(12), 1019–1030. https://doi.org/10.1016/j.plaphy.2008.07.006

Jun, L. I., Ximing, G., Huiqiong, L. I. N., Quanbo, S., Jia, C., & Xuechen, W. (2005). DGP1 , a drought-induced guard cell-specific promoter and its function analysis in tobacco plants, *48*(2), 181–186. https://doi.org/10.1360/04yc0004

Kaplan, B., Davydov, O., Knight, H., Galon, Y., Knight, M. R., Fluhr, R., & Fromm, H. (2006). Rapid Transcriptome Changes Induced by Cytosolic Ca2+ Transients

Reveal ABRE-Related Sequences as Ca2+-Responsive cis Elements in Arabidopsis. *THE PLANT CELL ONLINE*. https://doi.org/10.1105/tpc.106.042713

Kodavali, P. K., Dudkiewicz, M., Pikuła, S., & Pawłowski, K. (2014). Bioinformatics analysis of bacterial annexins - Putative ancestral relatives of eukaryotic annexins. *PLoS ONE*, *9*(1). https://doi.org/10.1371/journal.pone.0085428

Konopka-Postupolska, D. (2007). Annexins: Putative linkers in dynamic membrane-cytoskeleton interactions in plant cells. *Protoplasma*, *230*(3–4), 203–215. https://doi.org/10.1007/s00709-006-0234-7

Konopka-Postupolska, D., Clark, G., Goch, G., Debski, J., Floras, K., Cantero, A., … Hennig, J. (2009). The Role of Annexin 1 in Drought Stress in Arabidopsis. *Plant Physiology*, *150*(3), 1394–1410. https://doi.org/10.1104/pp.109.135228

Kumar, K. R. R., & Kirti, P. B. (2011). Differential gene expression in Arachis diogoi upon interaction with peanut late leaf spot pathogen, Phaeoisariopsis personata and characterization of a pathogen induced cyclophilin. *Plant Molecular Biology*, *75*(4–5), 497–513. https://doi.org/10.1007/s11103-011-9747-3

Kumari, S., Singh, P., Singla-Pareek, S. L., & Pareek, A. (2009). Heterologous expression of a salinity and developmentally regulated rice cyclophilin gene (OsCyp2) in E. coli and S. cerevisiae confers tolerance towards multiple abiotic stresses. *Molecular Biotechnology*, *42*(2), 195–204. https://doi.org/10.1007/s12033-009-9153-0

Laohavisit, A., & Davies, J. M. (2011). Annexins. *New Phytol*, *189*(1), 40–53. https://doi.org/10.1111/j.1469-8137.2010.03533.x

Lim, E. K., Roberts, M. R., & Bowles, D. J. (1998). Biochemical characterization of tomato annexin p35. Independence of calcium binding and phosphatase activities. *Journal of Biological Chemistry*, *273*(52), 34920–34925. https://doi.org/10.1074/jbc.273.52.34920

Lindermayr, C., Saalbach, G., & Durner, J. (2005). Proteomic Identification of S - Nitrosylated Proteins. *Plant Physiology*, *137*(March), 921–930. https://doi.org/10.1104/pp.104.058719.1

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Mahajan, S., Mahajan, S., Tuteja, N., & Tuteja, N. (2005). Cold, salinity and drought stresses: an overview. *Archives of Biochemistry and Biophysics*, *444*, 139–158. https://doi.org/10.1016/j.abb.2005.10.018

Mcclung, A. D., Carroll, A. D., & Battey, N. H. (1994). Identification and characterization of ATPase activity associated with maize, *712*.

Medvedev, S. S. (2005). Calcium Signaling System in Plants, *52*(2), 249–270.

Modell, H., Cliff, W., Michael, J., Mcfarland, J., Wenderoth, M. P., Wright, A., … Physiol, A. (2019). A physiologist ' s view of homeostasis, *98115*, 259–266. https://doi.org/10.1152/advan.00107.2015

Mortimer, J. C., Laohavisit, A., Macpherson, N., Webb, A., Brownlee, C., Battey, N. H., & Davies, J. M. (2008). Annexins: Multifunctional components of growth and adaptation. *Journal of Experimental Botany*, *59*(3), 533–544. https://doi.org/10.1093/jxb/erm344

Moss, S. E., & Morgan, R. O. (2004). The annexins. *Genome Biology*, *5*(4), 219. https://doi.org/10.1186/gb-2004-5-4-219

Mýtinová, Z., Motyka, V., Haisel, D., Gaudinová, A., Lubovská, Z., & Wilhelmová, N. (2010). Effect of abiotic stresses on the activity of antioxidative enzymes and contents of phytohormones in wild type and AtCKX2 transgenic tobacco plants. *Biologia Plantarum*. https://doi.org/10.1007/s10535-010-0082-3

Plesch, G., Ehrhardt, T., & Mueller-Roeber, B. (2001). Involvement of TAAAG elements suggests a role for Dof transcription factors in guard cell-specific gene expression. *Plant Journal*. https://doi.org/10.1046/j.1365-313X.2001.01166.x

Proust, J., Houlné, G., Schantz, M. L., Shen, W. H., & Schantz, R. (1999). Regulation of biosynthesis and cellular localization of Sp32 annexins in tobacco BY2 cells. *Plant Molecular Biology*, *39*(2), 361–372. https://doi.org/10.1023/A:1006199814795

Ramachandra Reddy, A., Chaitanya, K. V., Jutur, P. P., & Sumithra, K. (2004). Differential antioxidative responses to water stress among five mulberry (Morus alba L.) cultivars. *Environmental and Experimental Botany*, *52*(1), 33–42. https://doi.org/10.1016/j.envexpbot.2004.01.002

Raynal, P., & Pollard, H. B. (1994). Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *BBA - Reviews on Biomembranes*, *1197*(1), 63–93. https://doi.org/10.1016/0304-4157(94)90019-1

Reddy, V. S., & Reddy, A. S. N. (2004). Proteomics of calcium-signaling components in plants. *Phytochemistry*. https://doi.org/10.1016/j.phytochem.2004.04.033

Robles, P., & Quesada, V. (2019). Transcriptional and Post-transcriptional Regulation of Organellar Gene Expression (OGE) and Its Roles in Plant Salt Tolerance, 1–16. https://doi.org/10.3390/ijms20051056

Seals, D. F., Parrish, M. L., & Randall, S. K. (1994). A 42-kilodalton annexin-like protein is associated with plant vacuoles. *Plant Physiology*, *106*(4), 1403–12. https://doi.org/106/4/1403 [pii]

Seals, D. F., & Randall, S. K. (1997). A Vacuole-Associated Annexin Protein, VCaB42, Correlates with the Expansion of Tobacco Cells. *Plant Physiology*, *115*(2), 753–761. https://doi.org/10.1104/pp.115.2.753

Seigneurin-Berny, D., Rolland, N., Dorne, J., & Joyard, J. (2000). Sulfolipid is a potential candidate for annexin binding to the outer surface of chloroplast. *Biochemical and Biophysical Research Communications*, *272*(2), 519–24. https://doi.org/10.1006/bbrc.2000.2805

Shen, C., Que, Z., Xia, Y., Tang, N., Li, D., He, R., & Cao, M. (2017). Knock out of the annexin gene OsAnn3 via CRISPR/Cas9-mediated genome editing decreased cold tolerance in rice. *Journal of Plant Biology*, *60*(6), 539–547. https://doi.org/10.1007/s12374-016-0400-1

Shi, J., Dixon, R. A., Gonzales, R. A., Kjellbom, P., & Bhattacharyya, M. K. (1995). Identification of cDNA clones encoding valosin-containing protein and other plant plasma membrane-associated proteins by a general immunoscreening strategy.

Proc Natl Acad Sci U S A, *92*(10), 4457–4461.

Shin, H., & Brown, R. M. (1999). GTPase activity and biochemical characterization of a recombinant cotton fiber annexin. *Plant Physiology*, *119*(3), 925–934. https://doi.org/10.1091/mbc.E06-01-0041

Smallwood, M. F., Gurr, S. J., McPherson, M. J., Roberts, K., & Bowles, D. J. (1992). The pattern of plant annexin gene expression. *The Biochemical Journal*, *281 (Pt 2*, 501–505.

Smallwood, M. F., Keen, J. N., & Bowles, D. J. (1990). Purification and partial sequence analysis of plant annexins. *The Biochemical Journal*, *270*, 157–161.

Smith, P. D., & Moss, S. E. (1994). Structural evolution of the annexin supergene family. *Trends in Genetics*, *10*(7), 246. https://doi.org/10.1016/0168-9525(94)90171-6

Sun, W., Cao, Z., Li, Y., Zhao, Y., & Zhang, H. (2007). A simple and effective method for protein subcellular localization using Agrobacterium-mediated transformation of onion epidermal cells. *Biologia*, *62*(5), 529–532. https://doi.org/10.2478/s11756-007-0104-6

Szabados, L., & Savouré, A. (2010). Proline: a multifunctional amino acid. *Trends in Plant Science*. https://doi.org/10.1016/j.tplants.2009.11.009

Szalonek, M., Sierpien, B., Rymaszewski, W., Gieczewska, K., Garstka, M., Lichocka, M., … Konopka-Postupolska, D. (2015). Potato annexin STANN1 promotes drought tolerance and mitigates light stress in transgenic Solanum tuberosum L. plants. *PLoS ONE*, *10*(7), 1–38. https://doi.org/10.1371/journal.pone.0132683

Wang, W., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. *Planta*. https://doi.org/10.1007/s00425-003-1105-5

Yadav, D., Ahmed, I., Shukla, P., Boyidi, P., & Kirti, P. (2016). Overexpression of Arabidopsis AnnAt8 Alleviates Abiotic Stress in Transgenic Arabidopsis and Tobacco. *Plants*, *5*(2), 18. https://doi.org/10.3390/plants5020018

Yadav, D., Boyidi, P., Ahmed, I., & Kirti, P. B. (2018). Plant annexins and their involvement in stress responses. *Environmental and Experimental Botany*, *155*, 293–306. https://doi.org/10.1016/J.ENVEXPBOT.2018.07.002

Yamaguchi-shinozakiaib, K., & Shinozaki, K. (1994). A Nove1 cis-Acting Element in an Arabidopsis Gene 1 s lnvolved in Responsiveness to Drought , Lowqemperature , or High-Salt Stress, *6*(February), 251–264.

Zhang, Y., Wang, Q., Zhang, X., Liu, X., Wang, P., & Hou, Y. (2011). Cloning and Characterization of an Annexin Gene from Cynanchum komarovii that Enhances Tolerance to Drought and Fusarium oxysporum in Transgenic Cotton. *Journal of Plant Biology*, *54*(5), 303–313. https://doi.org/10.1007/s12374-011-9167-6

Zhou, C., Chen, R. J., Gao, X. L., Li, L. H., & Xu, Z. J. (2014). Heterologous expression of a rice RNA-recognition motif gene OsCBP20 in escherichia coli confers abiotic stress tolerance. *Plant OMICS*, *7*(1), 28–34.

Zhou, L., Duan, J., Wang, X. M., Zhang, H. M., Duan, M. X., & Liu, J. Y. (2011). Characterization of a Novel Annexin Gene from Cotton (Gossypium hirsutumcv CRI 35) and Antioxidative Role of its Recombinant Protein. *Journal of Integrative Plant Biology*, *53*(5), 347–357. https://doi.org/10.1111/j.1744-7909.2011.01034.x

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3. Plant Science colloquium, University of Hyderabad, March 19th, 2016

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Overexpression of *Arabidopsis AnnAt8* Alleviates Abiotic Stress in Transgenic *Arabidopsis* and Tobacco

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Abstract: Abiotic stress results in massive loss of crop productivity throughout the world. Because of our limited knowledge of the plant defense mechanisms, it is very difficult to exploit the plant genetic resources for manipulation of traits that could benefit multiple stress tolerance in plants. To achieve this, we need a deeper understanding of the plant gene regulatory mechanisms involved in stress responses. Understanding the roles of different members of plant gene families involved in different stress responses, would be a step in this direction. *Arabidopsis*, which served as a model system for the plant research, is also the most suitable system for the functional characterization of plant gene families. Annexin family in *Arabidopsis* also is one gene family which has not been fully explored. Eight annexin genes have been reported in the genome of *Arabidopsis thaliana*. Expression studies of different *Arabidopsis* annexins revealed their differential regulation under various abiotic stress conditions. *AnnAt8* (At5g12380), a member of this family has been shown to exhibit ~433 and ~175 fold increase in transcript levels under NaCl and dehydration stress respectively. To characterize Annexin8 (AnnAt8) further, we have generated transgenic *Arabidopsis* and tobacco plants constitutively expressing AnnAt8, which were evaluated under different abiotic stress conditions. *AnnAt8* overexpressing transgenic plants exhibited higher seed germination rates, better plant growth, and higher chlorophyll retention when compared to wild type plants under abiotic stress treatments. Under stress conditions transgenic plants showed comparatively higher levels of proline and lower levels of malondialdehyde compared to the wild-type plants. Real-Time PCR analyses revealed that the expression of several stress-regulated genes was altered in AnnAt8 over-expressing transgenic tobacco plants, and the enhanced tolerance exhibited by the transgenic plants can be correlated with altered expressions of these stress-regulated genes. Our findings suggest a role for AnnAt8 in enhancing abiotic stress tolerance at different stages of plant growth and development.

Keywords: annexin; seedling growth; osmotic stress; methyl viologen stress; salt stress; seed germination

1. Introduction

Abiotic stress includes physical and chemical changes in the environment, which affect normal growth and productivity of the plant. Due to shifts in the climatic conditions and improper management in farming, abiotic stress has become a challenge for the global agriculture industry. Abiotic stress ultimately could lead to the loss of cell homeostasis and cell death. In order to maintain stability in cell structure and functional machinery inside the cell [1], plants have evolved a group of genes, whose expression helps them survive the adverse conditions [2]. Salinity and drought are common stresses, which plants face during their life cycle. Few plant species have evolved in such a way that they have developed adaptations, and can complete their life cycle under these stressful conditions. However, in other species, their defense system becomes operational when exposed to the stress [3–5]. The defense responses also vary with the severity and duration of stress given to a plant. In mild stress conditions, expressions of stress-induced genes do not overlap each other. However, under severe stress conditions, a common set of genes may upregulate, which could be due to the generation of reactive oxygen species (ROS) [6]. Many genes involved in defense mechanisms against abiotic stresses have been identified so far. Annexins are also among them. They are present throughout plants, animals and fungi. Recently their presence has also been reported in prokaryotes. Annexin is a superfamily of genes, and plant annexins which belong to family D, have been further grouped into different subfamilies [7,8]. In the course of evolution, plant annexins have become diverse in their structure and function [8]. They have been shown to play a significant role in plant growth and development under normal and stress conditions [8–11]. In plants, the first multigene annexin family was identified in *Arabidopsis* with eight members representing this family, and expression and characterization studies have established them as a multifunctional protein family [8,11–18]. They possess different properties like Ca^{2+} binding, ion permeability, and peroxidase activity, which have been correlated with their responses during plant development and stress condition [19]. An increase in plant annexin abundance and their recruitment to membrane has been reported under different stress conditions [20–23]. This recruitment of annexins to the membrane has been linked to many functions like channel properties, protection of membrane and ROS-induced signaling [24,25]. Another important property of annexin is Ca^{2+} dependent lipid binding. Recent reports suggest that annexins can mediate the ROS-induced changes in Ca^{2+} permeability of membrane [26–29]. This annexin-mediated $Ca^{2+}{}_{(Cyt)}$ modulation is supposed to play a significant role in abiotic stress signaling and gene regulation. Annexin regulatory responses can also be mediated by changes in phytohormone level during plant growth and development in stress conditions [14]. There is limited information on how these properties work in a cumulative manner during stress responses. Previous expression studies of annexin family members in different plants showed differential expression pattern under normal and stress conditions [12,16,30–37]. Proteomic studies in many plant species also displayed their upregulation under salinity [18,38,39] and heat stress [15,40,41]. In a transcriptome study in *Arabidopsis*, annexin members showed a differential expression pattern during seed and seedling stages, and most of them were shown to be up-regulated in various abiotic stress treatments [31]. Previous studies on AnnAt1, AnnAt2, and AnnAt4 have indicated their role in salt and drought tolerance [14,20,21], whereas AnnAt5 plays a role in pollen development [16,17]. Among the other members of *Arabidopsis* annexin family, *AnnAt8* (AT5G12380) has been reported for its high fold transcript accumulation during seedling stage under salt and dehydration stress and showed similar response comparable to *Rd29* and *P5Cs* (two marker genes for salt stress in *Arabidopsis*) [31]. Some tissue-specific gene expression studies in *Arabidopsis* also showed the presence of *AnnAt8* transcript in female gametophyte [42,43]. In a recent study, it has been demonstrated that *AnnAt8* expression in *Arabidopsis* roots does not change on treatment with H_2O_2 [29]. Above mentioned expression studies suggest a possible role of AnnAt8 in plant growth and development in normal as well as in stress conditions. Since gene expression studies do not always lead to conclusions about the gene function, we followed the overexpression strategy of gene characterization. Further to this, we generated *AnnAt8* overexpressing transgenic tobacco and *Arabidopsis* plants and analyzed their performance under different stress conditions. The current study provides evidence for the involvement of AnnAt8 in alleviating abiotic stress in transgenic *Arabidopsis* and tobacco.

2. Materials and Methods

2.1. AnnAt8 Construct Preparation

The full-length cDNA of the *AnnAt8* gene (Locus: At5g12380 and NCBI GenBank Accession No: NM_121276) was amplified by using following primers (ORF1 For and ORF1 Rev harboring *Nco*I and *Xba*I sites respectively) and cloned in plant expression vector pRT100. Primers employed for the amplification of ORFI are listed in Supplementary Table S1. Expression cassette with CaMV35S promoter and poly-adenylation signal was released from the pRT100 using *Pst*I digestion, and the same site was used to clone the expression cassette into the binary vector pCAMBIA2300.

2.2. Plant Material and Experimental Conditions

Arabidopsis thaliana ecotype Col 0 was used for generating the transgenic plants constitutively expressing AnnAt8. The *Arabidopsis* plants were grown in a growth room under standard controlled light conditions. For vegetative growth of plants controlled temperature (21 ± 2 °C) and light conditions (8 h of light/16 h of dark period) were used in the growth chamber. For reproductive growth, 16 h of light/8 h of dark period was used. Light intensity in growth chamber was maintained at 100–150 $\mu mol\cdot m^{-2}\cdot s^{-1}$.

Nicotiana tabacum Linn cultivar Samsun was used for transgenic generation. The tobacco plants were grown in the greenhouse under natural light conditions. Seed germination was performed, and seedlings were maintained in a growth room at 26 ± 2 °C with 16 h of light/8 h of dark.

2.3. Plant Transformation

Arabidopsis plant transformation was performed by the floral dip method with minor modifications [44]. *Agrobacterium* strain EHA 105 harboring the construct pCAMBIA2300::AnnAt8 was used to transform the *Arabidopsis* following standard *in planta* protocol [44]. For the floral dip transformation, an appropriate flowering stage was selected, and a bunch of flowering stalks was inverted to dip in *Agrobacterium* cell (harboring pCAMBIA2300::AnnAt8) suspension containing 5% sucrose and 0.05% Silwet L-77 for two min. Further, the plants were wrapped with plastic films to maintain high humidity for 16–24 h in the dark. Finally, the plastic covers were removed, and plants were allowed to grow to silique formation and maturity, which was followed by drying and harvesting of seeds. Primary transformants were selected in T_1 generation using kanamycin (50 mg/L). The seeds from the putative transgenics were again selfed to obtain T_3 progenies through T_2.

Tobacco plant transformation was performed by leaf disc transformation method with minor modifications [45]. Small pieces of fully grown leaves were surface sterilized by 0.01% $HgCl_2$ for 3–5 min, which was followed by 4–5 washes with sterilized water. The explants were then incubated in *Agrobacterium* (strain EHA105) cell (harboring pCAMBIA2300::AnnAt8 construct) suspension. Subsequently, the treated explants were dried with sterilized tissue paper and placed on full MS-Agar medium (with 2.0 mg/L BAP and 0.1 mg/L NAA) for cocultivation. After two days of cocultivation, the explants were transferred to the shoot initiation medium (MS-Agar medium with 2.0 mg/L BAP and 0.1 mg/L NAA) with kanamycin (125 mg/L) for transformed shoot selection and Cefotaxime (250 mg/L) to check *Agrobacterium* overgrowth. After four weeks on the shoot induction medium, newly formed shoots from the callus were transferred to shoot elongation (MS-Agar) medium. Further, the elongated shoots were transferred to a root initiation medium (MS-Agar medium with 0.1 mg/L NAA); both media contained Kanamycin for selection. Finally, the selected putative transgenic plantlets were kept for hardening in the growth room and then transferred to the greenhouse for further growth and analysis. The seeds from the putative primary transgenic plants were collected through self-pollination and were germinated on Kanamycin containing medium to screen the T_1 plants, the confirmed plants were selfed to obtain T_2 progeny.

2.4. Seed Germination Analysis in Salt and Osmotic Stress

For seed germination assay, *AnnAt8* overexpressing transgenic (T_3 generation) and WT *Arabidopsis* seeds were surface sterilized by 1% sodium hypochlorite (NaOCl) for one min and then washed with sterile water 4–5 times. The seeds were transferred to the ½ MS-Agar medium and maintained in the growth chamber with a photoperiod of 8/16 h light/dark regime at 21 ± 2 °C room temp. For salt treatment, we used 100 mM of NaCl in half strength MS medium; for osmotic stress treatment, 250 mM sorbitol and for ABA treatment, 5 μM of ABA was used.

To test the seed germination rates in tobacco, T_2 transgenic (T-14.3 and T-15.2) and WT (Control) seeds were surface sterilized in 75% ethanol for one min, which was followed by treatment with sodium hypochlorite (NaOCl) for ten min. After sterilization, seeds were washed 4–5 times with sterile water and transferred to the half strength MS + 0.8% Agar (HiMedia Lab. Pvt. Ltd. Mumbai, India) plates and maintained in a culture room with a photoperiod of 16 h light and 8 h dark at room temp 27 ± 1 °C. For salt treatment, we used 0, 100, 150 and 200 mM of NaCl in half strength MS-Agar medium. For inducing osmotic stress, 0, 300 and 400 mM sorbitol concentrations were used.

2.5. Seedling Stress Assay

For *Arabidopsis* seedling assays, *AnnAt8* overexpressing *Arabidopsis* transgenic lines and WT seeds were surface sterilized by NaOCl and were allowed to germinate in a growth chamber maintained at 21 ± 2 °C with a photoperiod of 8/16 h light/dark. After seven days of post-germination growth on half strength MS-Agar medium, WT and *AnnAt8* overexpressing seedlings were transferred to stress media and were grown on vertically placed agar plates. Seedlings of WT and transgenic lines were allowed to grow further on only half strength MS medium for control, and half strength MS media with 100 mM NaCl for salt stress and 250 mM sorbitol for osmotic stress respectively. For all experiments, except the NaCl treatment, seedlings were grown on semi-vertical agar plates.

To evaluate the stress tolerance of transgenic tobacco plants at the seedling stage, we exposed the transgenic and WT seedlings to different stress conditions for different time periods. For all the bioassays performed in the current study; we used T_2 generation seeds. For seedling assay, T_2 generation seeds of transgenic lines, and WT were surface sterilized by NaOCl and placed on half strength MS (without organics) + 0.8% Agar (HiMedia Lab. Pvt. Ltd. India). Seedlings were allowed to grow for ten days in a growth chamber maintained at 27 ± 1 °C with a photoperiod of 16/8 h light/dark. Ten days old seedlings of WT and transgenic lines were transferred on half strength MS-Agar medium with 100 and 200 mM NaCl for salt stress. Dehydration stress was given by 200 and 300 mM sorbitol in half strength MS-Agar medium. For photooxidative stress, 10 μM Methyl Viologen (MV) was used in half strength MS-Agar medium. For each experiment, seedlings were grown on horizontal agar plates.

2.6. Leaf Disc Senescence Assay

Fully grown leaves from two-month old tobacco plants were taken for leaf disc senescence assays. The leaf discs of 1 cm diameter were excised with the help of a cork borer and used further in stress treatments. Sorbitol (300, 400 and 500 mM conc) was used to induce dehydration stress. Similarly, NaCl solutions of 100, 200 and 300 mM concentrations were used for salt stress. Water was used as a control. The treatments were carried out in continuous light until identifiable differences were observed.

2.7. Biochemical Analyses: Determination of Chlorophyll Content and Lipid Peroxidation Assay

To measure the total chlorophyll content of seedlings and leaf discs, equal quantities of treated and non-treated samples were taken. The total chlorophyll content of seedlings and senescing leaf discs was estimated according to Hiscox and Israelstam [46]. Lipid peroxidation in the form of thiobarbituric acid reactive substances was estimated following the MDA estimation protocol [47].

2.8. Estimation of Proline Content in Transgenic and WT Tobacco Seedlings

Fifteen day-old seedlings of WT and transgenic lines were transferred onto half strength MS-Agar medium with 200 mM NaCl, and seedlings grown only on half strength MS-Agar medium were used as a control. After four days of NaCl treatment, seedlings were used for estimation of proline according to Bates *et al.* [48].

2.9. Real Time Expression Analysis of Stress-Inducible Genes in WT and Transgenic Tobacco Plants

For the evaluation of transcript levels of different stress marker genes, 15 day-old transgenic, and WT tobacco seedlings were incubated with half strength MS (control) and 200 mM NaCl (treatment) for 6 h and samples were collected for RNA isolation. Total RNA was extracted using TRIZOL (Invitrogen, Waltham, MA, USA). The first strand of cDNA was prepared from 2 μg of total RNA using MMLV Reverse Transcriptase (Clontech, Mountain View, CA, USA) in a reaction volume of 20 μL. For the real-time expression analysis, FAST START-SYBR MIX (Roche, Penzberg, Germany) was used. All the reactions were performed in Mastercycler® 204 ep realplex4 (Eppendorf, Hamburg, Germany). PCR program used for all the amplifications was 95 °C for 10 min and 40 cycles of 95 °C for 15 s (denaturation), 57 °C for 30 s (annealing), 72 °C for 30 s (elongation). Primers used in the qPCR analysis are listed in Supplementary Table S3. Each sample was amplified in three independent biological replicates with three technical replicates. Relative gene expression was calculated according to the $\Delta\Delta C_T$ method [49]. *NtActin* was used as a reference gene to normalize gene expression in tobacco. ΔC_T for WT (untreated) was used as a control for the calculation of relative changes in expression of transgenic lines (untreated) and NaCl-treated WT and transgenic lines.

2.10. Subcellular Localization of AnnAt8 Protein

To check the subcellular localization of AnnAt8 protein, the ORF was reamplified with ORF2-F and ORF2-R primers harboring *Eco*RI and *Sma*I sites respectively. The amplified product was further digested and cloned in the pEGAD vector at the same sites, as a C-terminal fusion protein. Primers used in the amplification of ORF2 are listed in Supplementary Table S1. Further, the confirmed vectors were mobilized into *Agrobacterium tumefaciens* strain EHA105. The *Agrobacterium* cell (harboring the pEGAD-AnnAt8 construct) suspension was infiltrated in tobacco leaves (*Nicotiana tabacum* Linn cultivar Samsun) and GFP expression was visualized under the confocal microscope (Carl Zeiss LSM 710 NLO ConfoCor 3, Germany) at 480 to 515 nm wavelength after a 48 h incubation period, according to Kumar and Kirti [50].

2.11. Statistical Analysis

All the data was analyzed by the statistical analysis of variance (one-way ANOVA) by SIGMA PLOT, St. Louis, MO, USA ver. 11.0.

3. Results

3.1. Molecular Analysis of Transgenic Arabidopsis and Tobacco Plants

For the molecular and functional characterization of AnnAt8, *AnnAt8* overexpressing transgenic *Arabidopsis* and tobacco plants were generated through *Agrobacterium*-mediated genetic transformation of *Arabidopsis thaliana* ecotype Col 0 and *Nicotiana tabacum* L. cultivar Samsun. Transgenic *Arabidopsis* plants were generated by the standard floral dip method [44]. In T_1 generation, putative transgenic plants were selected on half strength MS medium containing Kanamycin (50 mg/L), and the putative transformed plants were analyzed for the presence of transgene by CaMV35S and AnnAt8 gene-specific PCR (Supplementary Figure S1). Along with this, the T-DNA integration was cross confirmed by *NptII* gene specific PCR (data not shown). Primer sequences used for the PCR analysis of transgenes are mentioned in the Supplementary Table S2. Further, on the basis of *AnnAt8* gene expression analysis in the T_1 generation (L-3, L-4, L-7, L-8, L-10, L-14, L-15 and L-19) (Figure 1), two transgenic lines (L-3, L-7) were finally selected for detailed analysis in the T_3 generation (obtained through selfing and cross checking).

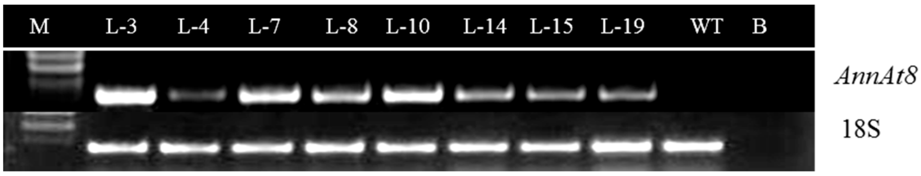

Figure 1. Expression analysis of *AnnAt8* by semi-quantitative RT-PCR in *AnnAt8* overexpressing transgenic *Arabidopsis* plants confirmed at T_1 generation. WT is taken as a non-transgenic control, B is non-template control. 18S used as reference gene.

Since the functional analysis of AnnAt8 in *Arabidopsis thaliana* transgenic showed the AnnAt8 involvement in abiotic stress tolerance, we proceeded to see whether *AnnAt8* can be used for the crop transformation purposes for the improving stress tolerance. Hence, tobacco was used as a model system, and we generated transgenic tobacco plants expressing *AnnAt8* in a constitutive manner and evaluated the plants for stress tolerance at different stages of growth. Tobacco transformation was done by standard leaf disc protocol [45]. In T_0 generation, 16 independent putative transformants were analyzed for the presence of transgenes by gene-specific PCR using *NptII* (Supplementary Figure S2) and *AnnAt8* gene specific primers. Primer sequences used for the PCR analysis of transgene are mentioned in the Supplementary Table S2. Further, on the basis of *AnnAt8* gene expression analysis using semi-quantitative RT-PCR in putative transgenic plants of T_0 generation (Figure 2), eight plants (T-11, 12, 14, 15, 16, 17, 19 and T-20) were selected for further analysis in T_1 generation. Based on the expression analysis and stress assay performed in the T_1 generation on T-11.1, T-12.1, T-14.3, T-15.2, T-17.1, T-19.1 and T-20.1, three transgenic lines were finally selected for detailed analysis (T-14.3, T-15.2, T-19.1) in the T_2 generation. Transgenic lines T-14.3 and T-15.2 were used in all assays while line T-19.1 was also included in some experiments.

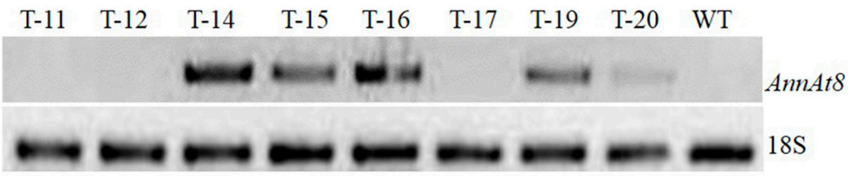

Figure 2. Expression analysis of *AnnAt8* in *AnnAt8* overexpressing transgenic tobacco plants by semi-quantitative RT-PCR. WT is taken as non-transgenic control. 18S was used as a reference.

3.2. Enhanced Seed Germination of Arabidopsis Transgenic Plants under Stress Conditions

Seed germination rates of WT and *AnnAt8* overexpressing *Arabidopsis* seeds were checked under different stress treatments. On regular half strength MS medium, more than 90% of WT and transgenic seeds completed germination at day 3. In the case of NaCl (100 mM) treatment, WT showed only 20% seed germination, while transgenic lines showed more than 90% of seed germination. At 5 µM ABA, a strong inhibition was observed in the germination of WT seeds, and only 20% seed germination was observed. However, the transgenic lines showed more than 90% seed germination at this concentration of ABA. The osmotic stress condition generated by adding sorbitol in the medium showed comparatively less inhibition in seed germination; 50% of seed germination was observed in the case of WT at 250 mM sorbitol concentration while *AnnAt8* overexpressing (OE) lines showed more than 95% seed germination at the same concentration. In each case, observations were made on day 3 after initiation of the treatment (Figure 3).

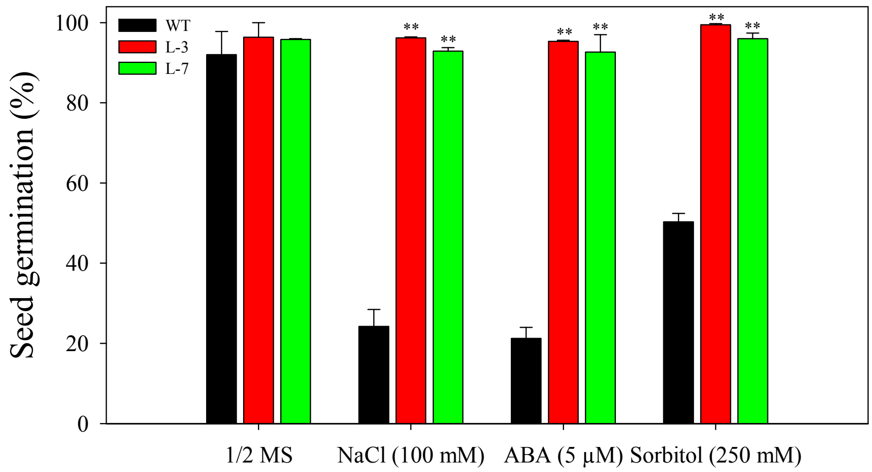

Figure 3. Seed germination of WT and *AnnAt8* overexpressing (OE) *Arabidopsis* plants under different stress conditions. Seed germination percentage of WT and *AnnAt8* OE *Arabidopsis* transgenic lines L-3 and L-7 under normal (half strength MS) and salt (100 mM), ABA (5 μM) and osmotic stress (sorbitol 250 mM). In each case, the observations were made on the third day after incubation under germination condition. For each biological replicate (n), 100 seeds of WT and transgenic lines were taken. Data represent the means of $n = 3 \pm$ SE. Two asterisks indicate that the mean values of transgenic lines were significantly higher than that of WT (control) when analyzed by one-way ANOVA ($p < 0.01$).

3.3. Salt and Dehydration Stress Tolerance of AnnAt8 Transgenic Arabidopsis Plants

Different growth parameters like root growth and fresh weight were assayed to observe the effect of stress conditions on the growth of seedlings and young plants of WT and *AnnAt8* OE lines L-3 and L-7. After 44 days of growth on NaCl (100 mM) medium, we observed that NaCl caused less inhibition on the growth of *AnnAt8* expressing plants in comparison to WT plants (Figure 4A,B).

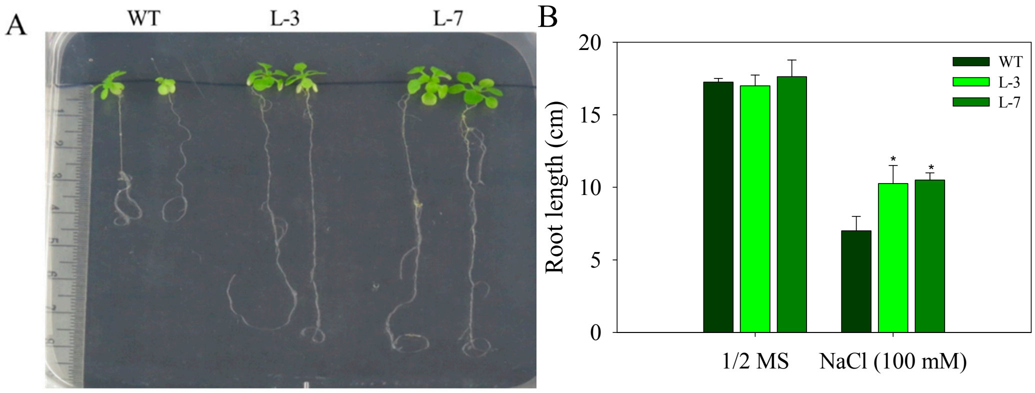

Figure 4. The growth of WT and *AnnAt8* overexpressing *Arabidopsis* plants under normal and salt (100 mM) stress conditions (**A**) Plant growth of WT and *AnnAt8* expressing *Arabidopsis* transgenic lines L-3 and L-7 under NaCl (100 mM) stress. Three replicates were used per treatment, and the experiment was repeated thrice. (**B**) Root length of WT and AnnAt8 expressing *Arabidopsis* transgenic plants under normal and salt stress. In each case, observations were made after 44 days from germination. For root length measurement, average value for 15 plantlets of WT and transgenic lines was taken for each biological replicate (n). Data represent means of $n = 3 \pm$ SE. One asterisk indicates that the mean values of transgenic lines were significantly higher than that of WT (control), when analyzed by one-way ANOVA ($p < 0.05$).

As it is known that NaCl imposes osmotic stress as well as ion toxicity on the plants, we used sorbitol in half strength MS-Agar medium to check for the osmotic stress effect and monitored the plant growth at different time points. At the early stage of observation after 15 days of growth, we found that the growth of WT seedlings was more hampered than *AnnAt8* expressing lines L-3 and L-7 (data not shown). We also noted that the growth inhibition caused by 250 mM Sorbitol was less than that of 100 mM NaCl treatment (data not shown). Another observation made at day 28 showed that the transgenic plants not only survived under high osmotic stress but also showed better root growth, with about threefold increase as compared to WT (Figure 5), while in the normal growth condition, a negligible difference was seen between the growth of WT and transgenic plant. With the advancement of time, we observed that the transgenic plants showed better adaptation to the dehydration stress while strong inhibition was observed in the growth of WT plants.

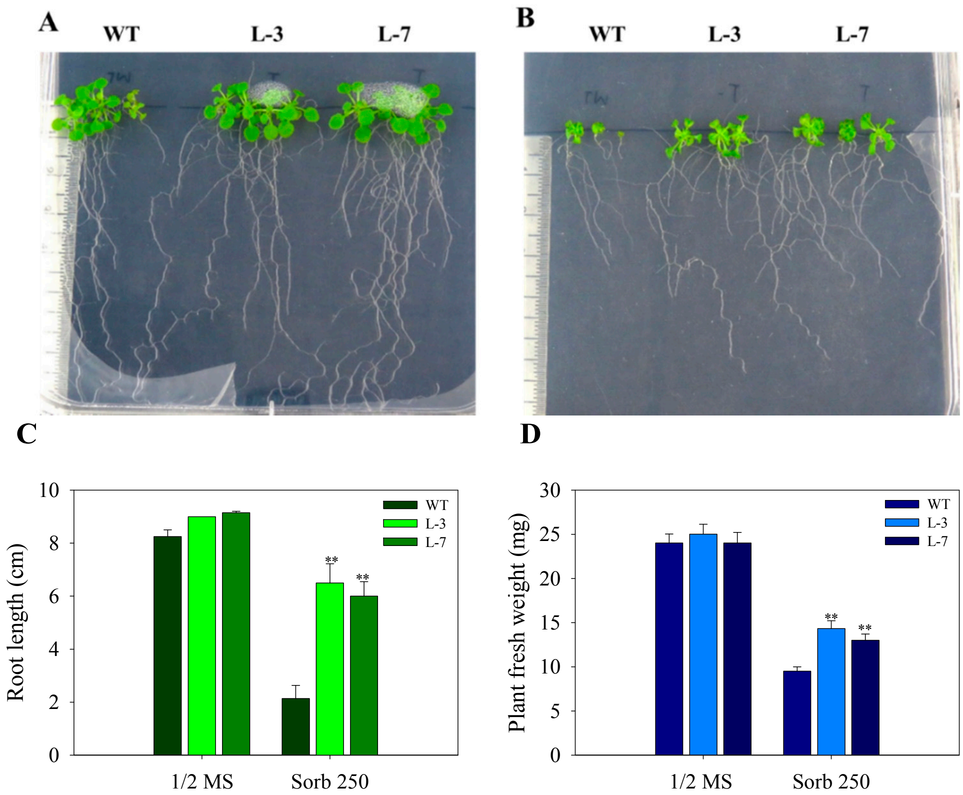

Figure 5. Effect of osmotic stress on the growth of WT and *AnnAt8* expressing *Arabidopsis* plants. (**A**,**B**) Root and shoot growth of plants under normal (half strength MS) and osmotic stress (sorbitol 250 mM), respectively. In each case, the observations were made on day 28 after germination. Three replicates were used per treatment, and the experiment was repeated thrice; (**C**) Root length of WT and transgenic lines L-3 and L-7 under normal and osmotic stress conditions; (**D**) Fresh weight of WT and transgenic lines under normal and osmotic stress conditions. For root length and fresh weight data, observations were made on day 28 and for each replicate (n) the average values for 15 plants of WT and transgenic lines were taken. Data represent means of $n = 3 \pm$ SE. Two asterisks indicate that the mean values of transgenic lines were significantly higher than that of WT (control) when analyzed by one-way ANOVA ($p < 0.01$).

An observation made at day 44 showed that the growth of WT was completely inhibited while the transgenic plants maintained their normal growth under osmotic stress (250 mM Sorbitol). Root length and fresh weight measurements of WT and lines L-3, L-7 were also performed (Figure 6C,D). We measured the lateral root density of plants and found that the lateral root density was higher in the transgenic plants compared to the WT under osmotic stress (data not shown). Fresh weight of plants was also measured at different time points of stress treatment (Figures 5D and 6D).

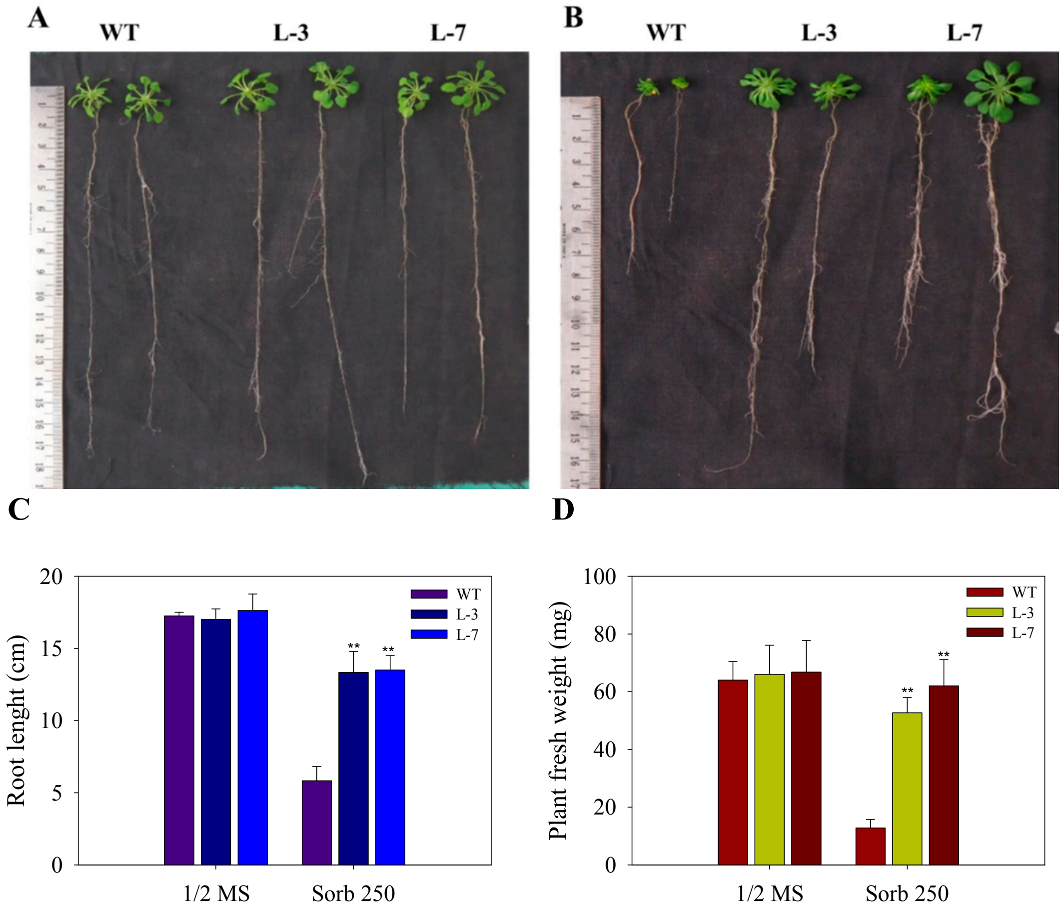

Figure 6. Effect of osmotic stress on the growth of WT and transgenic *Arabidopsis* plants (**A**,**B**) growth of plant under control (half strength MS) and osmotic stress (sorbitol 250 mM) conditions, respectively. Three replicates were used per treatment and the experiment was repeated thrice. (**C**) Root length of WT and transgenic L-3 and L-7 under normal and osmotic stress conditions; (**D**) Fresh weight of plants. In each case observation was made on day 44 after germination. For root length data, the average values of 15 plants of WT and transgenic lines were taken for each biological replicate (n). Data represent means of $n = 3 \pm$ SE. Two asterisks indicate that the mean values of transgenic lines were significantly higher than that of WT (control) when analyzed by one-way ANOVA ($p < 0.01$).

3.4. Osmotic and Salt Stress Tolerance of AnnAt8 Transgenic Tobacco Plants

We evaluated the tolerance of *AnnAt8* transgenic tobacco plants under osmotic stress at different stages of plant growth. Sorbitol was used in this study to induce osmotic stress. Seed germination, seedling, and mature plant stages were observed in the present study. The germination percentages of WT and transgenic lines were almost similar under control condition (without sorbitol), (Figure 7C). More than 95 percent of the seeds of the WT and transgenic lines germinated after 3 days of plating on the control medium, while in 300 and 400 mM sorbitol containing half strength MS media, the overall germination was delayed in WT and transgenic lines, in comparison to the control condition. At 300 mM sorbitol concentration, the seed germination started on day 4, and it continued up to the day 8 of observation. The day wise observations showed that the transgenic lines showed a significantly higher percentage of germination compared to WT (Figure 7A). Similarly, on 400 mM sorbitol medium, the day wise percent germination of transgenic seeds was higher than the WT seeds (Figure 7B).

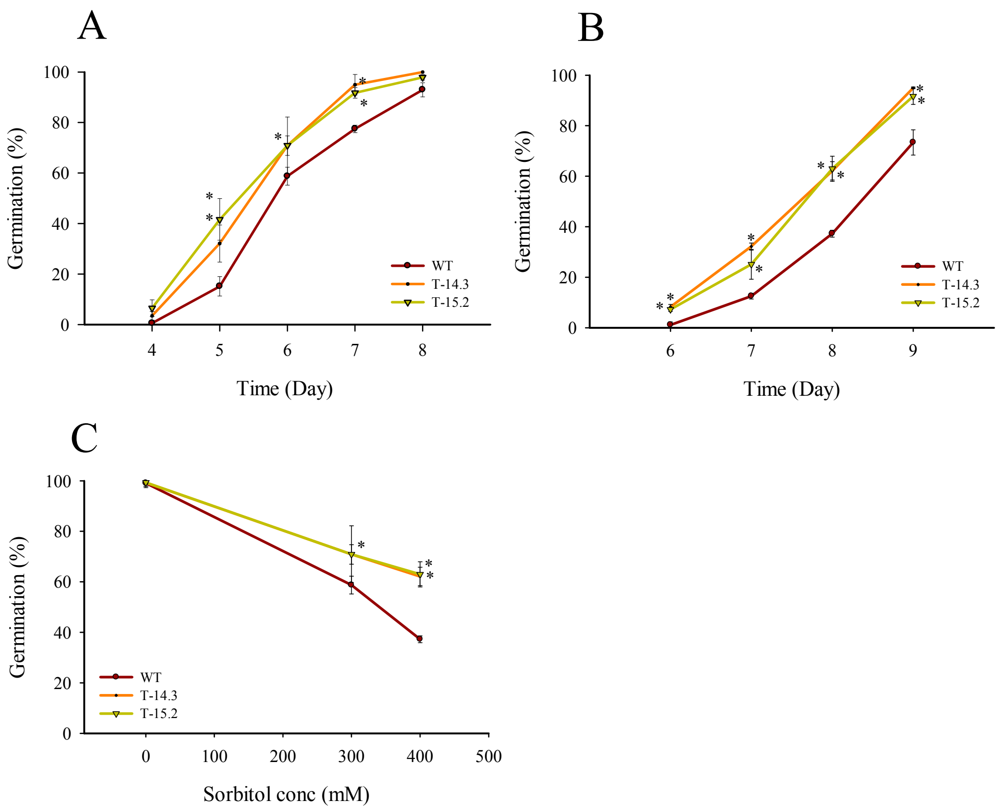

Figure 7. Seed germination rates of WT and *AnnA8* overexpressing transgenic tobacco plants under osmotic stress condition. (**A**, **B**) are day wise percentages of seed germination respectively on 300 and 400 mM sorbitol; (**C**) Sorbitol dose response on the seed germination, percentage value of seed germination on 0, 300 and 400 mM sorbitol were taken on day 4, 7 and 8, respectively, from the date of plating on the media, for each biological replicate (n) 150 seeds of WT and transgenic lines were taken. Data represent means of $n = 3 \pm$ SE. An asterisk indicates that the mean values of transgenic lines were significantly higher than that of WT (control) when analyzed by one-way ANOVA ($p < 0.05$).

For seedling stress assay, 10 day-old seedlings were transferred to the half strength MS medium with different concentrations (100–200–300 mM) of sorbitol. No differences were observed between the WT and the transgenic lines at 100 mM sorbitol concentration. At 200 mM, there were significant differences in the root and shoot growth of transgenic seedlings compared to WT (data not shown). Increased root and shoot growth was observed in the transgenic lines with enhanced root branching. At 300 mM sorbitol concentration, the seedling growth of WT and transgenic plants was slow in the beginning. However, the transgenic lines resumed active growth with the passage of time showing overall enhanced growth compared to the WT (Figure 8). A significant difference in the seedlings biomass was also observed under osmotic stress with a higher fresh weight of transgenic lines in comparison to WT (Figure 9A).

Leaf disc senescence assay was performed to evaluate the total chlorophyll content of WT and transgenic lines under normal and stress conditions; leaf discs from the mature leaves of two-month-old transgenic and WT plants were taken for analysis. At 300 mM concentration, we did not find any significant difference in chlorophyll content of WT and transgenic lines. At 400 mM concentration, one transgenic line showed significantly higher chlorophyll content compared to WT. The total chlorophyll content of both transgenic lines was significantly higher than WT at 500 mM sorbitol concentration (Figure 9B).

As salt tolerance in crop plants is of commercial significance, we studied the response of *AnnAt8* expressing transgenic tobacco plants towards sodium chloride treatment. In the case of seed germination, three different concentrations of sodium chloride (100, 150 and 200 mM) were used to check the germination rates. We observed a delay in the germination of WT and transgenic seeds on sodium chloride containing germination media. On the germination medium without NaCl, seeds of all the transgenic lines including WT, almost completed their germination on day 3 (Figure 10). Transgenic lines showed higher germination rates compared to WT at NaCl concentration of 100, 150 and 200 mM (Figure 11). At 200 mM conc, about 75%–85% seeds of the transgenic lines germinated by day 7, while the seed germination of WT was only around 25%. Percent germination observed on each day showed a significant difference between WT and transgenic lines (Figure 11A, B). At 200 mM NaCl, we found a very significant difference in the germination percentage from day 6 onwards itself (Figure 11C). The germination percentages of WT and transgenic lines on control media at day 4, and on the stress media (with 100, 150 and 200 mM NaCl concentration respectively) at day 6 were used to check the NaCl doses response (Figure 11D).

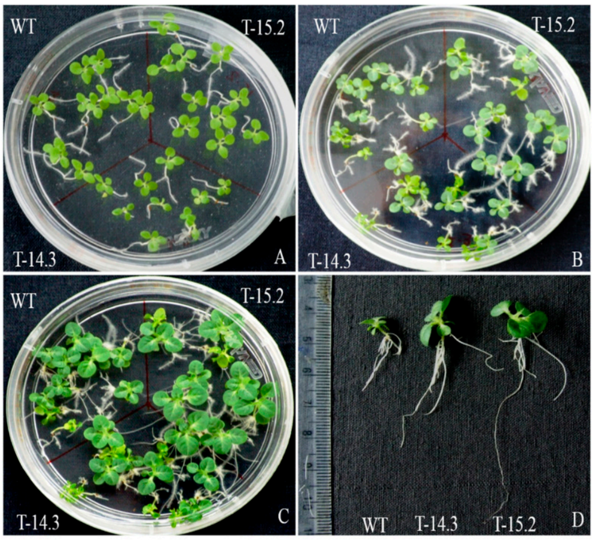

Figure 8. Seedling growth of WT and *AnnAt8* transgenic tobacco plants under osmotic stress. (**A**) Seedlings on day 15 after transfer on half strength MS medium; (**B**,**C**) seedlings on day 35 and 63, respectively after transfer on half strength MS medium supplemented with 300 mM of sorbitol; (**D**) comparison of root and shoot growth after two months of osmotic stress (300 mM sorbitol). Three replicates were used per treatment and the experiment was repeated thrice.

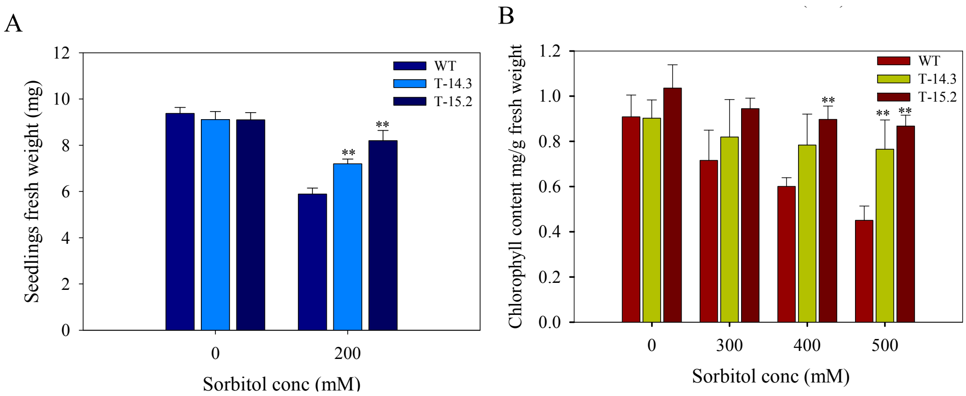

Figure 9. Fresh weight and estimation of total chlorophyll content of WT and transgenic tobacco plants under osmotic stress condition (**A**) Seedling weight under normal and osmotic stress after 26 days of growth. For seedling weight, 10 seedlings for each biological replicate (n) were used, (**B**) Total chlorophyll content of WT and transgenic lines on 0, 300, 400 and 500 mM sorbitol solution. Data represent means of $n = 3 \pm$ SE. Two asterisks indicate that the mean values of transgenic lines were significantly higher than that of WT when analyzed by one-way ANOVA ($p < 0.01$).

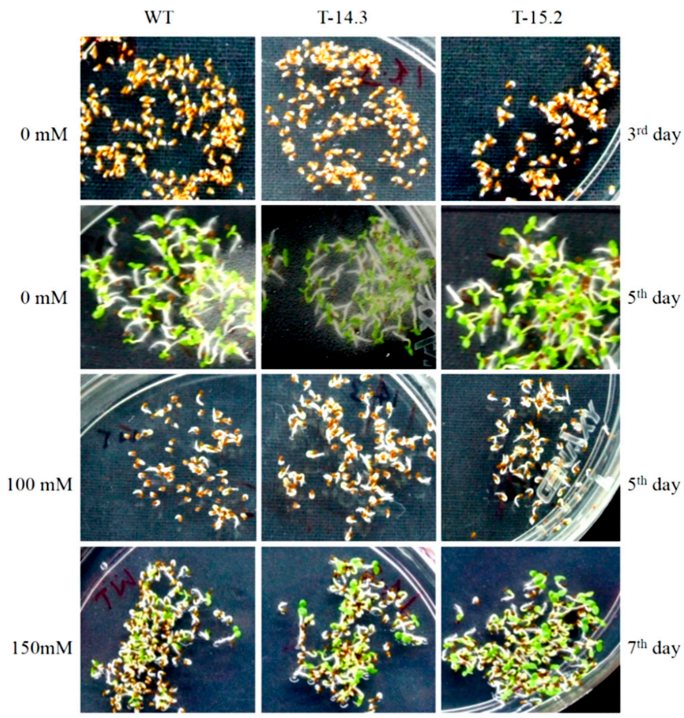

Figure 10. Seed germination of WT and *AnnAt8* transgenic tobacco plants under normal and salt stress conditions. There were no differences in seed germination rates of WT and transgenic lines on half strength MS medium. On NaCl (100 and 150 mM concentrations) media, transgenic lines showed a higher rate of seed germination compared to WT. Three replicates were used per treatment and the experiment repeated three times.

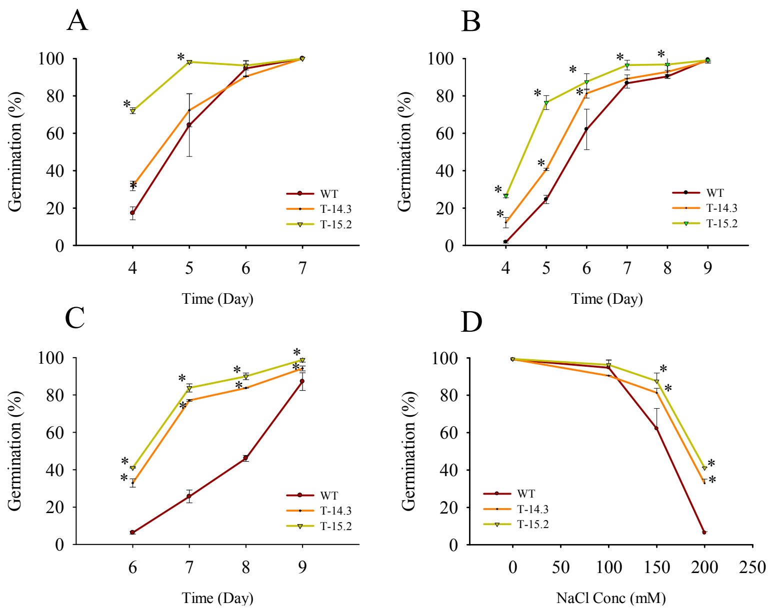

Figure 11. Seed germination rates of WT and *AnnAt8* overexpressing transgenic tobacco plants under salt stress. (**A**–**C**) are day wise seed germination percentages on 100, 150 and 200 mM NaCl concentrations, respectively; (**D**) NaCl dose response on seed germination. For each biological replicate (n) 150 seeds of WT and transgenic lines were taken. Data represent means of $n = 3 \pm$ SE. An asterisk indicates that the mean values of transgenic lines were significantly higher than that of WT, when analyzed by one-way ANOVA ($p < 0.05$).

Initially the seedling growth of WT and transgenic plants was normal on 100 mM concentration. With the advancement of time, WT showed retardation in the growth. After eight weeks of growth on 100 mM NaCl medium, transgenic lines showed better root and shoot growth compared to WT (Figure 12B). At 200 mM concentration, both WT and transgenic lines showed slow growth in the initial stages. Subsequently, the transgenic seedlings showed better adaptation in root and shoot growth compared to WT (Figure 12C). Seedling weight measurement of NaCl-treated seedlings also showed a higher fresh weight of transgenic lines in comparison to WT (Figure 13A). Additionally, to check the tolerance at a higher level of NaCl concentration, seedlings were transferred on 300 mM NaCl containing half strength MS-Agar medium for 16 days. A uniform growth inhibition and bleaching were observed in WT and transgenic lines. After stress treatment, WT and transgenic seedlings were transferred to the recovery medium and we observed that transgenic seedlings showed 90% recovery with a quick resumption of growth, while only 50% of WT seedlings did so (data not shown).

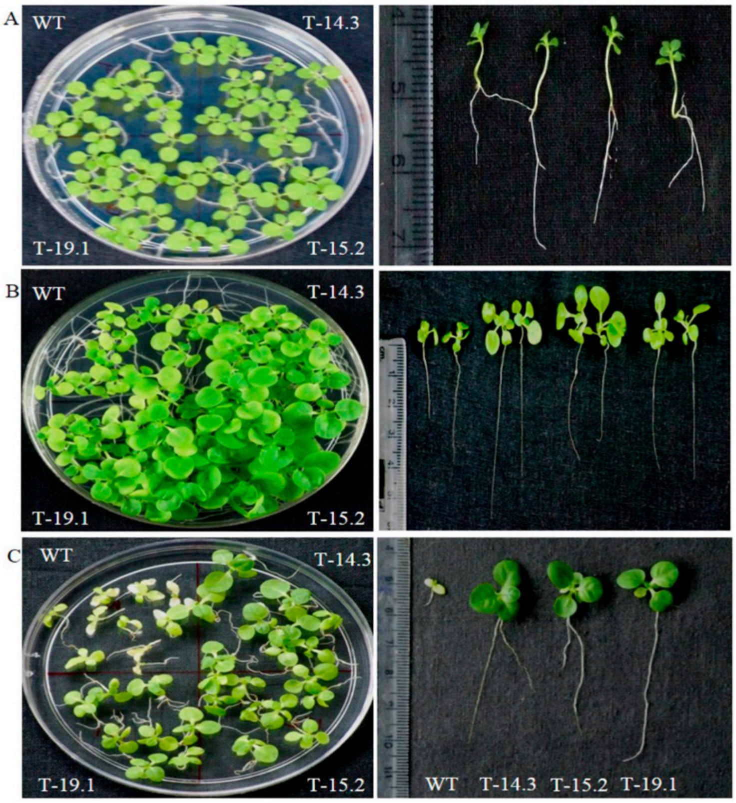

Figure 12. Seedling growth under salt stress. (**A**) Seedlings after 18 days of transfer on half strength MS medium; (**B**) seedlings of WT and *AnnAt8* transgenic lines after 56 days of growth on 100 mM NaCl; (**C**) seedlings of WT and transgenic lines after 42 days of growth on 200 mM NaCl. Three replicates were used per treatment and the experiment repeated three times.

Proline estimation showed a several-fold increase in proline content of the transgenic and WT seedlings after four days of NaCl treatment. However, the proline content of the transgenic seedlings was significantly higher than WT (Figure 13B). Lipid peroxidation assay, performed with the NaCl-treated transgenic and WT seedlings also showed lower lipid peroxidation in the transgenic plants compared to the WT plant (Figure 13C). Total chlorophyll contents of WT and transgenic lines were estimated after a leaf disc senescence assay and WT showed less total chlorophyll content in comparison to transgenic lines (Figures 13D and 14).

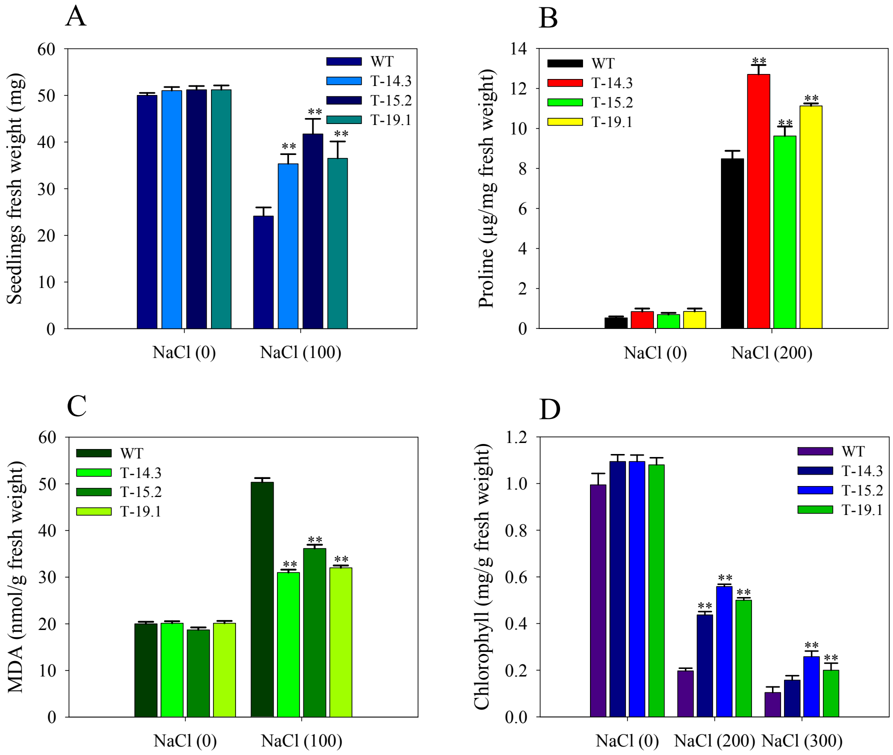

Figure 13. Seedling biomass of *AnnAt8* transgenic tobacco plants under salt stress and measurement of chlorophyll, malondialdehyde (MDA) and proline content under salt stress. (**A**) Fresh weight of transgenic and WT seedlings after nine weeks of growth on half strength MS (with 0 and 100 mM NaCl). For seedling weight average, 10 seedlings for each biological replicate (n) were used; (**B**) Proline content of transgenic and WT seedlings with (200 mM NaCl) and without salt treatment; (**C**) MDA content of transgenic and WT seedlings with (100 mM NaCl) and without salt treatment (**D**) Total chlorophyll content of leaf discs from WT and transgenic tobacco plants with and without NaCl treatment. The data represent the means of $n = 3 \pm$ SE. Two asterisks indicate that the mean values of transgenic lines were significantly higher than that of WT when analyzed by one-way ANOVA ($p < 0.01$).

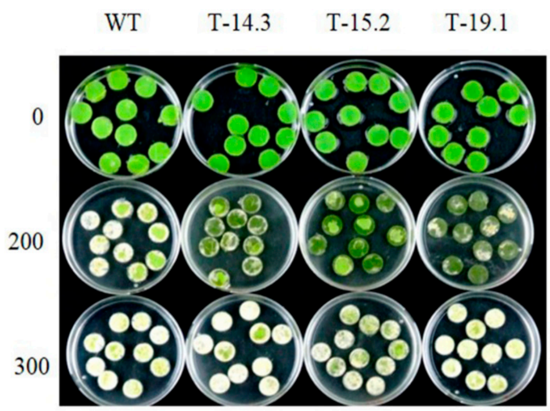

Figure 14. Leaf disc senescence assay under salt stress. Leaf discs from fully expanded leaves of two-month-old WT and *AnnAt8* transgenic tobacco plants were floated on NaCl solution for the stress treatment. The treatments were carried out in continuous light until identifiable differences were observed. Leaf discs of the WT plants bleached out faster in the stress medium (0, 200, 300 mM NaCl). However, the chlorophyll loss was slow in the transgenic lines. Three replicates were used per treatment, and the experiment was repeated at least three times and similar findings were observed.

3.5. Alleviation of Methyl Viologen Stress in AnnAt8 Transgenic Tobacco Plants

To check the photooxidative stress tolerance in transgenic plants, we subjected the WT and transgenic seedlings to 10 µM Methyl Viologen (MV) on half strength MS medium. We observed that the green tissue that directly came in contact with the medium bleached completely in 3–4 days of exposure (data not shown). The seedlings in which the green tissues did not come into contact with the medium also bleached but at a slower rate, and continued their growth with new adventitious root formation. The WT did not show any new root growth and adventitious root formation up to 18 days of observation (Figure 15 A). For recovery assay, seedlings were transferred to half strength MS-Agar medium after 14 days of MV stress. Transgenic seedlings showed faster recovery compared to the WT seedlings (Figure 15B). After recovery, the total chlorophyll contents of the transgenic lines were higher in comparison to WT (Figure 15C). Seedling weight after 12 days of treatment with MV and then nine days of recovery on half strength MS also showed higher fresh weight of transgenic lines in comparison to WT (Figure 15D).

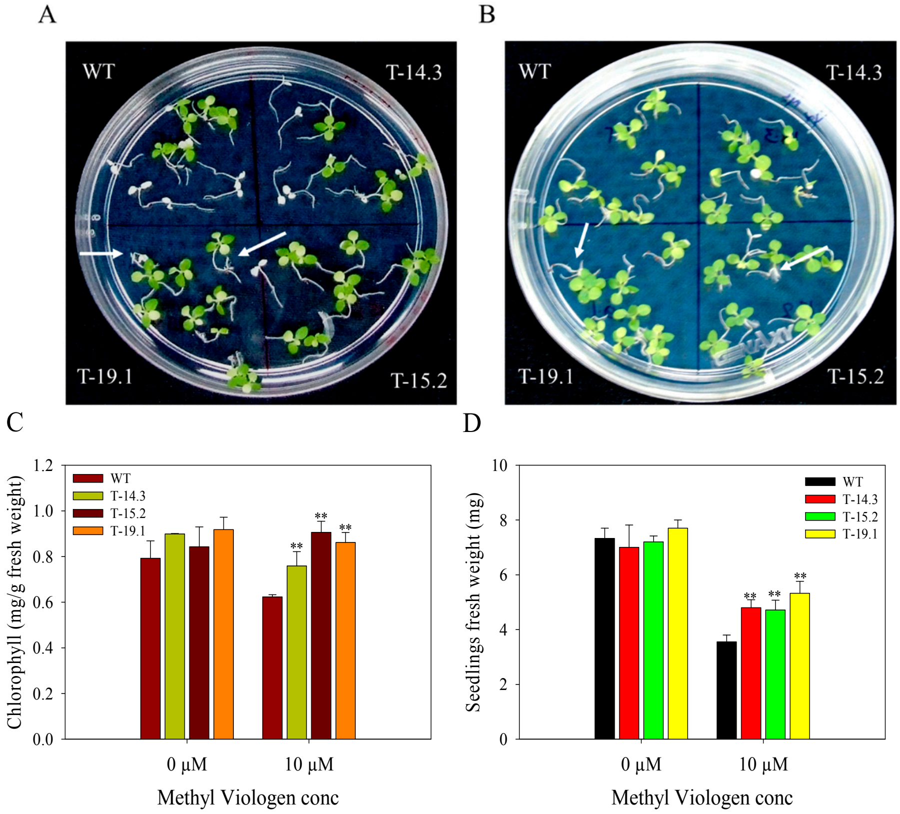

Figure 15. Seedling growth of *AnnAt8* transgenic tobacco plants under Methyl Viologen (MV) stress. (**A**) Seedlings on the 18th day after transfer on stress medium, transgenic lines showed better adaptation with the formation of new adventitious roots while WT showed bleaching and complete inhibition of growth; (**B**) Seedlings on recovery medium after 14 days of MV stress; transgenic lines showed fast recovery compared to WT. (White arrows indicate adventitious root formation in the seedlings). All experiments were performed in triplicate and repeated three times; (**C**) Total chlorophyll content of the recovered seedlings after a prolonged exposure on MV (10 µM) containing half strength MS medium; (**D**) fresh weight of *AnnAt8* transgenic and WT seedlings with and without MV (10 µM) treatment. Seedlings were treated with MV for 12 days and then transferred to the recovery medium for nine days, seedlings grown on half strength MS medium without MV were used as a control; for seedling weight, 10 seedlings from each biological replicate (n) were used and data represent means of $n = 3 \pm$ SE. Two asterisks indicate that the mean values of transgenic lines were significantly higher than that of WT when analyzed by one-way ANOVA ($p < 0.01$).

3.6. Enhanced Tolerance of *AnnAt8* Transgenic Tobacco Plants to Osmotic Stress Condition

To check the effect of simulated drought condition on the seedling growth of WT and transgenic plants, 10% Polyethylene glycol (PEG) 4000 in half strength MS medium was used and half strength MS medium without PEG was used as a control. Initially, both WT and transgenic seedlings showed slow root and shoot growth on the PEG containing medium. The transgenic seedlings showed better adaptation at later stages of growth, with better root and shoot growth compared to WT (Figure 16).

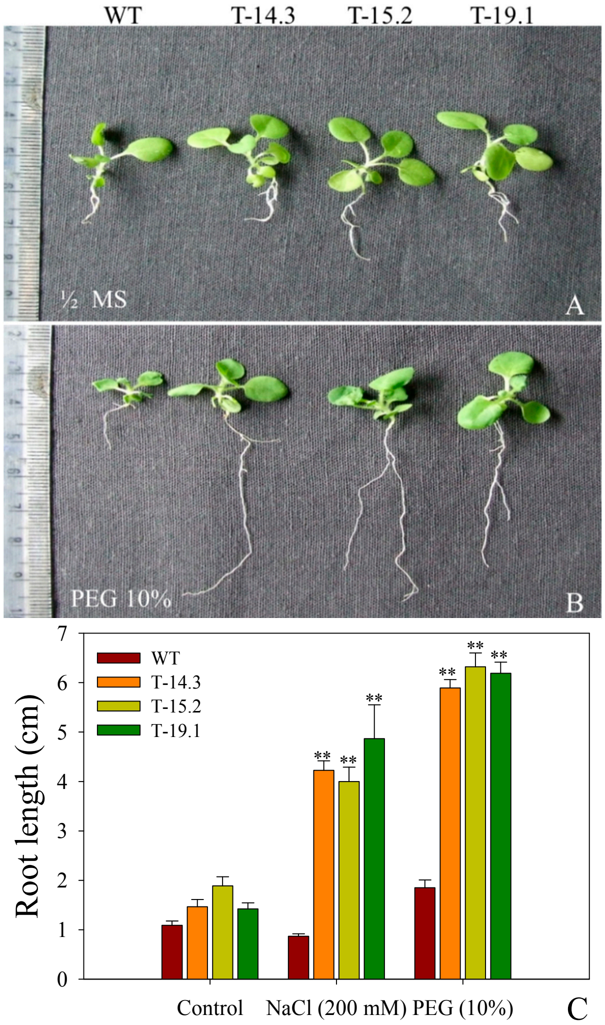

Figure 16. Seedling growth under salt and dehydration stress. (**A**, **B**) Seedlings of WT and *AnnAt8* overexpressing transgenic tobacco plants after five weeks of growth on half strength MS and 10% polyethylene glycol PEG; (**C**) Root lengths of WT and AnnAt8 transgenic seedlings after five weeks of growth on half strength MS, 200 mM NaCl and 10% PEG, respectively, for each biological replicate (n) 15 seedlings of WT and transgenic lines were taken. Data represent means of $n = 3 \pm$ SE. Two asterisks indicate that the mean values of transgenic lines were significantly higher than that of WT (control) when analyzed by one-way ANOVA ($p < 0.01$).

3.7. Enhanced Root Growth of *AnnAt8* Transgenic Tobacco Seedlings under Salt and Dehydration Stress

Evidence of better root growth of seedlings under salt and dehydration stresses was provided by carrying out statistical analysis of root growth of the seedlings after five weeks of stress treatment (Figure 16C).

3.8. Expression Analysis of Stress Related Genes in Transgenic and WT Tobacco Seedlings

Since the AnnAt8 transgenic lines showed enhanced tolerance to several stresses as detailed earlier, we studied the co-expression of some stress tolerance related genes, whose involvement in stress tolerance was demonstrated in previous reports. Real-time RT-PCR analysis was carried out for stress-related genes, which showed co-expression with AnnAt8 (Figure 17). Under stress conditions, the expression of *AnnAt8* in transgenic tobacco was accompanied by a significant upregulation of *NtMnSOD* (Manganese superoxide dismutase), *NtERD10C* (early response to dehydration 10C), *NtERD10D* (early response to dehydration 10D), *NtDREB3* (dehydration-responsive element binding protein), *NtP5CS* (Δ^1pyrolline-5-corboxylate synthetase), *NtNCED3* (9-cis-epoxy carotenoid dioxygenase), *NtAPX* (Ascorbate peroxidase), *NtSUSY* (Sucrose synthase), *NtSAMDC* (S-adenosylmethionine decarboxylase), *NtSOS1* (salt overly sensitive 1), *NtCAT* (Catalase), and *NtERF5* (Ethylene responsive factor 5) transcripts (Figure 17). Both the high expression transgenic lines (T-14.3 and T-15.2) with enhanced stress tolerance showed higher expression of the stress related genes under NaCl treatment; the expression of these genes was always higher in these lines in comparison to WT under NaCl treatment (Figure 17).

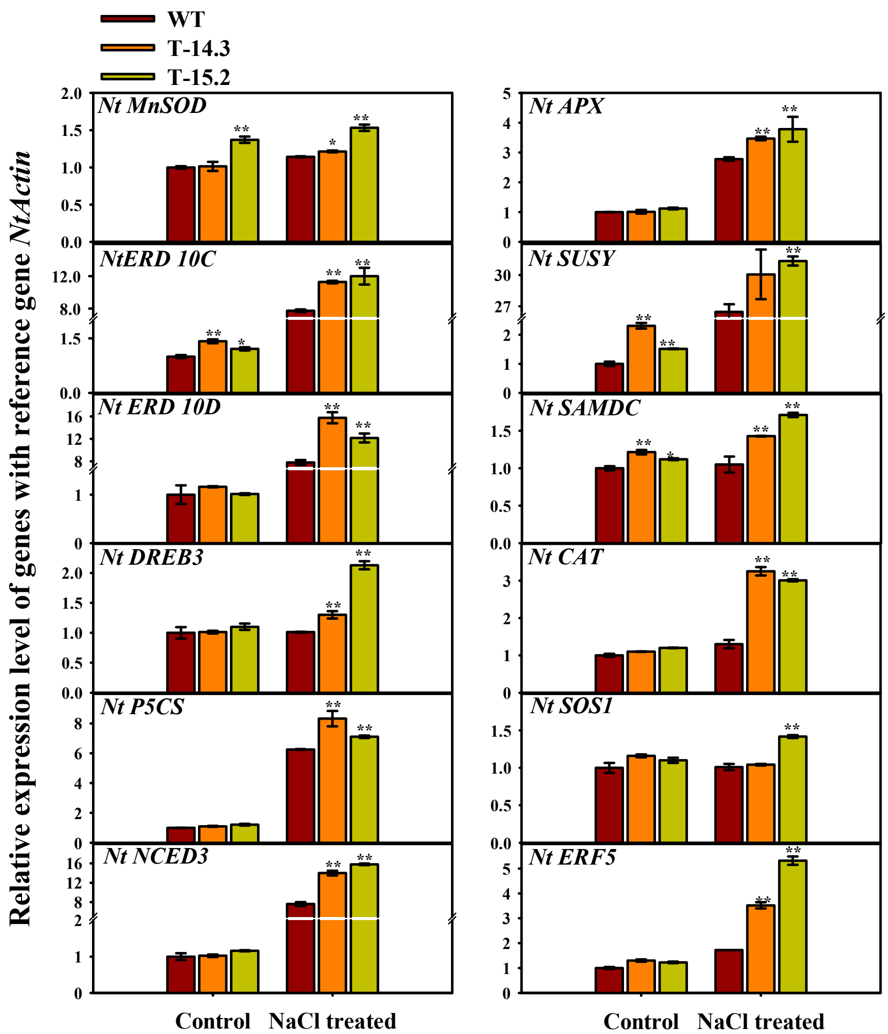

Figure 17. Real-time expression analysis of stress responsive genes in WT and *AnnAt8* transgenic tobacco seedlings before and after salt treatment to the seedlings, data represent means of $n = 3 \pm$ SE. An asterisk indicates that the mean values of transgenic lines were significantly higher than those of WT when analyzed by one-way ANOVA at $p < 0.05$ and two asterisks at $p < 0.01$.

3.9. Subcellular Localization of GFP-Tagged AnnAt8 Protein in Tobacco Cells

To examine the subcellular localization of GFP-AnnAt8, 35Spro::GFP-AnnAt8 and an empty vector control (35Spro::GFP) were introduced individually into *Agrobacterium tumefaciens* EHA105. For the transient gene expression, tobacco leaf was infiltrated with the *Agrobacterium* cells. Then the tobacco leaf epidermal cells were observed for transient expression of the GFP Tagged protein under the confocal microscope. The fluorescence signal of the GFP control was observed all over the cell whereas the localization of the GFP-AnnAt8 fusion protein driven by the 35S promoter was observed at the cell periphery, but not in the nucleus of tobacco leaf epidermal cells (Figure 18).

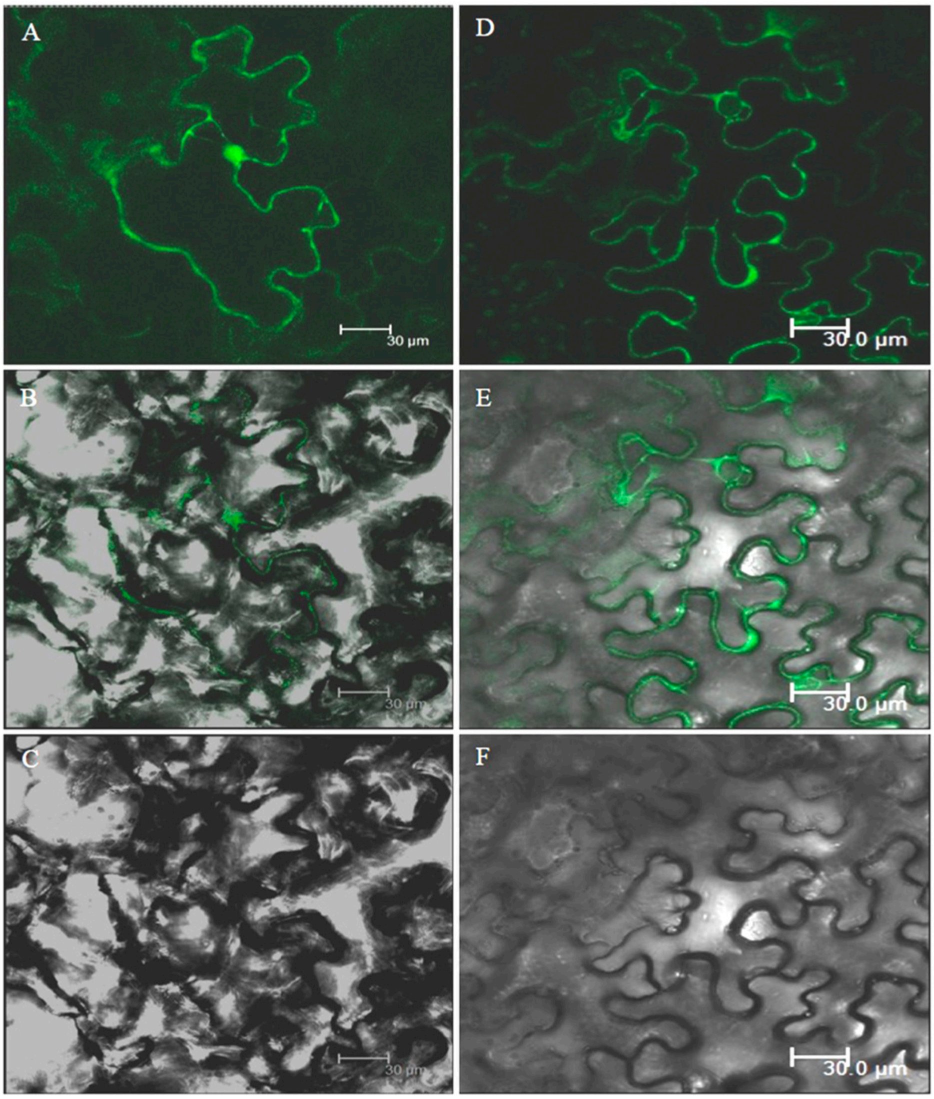

Figure 18. Subcellular localization of GFP-AnnAt8 by transient expression in tobacco leaf epidermal cells. (**A**–**C**) Subcellular localization of GFP control; (**D**–**F**) Subcellular localization of GFP-tagged AnnAt8.

4. Discussion

Arabidopsis has eight annexin genes in its genome and the expression analysis of all these genes under stress treatments has been reported earlier [31]. Few members (AnnAt1, AnnAt2, and AnnAt4) of *Arabidopsis* annexin family are well known for their role in abiotic stress tolerance [14,20,21]. Recently, AnnAt5 has been reported for its role in pollen development [16]. In an expression analysis, a very high level increase in *AnnAt8* transcripts was observed under salt and dehydration stresses in *Arabidopsis* seedlings [31]. However, the level of expression of a gene may not always lead to a positive or direct link to a particular phenotype. In the case of *AnnAt4*, the overexpression lines became more sensitive than WT under drought and salt stress treatments, and the *AnnAt4* mutant exhibited less sensitivity than WT to similar stress conditions [14]. Consequently, characterization of the annexin genes through expression in transgenic plants is necessary for linking their possible role in stress responses. In the present study, we generated transgenic *Arabidopsis* and tobacco plants expressing *AnnAt8* in a constitutive manner and assayed them for stress tolerance at different stages of the plant growth and development.

4.1. AnnAt8 Is Involved in Salt and Dehydration Stress Tolerance

Arabidopsis transgenic plants showed tolerance to NaCl stress at seed germination stage and further at seedling growth stage. Corroborating the *Arabidopsis* results, our observations in tobacco indicated that the seeds of *AnnAt8* transgenic tobacco plants germinated well under mild to higher concentrations of sodium chloride in the germination medium. Similarly, the transgenic tobacco seedlings also showed better survival and growth up to 300 mM NaCl. This indicates that AnnAt8 not only helps in seed germination but also in the further growth of the seedlings in salt stress conditions. The importance of seed germination under salinity cannot be underestimated as seeds fail to germinate in this condition. This is a major problem in saline soils and the plant stand after seed sowing would be poor. Seed germination was reduced under osmotic stress in WT and *AnnAt8* transgenic plants. However, the germination percentage of the transgenic seeds was significantly higher in comparison to WT plants in *Arabidopsis* and tobacco. But these differences in seed germination rates were less, compared to those observed in cases of salt stress given to *Arabidopsis* and tobacco seeds, which could be due to the additional ion (Na^+) toxicity imposed during salt stress. The expression of AnnAt8 appears to ameliorate salt stress in the seed germination stage. Previous reports [14,21] showed similar beneficiary effects of annexin in salt stress at the seed germination stage. As salt stress has osmotic as well ion toxicity effects on the cell, there is a strong possibility of the involvement of AnnAt8 in defense against dual effects caused by salt stress at the germination and seedling stages.

Recent reports suggest that maize annexins and AnnAt1 can mediate the Ca^{2+} flux across the plasma membrane [26–29,51]. This Ca^{2+} modulation property of annexin might play an important role in the regulation of various stress-responsive pathways. Membrane binding and Ca^{2+} binding are important properties of annexin proteins, which are probably responsible for membrane integrity and modulation of the various abiotic stress responses, and the up-regulation of stress associated genes in *AnnAt8* transgenic plants. Ionic stress causes membrane damage, which can be due to high rates of membrane lipid peroxidation and we observed a lower level of lipid peroxidation in tobacco transgenic lines compared to WT, which shows that AnnAt8 has a role in membrane protection.

Root elongation is an adaptive mechanism during salt and drought stress. During salt and drought stress, roots are directly affected and a plant can sustain its shoot growth by maintaining normal root growth, by increasing water and nutrient uptake. We also found similar results in salt, dehydration and drought stress treatments. The transgenic *Arabidopsis* and tobacco seedlings grown on different stress media and the observations made at different time points indicate that AnnAt8 has an additive role in the normal adaptive mechanism of the seedlings under salt and dehydration stresses. This helps seedlings to maintain the integrity of cells and tissues during the early phase of stress, during the later stages of stress response, leading to better root growth in *AnnAt8* transgenic seedlings compared to WT.

4.2. AnnAt8 Is Involved in Alleviation of Methyl Viologen Induced Stress

Methyl Viologen (MV) stress inhibits the photosynthesis by interrupting regular electron flow in PSII causing formation of free radicals (peroxide) leading to membrane damage and ion leakage [52]. *AnnAt8* transgenic tobacco plants showed tolerance to MV at seedling stage; they not only survived on MV containing medium for a longer duration but also showed new adventitious root formation, which was not observed in WT seedlings (Figure 15A,B). *AnnAt8* transgenic showed reduced lipid peroxidation compared to WT (data not shown). Lipid peroxidation causes membrane damage, which leads to ion leakage. The higher chlorophyll content of MV stressed *AnnAt8* transgenic seedlings is also in support of stress alleviating effect of AnnAt8. Seedling biomass of transgenic tobacco lines was also higher than WT in MV stress. It suggests that AnnAt8 helped transgenic seedlings not only in surviving the MV stress, but also in maintaining their growth under MV-induced stress. A recent report also suggests that AnnBj3 of mustard plays a role in the alleviation of MV-mediated photooxidative stress in *Arabidopsis* [53]. The mechanism behind the AnnAt8 mediated MV stress tolerance needs to be studied further.

4.3. Localization Patterns of AnnAt8

Subcellular localization of GFP-AnnAt8 showed that the distribution of GFP-AnnAt8 fusion protein is peripheral in the cell and close to the membrane. Our observations are in line with the previous reports, which support annexin localization to the cell peripheral region [21,22,54]. We have not observed any nuclear localization in the present case. However, there are reports which support the distribution of some annexins to the nucleus and other organelles as well [55,56].

4.4. How Does AnnAt8 Respond to Abiotic Stress?

Co-expression studies of different stress responsive genes performed in *Arabidopsis* annexin knockout mutants and overexpressing transgenic mutants showed their altered expression under normal and stress conditions [14,29,51]. Expression studies of stress responsive genes performed on the WT and *AnnAt8* transgenic tobacco seedlings also showed an altered expression of different stress-responsive genes under normal and stress conditions.

Drought and salt stress responses overlap each other in many ways. Drought is mainly composed of osmotic stress whereas NaCl exerts osmotic as well as Na^+ toxicity, which is controlled by a separate signaling pathway known as SOS (salt overly sensitive) [57]. Plants respond to these stress conditions with a change in its transcriptome. Genes expressed during salt and drought stress show an overlap and expression of these genes, regulated by many transcription factors which are common to both stresses.

Comparatively higher levels of transcripts of different stress responsive genes in *AnnAt8* transgenic lines compared to WT under salt stress condition show that AnnAt8 is associated with the differential regulation of these genes directly or indirectly. DREB proteins have been very well characterized for their role in drought tolerance [58]. *NtDREB3*, a member of the DREB family, showed a higher transcript level in *AnnAt8* transgenic lines compared to the WT seedlings in NaCl stress. This increase in transcript levels could be one reason for salt and dehydration tolerance responses exhibited by *AnnAt8* overexpressing tobacco transgenic seedlings. Co-expression of *DREB2A* in *AnnAt4* overexpression line also showed an upregulation of *DREB2A* in normal condition, while in NaCl stress it showed a down regulation which could be the reason of drought sensitive phenotype of 35S:AnnAt4 transgenic plants [14]. Possible reasons for the different behaviors of AnnAt4 and AnnAt8 could be the different regulatory mechanisms involved under stress condition. NCED3 is the rate-limiting enzyme of ABA biosynthesis pathway. The higher *NtNCED3* transcript level in *AnnAt8* transgenic seedlings possibly increased the ABA level, inducing ABA-dependent abiotic stress defense pathways. However, AnnAt4 responses shown in drought stress was different where *AtNCED3* showed an upregulation under normal conditions, while it showed a down regulation on drought treatment [14]. This different gene regulatory mechanism needs further investigation.

Osmolytes like proline and sucrose help in maintaining cytosolic osmotic potential during stress conditions [59]. An increase in the *NtP5CS* transcript level, which was consistent with enhanced proline content of *AnnAt8* transgenic lines under normal and stress condition, suggests a possibility of AnnAt8 mediated modulation of *NtP5CS* expression. Previous expression studies made on *AnnAt4* overexpression line in *Arabidopsis* showed a down regulation of *P5CS1* on NaCl treatment [14] and conferred a stress-sensitive phenotype. Sucrose synthase (SUSY) is a key enzyme of the sucrose synthesis pathway. It has been reported that different abiotic stress conditions induce the expression of SUSY [60,61]. Consistent with these reports, the relatively higher expression of *NtSUSY* in the *AnnAt8* transgenic seedlings in comparison to WT under salt stress, also favors stress tolerant phenotype of tobacco transgenic lines.

ERF is a transcription factor which binds to GCC box in the promoter region of stress-inducible genes. Previous reports claim that it responds specifically to pathogen-induced ethylene-mediated signaling. However, recent findings suggest that there is crosstalk between the ERF mediated responses [62]. NtERF5 is a transcription factor, which works in ethylene-dependent and independent manner during different abiotic stress (high salinity, cold, and drought) conditions [63]. Our findings also showed its upregulation in the *AnnAt8* transgenic tobacco plants. Earlier overexpression studies of *ERF* genes support its role in biotic and abiotic stress tolerance [58,64,65].

Expression of NtERD10C and NtERD10D generally increases under osmotic stress. We observed a significantly higher expression of *NtERD10C* and *NtERD10D* in the *AnnAt8* transgenic tobacco plants compared to WT under salt stress treatment. ERD10C and ERD10D belong to LEA family of proteins, which are well known for their role in water holding capacity, and they protect the labile enzymes and the macromolecular structure of the cells, thus help during the dehydration stress. The upregulation of these genes in the *AnnAt8* transgenic plants under NaCl stress condition probably led to better adaptation in early stages of dehydration stress. We also observed a higher expression of *NtMnSOD*, *NtCAT* and *NtAPX* in the transgenic seedlings in comparison to the WT under salt stress condition. Higher accumulation of the transcripts of these genes during stress condition with marginal changes in transcript level under normal growth condition indicates that there might be some possibility of post-translational changes in the AnnAt8 protein, which may enhance its activity under stress condition [20].

SAMDC is a key enzyme of the polyamine biosynthesis pathway [66]. In different stress conditions like drought, salinity and cold, polyamine synthesis is developed [67]. *SAMDC* gene expression is regulated through ABRE and other stress responsive transcription factors [68]. Increased levels of *NtNCED3* in seedling stage in the present analysis correlates well with the increased expression of *SAMDC* gene [69,70]. Another important salt stress inducible gene is *SOS1*, which is regulated by Ca^{2+} mediated signaling. Its antiporter activity is regulated by SOS2 and SOS3, which are upstream components of the SOS signaling; SOS2 is a kinase and SOS3 is a Ca^{2+} binding protein [71]. Upregulation of *SOS1* in *AnnAt8* transgenic lines was observed under salt stress. Previous report on the coexpression of *AtSOS1* in *Atann1 Arabidopsis* root showed a down regulation in relation to WT under salt stress and it was concluded that the down regulation of *AtSOS1* in a*nnAt1* mutant possibly contributed to poor germination of the mutant under saline condition [21,51]. An increased seed germination rate observed in *AnnAt8* transgenic under salt stress suggests the possibility of AnnAt8 mediated regulation of *NtSOS1*, which may possibly minimize the Na^{+} toxicity effect on seed germination.

5. Conclusions

In conclusion, the *AnnAt8* overexpressing tobacco and *Arabidopsis* transgenic plants showed enhanced tolerance to some abiotic stresses. Seed germination under stress is an important phenomenon, which affects the plant stand in stressed soils after germination, which ultimately leads to varied productivity. The transgenic plants showed enhanced seed germination and seedling growth, particularly under salt and dehydration stresses. AnnAt8 expression also mitigated oxidative stress condition. The enhanced stress tolerance is possibly associated with the upregulation of some stress-regulated genes in *AnnAt8* transgenic lines under stress treatments. The expression of these genes has been shown to be involved in stress tolerances in previous studies. The gene-regulatory response of AnnAt8 appears to be similar to AnnAt1 but looks different from AnnAt4 with respect to salt and drought tolerant properties (as reported in earlier studies). These different gene regulatory responses exhibited by annexins in *Arabidopsis* needs further investigation for a better understanding of their mechanism of action under normal and stress conditions, which can vary among the *Arabidopsis* annexin family. This study will help understand the functional redundancy and diversity present within the *Arabidopsis* annexin family.

Supplementary Materials: Supplementary Materials can be found at www.mdpi.com/2223-7747/5/2/18/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ABA, Abscisic acid; PEG, Polyethylene glycol; *AnnAt8,* Arabidopsis annexin-8; APX , Ascorbate peroxidase; CAT, Catalase; MV, Methyl Viologen; PSII, photosystem II; SOD, Superoxide dismutase.

References

1. Wang, W.; Vinocur, B.; Altman, A. Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. *Planta* **2003**, *218*, 1–14. [CrossRef] [PubMed]
2. Apel, K.; Hirt, H. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **2004**, *55*, 373–399. [CrossRef] [PubMed]
3. Hasegawa, P.M. Sodium (Na^+) homeostasis and salt tolerance of plants. *Environ. Exp. Bot.* **2013**, *92*, 19–31. [CrossRef]
4. Hirayama, T.; Shinozaki, K. Research on plant abiotic stress responses in the post-genome era: Past, present and future. *Plant J.* **2010**, *61*, 1041–1052. [CrossRef] [PubMed]
5. Shavrukov, Y. Salt stress or salt shock: Which genes are we studying? *J. Exp. Bot.* **2013**, *64*, 119–127. [CrossRef] [PubMed]
6. Claeys, H.; Van Landeghem, S.; Dubois, M.; Maleux, K.; Inzé, D. What is stress? Dose-response effects in commonly used *in vitro* stress assays. *Plant Physiol.* **2014**, *165*, 519–527. [CrossRef] [PubMed]
7. Moss, S.; Morgan, R. The annexins. *Genome Biol.* **2004**, *5*. [CrossRef] [PubMed]
8. Clark, G.B.; Morgan, R.O.; Fernandez, M.P.; Roux, S.J. Evolutionary adaptation of plant annexins has diversified their molecular structures, interactions and functional roles. *New Phytol.* **2012**, *196*, 695–712. [CrossRef] [PubMed]
9. Gerke, V.; Creutz, C.E.; Moss, S.E. Annexins: Linking Ca^{2+} signalling to membrane dynamics. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 449–461. [CrossRef] [PubMed]
10. Jami, S.K.; Clark, G.B.; Ayele, B.T.; Ashe, P.; Kirti, P.B. Genome-wide comparative analysis of annexin superfamily in plants. *PLoS ONE* **2012**, *7*, e47801. [CrossRef] [PubMed]
11. Davies, J. Annexin-mediated calcium signalling in plants. *Plants* **2014**, *3*, 128–140. [CrossRef]
12. Clark, G.B.; Sessions, A.; Eastburn, D.J.; Roux, S.J. Differential expression of members of the annexin multigene family in *Arabidopsis*. *Plant Physiol.* **2001**, *126*, 1072–1084. [CrossRef] [PubMed]
13. Hofmann, A. Annexins in the plant kingdom: Perspectives and potentials. *Annexins* **2004**, *1*, 51–61.
14. Huh, S.M.; Noh, E.K.; Kim, H.G.; Jeon, B.W.; Bae, K.; Hu, H.C.; Kwak, J.M.; Park, O.K. Arabidopsis annexins AnnAt1 and AnnAt4 interact with each other and regulate drought and salt stress responses. *Plant Cell Physiol.* **2010**, *51*, 1499–1514. [CrossRef] [PubMed]
15. Wang, X.; Ma, X.; Wang, H.; Li, B.; Clark, G.; Guo, Y.; Roux, S.; Sun, D.; Tang, W. Proteomic study of microsomal proteins reveals a key role for Arabidopsis annexin 1 in mediating heat stress-induced increase in intracellular calcium levels. *Mol. Cell. Proteom.* **2015**, *14*, 686–694. [CrossRef] [PubMed]

16. Zhu, J.; Yuan, S.; Wei, G.; Qian, D.; Wu, X.; Jia, H.; Gui, M.; Liu, W.; An, L.; Xiang, Y. Annexin5 is essential for pollen development in arabidopsis. *Mol. Plant* **2014**, *7*, 751–754. [CrossRef] [PubMed]
17. Zhu, J.; Wu, X.; Yuan, S.; Qian, D.; Nan, Q.; An, L.; Xiang, Y. Annexin5 plays a vital role in *Arabidopsis* pollen development via Ca^{2+}-dependent membrane trafficking. *PLoS ONE* **2014**, *9*, e102407.
18. Jia, F.; Wang, C.; Huang, J.; Yang, G.; Wu, C.; Zheng, C. SCF E3 ligase PP2-B11 plays a positive role in response to salt stress in *Arabidopsis*. *J. Exp. Bot.* **2015**, *66*, 4683–4897. [CrossRef] [PubMed]
19. Mortimer, J.C.; Laohavisit, A.; Macpherson, N.; Webb, A.; Brownlee, C.; Battey, N.H.; Davies, J.M. Annexins: Multifunctional components of growth and adaptation. *J. Exp. Bot.* **2008**, *59*, 533–544. [CrossRef] [PubMed]
20. Konopka-Postupolska, D.; Clark, G.; Goch, G.; Debski, J.; Floras, K.; Cantero, A.; Fijolek, B.; Roux, S.; Hennig, J. The role of Annexin 1 in drought stress in Arabidopsis. *Plant Physiol.* **2009**, *150*, 1394–1410. [CrossRef] [PubMed]
21. Lee, S.; Lee, E.J.; Yang, E.J.; Lee, J.E.; Park, A.R.; Song, W.H.; Park, O.K. Proteomic identification of annexins, calcium-dependent membrane binding proteins that mediate osmotic stress and abscisic acid signal transduction in Arabidopsis. *Plant Cell Online* **2004**, *16*, 1378–1391. [CrossRef] [PubMed]
22. Qiao, B.; Zhang, Q.; Liu, D.; Wang, H.; Yin, J.; Wang, R.; He, M.; Cui, M.; Shang, Z.; Wang, D.; *et al.* A calcium-binding protein, rice annexin OsANN1, enhances heat stress tolerance by modulating the production of H_2O_2. *J. Exp. Bot.* **2015**, *66*, 5853–5866. [CrossRef] [PubMed]
23. 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. Induction of annexin by heavy metals and jasmonic acid in *Zea mays*. *Funct. Integr. Genom.* **2013**, *13*, 241–251. [CrossRef] [PubMed]
24. Laohavisit, A.; Davies, J.M. Annexins. *New Phytol.* **2011**, *189*, 40–53. [CrossRef] [PubMed]
25. Balasubramanian, K.; Bevers, E.M.; Willems, G.M.; Schroit, A.J. Binding of annexin v to membrane products of lipid peroxidation. *Biochemistry* **2001**, *40*, 8672–8676. [CrossRef] [PubMed]
26. Laohavisit, A.; Mortimer, J.C.; Demidchik, V.; Coxon, K.M.; Stancombe, M.A.; Macpherson, N.; Brownlee, C.; Hofmann, A.; Webb, A.A.R.; Miedema, H.; *et al.* *Zea mays* Annexins modulate cytosolic free Ca^{2+} and generate a Ca^{2+}-permeable conductance. *Plant Cell Online* **2009**, *21*, 479–493. [CrossRef] [PubMed]
27. Laohavisit, A.; Brown, A.T.; Cicuta, P.; Davies, J.M. Annexins: Components of the calcium and reactive oxygen signaling network. *Plant Physiol.* **2010**, *152*, 1824–1829. [CrossRef] [PubMed]
28. Laohavisit, A.; Shang, Z.; Rubio, L.; Cuin, T.A.; Véry, A.A.; Wang, A.; Mortimer, J.C.; Macpherson, N.; Coxon, K.M.; Battey, N.H.; *et al.* Arabidopsis Annexin1 mediates the radical-activated plasma membrane Ca^{2+}- and K^+-permeable conductance in root cells. *Plant Cell Online* **2012**, *24*, 1522–1533. [CrossRef] [PubMed]
29. Richards, S.L.; Laohavisit, A.; Mortimer, J.C.; Shabala, L.; Swarbreck, S.M.; Shabala, S.; Davies, J.M. Annexin 1 regulates the H_2O_2-induced calcium signature in *Arabidopsis thaliana* roots. *Plant J.* **2014**, *77*, 136–145. [CrossRef] [PubMed]
30. Arpat, A.; Waugh, M.; Sullivan, J.P.; Gonzales, M.; Frisch, D.; Main, D.; Wood, T.; Leslie, A.; Wing, R.; Wilkins, T. Functional genomics of cell elongation in developing cotton fibers. *Plant Mol. Biol.* **2004**, *54*, 911–929. [CrossRef] [PubMed]
31. Cantero, A.; Barthakur, S.; Bushart, T.; Chou, S.; Morgan, R.; Fernandez, M.; Clark, G.; Roux, S. Expression profiling of the *Arabidopsis* annexin gene family during germination, de-etiolation and abiotic stress. *Plant Physiol. Biochem.* **2006**, *44*, 13–24. [CrossRef] [PubMed]
32. Feng, Y.M.; Wei, X.K.; Liao, W.X.; Huang, L.H.; Zhang, H.; Liang, S.C.; Peng, H. Molecular analysis of the annexin gene family in soybean. *Biol. Plant.* **2013**, *57*, 655–662. [CrossRef]
33. He, M.; Yang, X.; Cui, S.; Mu, G.; Hou, M.; Chen, H.; Liu, L. Molecular cloning and characterization of annexin genes in peanut (*Arachis hypogaea* L.). *Gene* **2015**, *568*, 40–49. [CrossRef] [PubMed]
34. Jami, S.K.; Dalal, A.; Divya, K.; Kirti, P.B. Molecular cloning and characterization of five annexin genes from Indian mustard (*Brassica juncea* l. Czern and coss). *Plant Physiol. Biochem.* **2009**, *47*, 977–990. [CrossRef] [PubMed]
35. Lu, Y.; Ouyang, B.; Zhang, J.; Wang, T.; Lu, C.; Han, Q.; Zhao, S.; Ye, Z.; Li, H. Genomic organization, phylogenetic comparison and expression profiles of annexin gene family in tomato (*Solanum lycopersicum*). *Gene* **2012**, *499*, 14–24. [CrossRef] [PubMed]
36. Yadav, D.; Ahmed, I.; Kirti, P.B. 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* **2015**, *4*, 109–124. [CrossRef]

37. Yan, H.; Luo, Y.; Jiang, Z.; Wang, F.; Zhou, B.; Xu, Q. Cloning and expression characterization of four annexin genes during germination and abiotic stress in *Brassica rapa* subsp. Rapa "tsuda". *Plant Mol. Biol. Report.* **2016**, *34*, 467–482. [CrossRef]
38. Ji, W.; Koh, J.; Li, S.; Zhu, N.; Dufresne, C.P.; Zhao, X.; Chen, S.; Li, J. Quantitative proteomics reveals an important role of gscbrlk in salt stress response of soybean. *Plant Soil* **2015**, 1–20. [CrossRef]
39. Zhang, H.; Han, B.; Wang, T.; Chen, S.; Li, H.; Zhang, Y.; Dai, S. Mechanisms of plant salt response: Insights from proteomics. *J. Proteome Res.* **2012**, *11*, 49–67. [CrossRef] [PubMed]
40. Chu, P.; Chen, H.; Zhou, Y.; Li, Y.; Ding, Y.; Jiang, L.; Tsang, E.W.T.; Wu, K.; Huang, S. Proteomic and functional analyses of *Nelumbo nucifera* annexins involved in seed thermotolerance and germination vigor. *Planta* **2011**, *235*, 1271–1288. [CrossRef] [PubMed]
41. Zhang, Y.; Xu, L.; Zhu, X.; Gong, Y.; Xiang, F.; Sun, X.; Liu, L. Proteomic analysis of heat stress response in leaves of radish (*Raphanus sativus* L.). *Plant Mol. Biol. Report.* **2012**, *31*, 195–203. [CrossRef]
42. Steffen, J.G.; Kang, I.H.; Macfarlane, J.; Drews, G.N. Identification of genes expressed in the *Arabidopsis* female gametophyte. *Plant J.* **2007**, *51*, 281–292. [CrossRef] [PubMed]
43. Wuest, S.E.; Vijverberg, K.; Schmidt, A.; Weiss, M.; Gheyselinck, J.; Lohr, M.; Wellmer, F.; Rahnenführer, J.; von Mering, C.; Grossniklaus, U. Arabidopsis female gametophyte gene expression map reveals similarities between plant and animal gametes. *Curr. Biol.* **2010**, *20*, 506–512. [CrossRef] [PubMed]
44. Clough, S.J.; Bent, A.F. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **1998**, *16*, 735–743. [CrossRef] [PubMed]
45. Horsch, R.B.; Fry, J.E.; Hoffmann, N.L.; Eichholtz, D.; Rogers, S.G.; Fraley, R.T. A simple and general method for transferring genes into plants. *Science* **1985**, *227*, 1229–1231.
46. Hiscox, J.D.; Israelstam, G.F. A method for the extraction of chlorophyll from leaf tissue without maceration. *Can. J. Bot.* **1979**, *57*, 1332–1334. [CrossRef]
47. Heath, R.L.; Packer, L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **1968**, *125*, 189–198. [CrossRef]
48. Bates, L.; Waldren, R.; Teare, I. Rapid determination of free proline for water-stress studies. *Plant Soil* **1973**, *39*, 205–207. [CrossRef]
49. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative pcr and the $2^{-\Delta\Delta ct}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
50. Kumar, K.; Kirti, P. Differential gene expression in *Arachis diogoi* upon interaction with peanut late leaf spot pathogen, *Phaeoisariopsis personata* and characterization of a pathogen induced cyclophilin. *Plant Mol. Biol.* **2011**, *75*, 497–513. [CrossRef] [PubMed]
51. Laohavisit, A.; Richards, S.L.; Shabala, L.; Chen, C.; Colaço, R.D.D.R.; Swarbreck, S.M.; Shaw, E.; Dark, A.; Shabala, S.; Shang, Z.; *et al.* Salinity-induced calcium signaling and root adaptation in Arabidopsis require the calcium regulatory protein Annexin1. *Plant Physiol.* **2013**, *163*, 253–262. [CrossRef] [PubMed]
52. Faize, M.; Burgos, L.; Faize, L.; Piqueras, A.; Nicolas, E.; Barba-Espin, G.; Clemente-Moreno, M.; Alcobendas, R.; Artlip, T.; Hernandez, J. Involvement of cytosolic ascorbate peroxidase and Cu/Zn-superoxide dismutase for improved tolerance against drought stress. *J. Exp. Bot.* **2011**, *62*, 2599–2613. [CrossRef] [PubMed]
53. Dalal, A.; Kumar, A.; Yadav, D.; Gudla, T.; Viehhauser, A.; Dietz, K.J.; Kirti, P.B. Alleviation of methyl viologen-mediated oxidative stress by *Brassica juncea* annexin-3 in transgenic *Arabidopsis*. *Plant Sci.* **2014**, *219*, 9–18. [CrossRef] [PubMed]
54. Clark, G.B.; Rafati, D.S.; Bolton, R.J.; Dauwalder, M.; Roux, S.J. Redistribution of annexin in gravistimulated pea plumules. *Plant Physiol. Biochem.* **2000**, *38*, 937–947. [CrossRef]
55. Clark, G.B.; Dauwalder, M.; Roux, S.J. Immunological and biochemical evidence for nuclear localization of annexin in peas. *Plant Physiol. Biochem.* **1998**, *36*, 621–627. [CrossRef]
56. Kovács, I.; Ayaydin, F.; Oberschall, A.; Ipacs, I.; Bottka, S.; Pongor, S.; Dudits, D.; Tóth, É.C. Immunolocalization of a novel annexin-like protein encoded by a stress and abscisic acid responsive gene in alfalfa. *Plant J.* **1998**, *15*, 185–197. [CrossRef] [PubMed]
57. Zhu, J.K. Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* **2002**, *53*, 247–273. [CrossRef] [PubMed]
58. Umezawa, T.; Fujita, M.; Fujita, Y.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Engineering drought tolerance in plants: Discovering and tailoring genes to unlock the future. *Curr. Opin. Biotechnol.* **2006**, *17*, 113–122. [CrossRef] [PubMed]

59. Sharma, S.; Villamor, J.G.; Verslues, P.E. Essential role of tissue-specific proline synthesis and catabolism in growth and redox balance at low water potential. *Plant Physiol.* **2011**, *157*, 292–304. [CrossRef] [PubMed]
60. Dejardin, A.; Sokolov, L.; Kleczkowski, L. Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in Arabidopsis. *Biochem. J.* **1999**, *344*, 503–509. [CrossRef] [PubMed]
61. An, X.; Chen, Z.; Wang, J.; Ye, M.; Ji, L.; Wang, J.; Liao, W.; Ma, H. Identification and characterization of the *Populus* sucrose synthase gene family. *Gene* **2014**, *539*, 58–67. [CrossRef] [PubMed]
62. Park, J.M.; Park, C.J.; Lee, S.B.; Ham, B.K.; Shin, R.; Paek, K.H. Overexpression of the tobacco tsi1 gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. *Plant Cell* **2001**, *13*, 1035–1046. [CrossRef] [PubMed]
63. Zhai, Y.; Wang, Y.; Li, Y.; Lei, T.; Yan, F.; Su, L.; Li, X.; Zhao, Y.; Sun, X.; Li, J. Isolation and molecular characterization of *Gmerf7*, a soybean ethylene-response factor that increases salt stress tolerance in tobacco. *Gene* **2013**, *513*, 174–183. [CrossRef] [PubMed]
64. Medina, J.; Catalá, R.; Salinas, J. The CBFs: Three *Arabidopsis* transcription factors to cold acclimate. *Plant Sci.* **2011**, *180*, 3–11. [CrossRef] [PubMed]
65. Robert-Seilaniantz, A.; Grant, M.; Jones, J.D. Hormone crosstalk in plant disease and defense: More than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* **2011**, *49*, 317–343. [CrossRef] [PubMed]
66. Rangan, P.; Subramani, R.; Kumar, R.; Singh, A.K.; Singh, R. Recent advances in polyamine metabolism and abiotic stress tolerance. *BioMed. Res. Int.* **2014**, *2014*. [CrossRef] [PubMed]
67. Groppa, M.; Benavides, M. Polyamines and abiotic stress: Recent advances. *Amino Acids* **2008**, *34*, 35–45. [CrossRef] [PubMed]
68. Basu, S.; Roychoudhury, A.; Sengupta, D. Deciphering the role of various cis-acting regulatory elements in controlling samdc gene expression in rice. *Plant Signal. Behav.* **2014**, *9*. [CrossRef]
69. Bitrián, M.; Zarza, X.; Altabella, T.; Tiburcio, A.F.; Alcázar, R. Polyamines under abiotic stress: Metabolic crossroads and hormonal crosstalks in plants. *Metabolites* **2012**, *2*, 516–528. [CrossRef] [PubMed]
70. Urano, K.; Yoshiba, Y.; Nanjo, T.; Igarashi, Y.; Seki, M.; Sekiguchi, F.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Characterization of *Arabidopsis* genes involved in biosynthesis of polyamines in abiotic stress responses and developmental stages. *Plant Cell Environ.* **2003**, *26*, 1917–1926. [CrossRef]
71. Isayenkov, S. Physiological and molecular aspects of salt stress in plants. *Cytol. Genet.* **2012**, *46*, 302–318. [CrossRef]

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Plant annexins and their involvement in stress responses

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ABSTRACT

Annexins, which form an evolutionarily conserved family of proteins are known to be involved in important biological processes such as membrane trafficking, cytoskeletal organization, cellular homeostasis and ion transport. They are widely known for mediating plant stress responses. Although, the mechanism involved in these responses is not deciphered clearly, several recent attempts in this direction have strengthened our understanding of the different components involved in annexin-mediated stress responses in plants, which prompted us to link and hypothesize their involvement in stress alleviation by predicting a possible relation with different elements participating in stress signaling. In the light of past and present findings, we discuss the structural and cellular properties of plant annexins emphasizing their stress-mediated roles and propose an annexin-mediated signaling cascade in this review, which has not been dealt with earlier. Annexin participation in diverse cellular functions highlight their essential role in plant growth and development and also their importance in crop improvement programs for enhancing multiple stress tolerance.

1. Introduction

Since the first isolation of an annexin from animal cells as a vesicle fusion protein (Creutz et al., 1978), annexins have been the subject of multidisciplinary research for nearly forty years. Their identification in other organisms including plants resulted in their expansion to a superfamily of proteins, which have been subgrouped into seven different families viz., ANXA-G (Fernandez et al., 2017). The family ANXA includes vertebrate annexins and ANXB comprises invertebrate annexins; annexins from fungi and unicellular eukaryotic organisms were assigned to ANXC. ANXD consists of plant annexins and it comprises 40 + subfamilies (Clark et al., 2012). The family ANXE includes protist annexins, ANXF represents bacterial annexins and ANXG includes putative archaeal annexins (Fernandez et al., 2017; Moss and Morgan, 2004). Separate attempts have also been made to understand the annexin phylogeny in vertebrates (Fernandez et al., 2017), plants (Clark et al., 2012), fungi (Khalaj et al., 2015), protists (Einarsson et al., 2016) and bacteria (Fernandez et al., 2017; Kodavali et al., 2014).

Initial research on annexins primarily focused on the vertebrate members and their association with membrane lipids and cytoskeletal proteins that indicated their proposed participation in intracellular transport processes leading to vesicle translocation, fusion and transcytosis (Fernandez et al., 2017; Potez et al., 2011; Tebar et al., 2014). Initially, annexins have been defined as calcium (Ca^{2+}) mediated, membrane lipid associated proteins, but the report of Ca^{2+} independent membrane interaction and the presence of other conserved structural motifs, distinct functional domains, unique amino-termini and varied forms of architecture broke this paradigm and proposed integrated roles and mechanisms for individual annexin functions (Fernandez et al., 2017).

Initially identified members of plants and vertebrate annexins mainly consist of a homologous tetrad of 68 aa ANX domains, which probably originated from a monomeric annexin (Fernandez et al., 2017). Later, many other alternative forms of architecture for the annexin proteins have also been reported, which included 1-20 ANX domains (Crompton et al., 1988; Fernandez et al., 2017). The members of ANXD family have < 45% amino acid (aa) identity with animal annexins. However, they still preserve the unique annexin fold with the secondary structure getting assembled into the characteristic tetrad of the four homologous domains (Hofmann, 2004; Moss and Morgan, 2004). Each annexin repeat comprises five α- helices (A-E) connected with loops forming a helix-loop-helix structure and the membrane binding sites are situated in the AB and DE loops. Plant annexins also have more surface area on the protein because of the presence of extra clefts and grooves when compared to the animal annexins suggesting a wider range of interaction partners for the annexin proteins and wider

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