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Transcriptional regulation of annexins in indian mustard, *Brassica juncea* and detoxification of ROS in transgenic tobacco plants constitutively expressing *AnnBj1*

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Annexins in plants constitute a multigene family and there is growing evidence for their involvement in response to various stress treatments. We have cloned and characterized six different annexin genes from mustard. The transcript regulation of these genes was studied in treatments with various signaling molecules, osmotic stress, oxidative stress conditions and wounding. All these annexins were found to be reponsive to Abscisic acid (ABA). Two genes (*AnnBj1* and *AnnBj3*) were found to respond to most of the stress conditions, which suggest their possible role in cross-talk in multiple signaling pathways. Wound response signal methyl jasmonate (mJA) caused rapid and high expression of *AnnBj4*. We extended our previous study and showed that transgenic tobacco plants heterologously expressing *AnnBj1* evidenced mannitol induced ROS detoxification. These results suggest Brassica annexins may have potential role in alleviating abiotic stress, which should be characterized by in vivo function based studies through silencing by RNAi or overexpression in transgenic plants.

Key words: abiotic stress, abscisic acid, brassica annexins, reactive oxygen species

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Abiotic stresses in the form of drought, salinity, cold and metal toxicity cause considerable loss to crop plants. Under these stress conditions, the level of cytosolic calcium (Ca^{2+}) increases thereby modulating the expression of genes of various calcium binding proteins.[1] One among those is classified as plant annexins. Plant annexins are Ca^{+2}-dependent phospholipid binding proteins that are evolutionary conserved in plant and animals. Structurally, they contain four annexin repeats of 70–75 amino acids each with a characteristic endonexin sequence designated as GxGT-[38 residues]-D/E. Unlike animal annexins, the type-II calcium binding sites are present in the first and fourth repeats in plants and this was supported by structural studies of cotton annexin binding calcium.[2] These proteins in plants belong to multigene family with Arabidopsis and rice containing eight and ten genes respectively.[3]

Recently, we have reported the cloning and characterization of five different annexins genes (*AnnBj1*, *AnnBj2*, *AnnBj3*, *AnnBj6* and *AnnBj7*) from *Brassica juncea*.[4] Based on the same approach of RACE-PCR, we isolated another annexin member, *AnnBj4* (GenBank accession no. GU584092) with its deduced amino acid sequence showing 86% identity to its Arabidopsis homolog (At2g38750). All six Brassica annexins have molecular masses of ~36.0 kDa and acidic p*I* except for AnnBj4, which has a neutral p*I*. Multiple alignments of these six deduced amino acid sequences presented AnnBj4 showing the least identity with other members of the gene family characterized so far. Domain analysis of the deduced proteins by SMART database showed the presence of four repeats with calcium binding sites in first and fourth repeats in all except for AnnBj4, which has only two annexin domain repeats in 2nd and 4th respectively. The two repeats in AnnBj4 also lack the calcium binding site. Structural variation

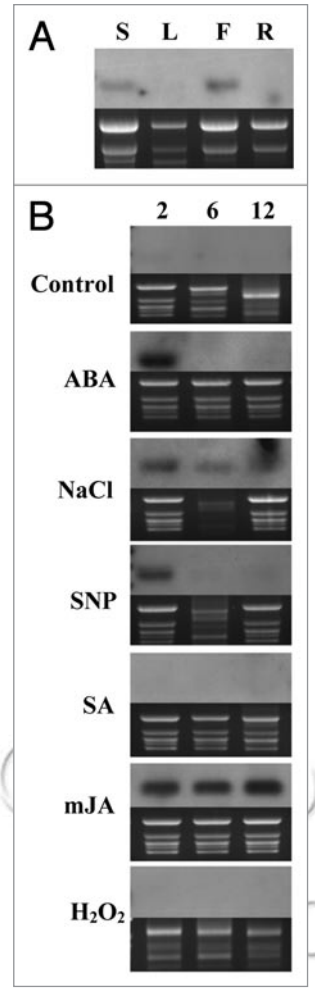

Figure 1. (A) Northern blot analysis showing tissue-specific expression *AnnBj4* gene. S, Stem; L, Leaf; F, Flower; R, Root. (B) Northern blot analysis showing the expression of *AnnBj4* upon different stress treatments. ABA (100 µM), NaCl (200 mM), SNP (100 µM), SA (100 µM), mJA (100 µM), H_2O_2 (10 mM). Control represents mock treatment with water. Lower panel shows equal sample loading by including ethidium bromide in the gel.

and divergence of this protein with rest of the members indicates that this protein may have varied functions.

Plant annexins are known to be regulated developmentally and upregulated in response to biotic and abiotic stresses (reviewed in ref. 5). In our recent study, we demonstrated the differential regulation of these genes upon external application of signaling molecules, abscisic acid (ABA), Ethephon, Salicylic acid (SA) and methyl jasmonate (mJA), oxidative stressors inducers hydrogen peroxide (H_2O_2) and methyl viologen (MV), salinity and wounding. All the signaling molecules appear to regulate the transcription of *AnnBj1*, *AnnBj2* and *AnnBj3* genes, indicating the possible cross-talk in multiple signaling pathways for an adaptive response to these stresses. Significant increase in the expression of *AnnBj1* and *AnnBj3* genes was also observed with other stresses applied.[4,6] In addition to ABA, *AnnBj6* and *AnnBj7* genes respond to ethephon and NaCl respectively. The transcripts of *AnnBj4* were found to be expressed only in stem and flower tissues (**Fig. 1A**). However, we found that the expression of this transcript in leaves upon treatment with some of the above stressors (**Fig. 1B**). Northern blot analyses using 3'-UTR as a probe showed rapid and strong *AnnBj4* upregulation upon treatment with mJA. The inducer of systemic acquired resistance—SA and oxidative stress inducer—H_2O_2 had no effect on *AnnBj4* expression. This gene was also transiently induced by nitric oxide generator, sodium nitroprusside (SNP) and ABA as early as 2 h post treatment. Jasmonic acid (JA) is an important signal molecule, which accumulates in planta during wounding, pathogen invasion and drought conditions.[7] Evidence also shows that mJA induces stomatal closure similar to ABA, which is mediated by NO and/or ROS messengers.[8,9] Low level of *AnnBj4* accumulation was also observed in treatment with NaCl. The mRNA levels of its Arabidopsis homolog, *AnnAt4* showed higher message levels with NaCl in young seedlings. Genetic studies have also demonstrated that *AnnAt4* along with *AnnAt1* play an important role in germination during osmotic and ABA signaling.[10] Annexin from tobacco (*NtAnn12*) was expressed during ABA and NaCl treatments but not with H_2O_2 indicating its involvement in osmotic stress signaling.[11] The in vivo function of this gene during stress by either pathogen, salt or drought awaits further study. Under abiotic stress conditions, endogenous ABA levels in plants increase, which in turn, induce the expression of stress-inducible genes. It is interesting to note that the phytohormone, ABA upregulated all the six annexin genes in Brassica. It has been reported that an in silico analysis of *AnnBj1* and *AnnAt1* promoter region revealed the presence of cis-acting element ABRE, that is responsive to ABA and thus the regulation of the genes by ABA.[4,12] The partial 5'-upstream regions of *AnnBj1*, *AnnBj2* and *AnnBj3* also contain motifs MYC and ERD1 similar to those present in Arabidopsis *rd22* and *ERD1* genes, which are essentail for ABA and drought regulated gene expression. Analysis of the *AnnBj1* promoter also showed the antioxidant responsive element-ARE1, a cis-element similar to that found in promoters of *CAT1*, *CAT3*, *GPX*, *GST* and *heme oxygenase-1* genes that are responsive to reactive oxygen species and regulation of gene expression in defense against oxidative stresses.[13-16] Sequence analysis of *AnnAt1* promoter did not reveal this ARE1 element.

Earlier observations on annexins demonstrated inherent peroxidase activity that was thought to be related to the presence of His40 within the heme motif and their ability to rescue mutant cell lines of *E. coli* compromised in oxidative stress conditions.[17] Mutagenesis of the His40 residue in AnnAt1 abolished the peroxidase activity.[18] In contrast to this, Laohavisit et al.[19] recently reported heme independent peroxidase activity with a maize annexin, while Konopka-Postupolska et al.[12] still showed low peroxidase activity with mutated AnnAt1 (H40A) protein, indicating that other residues are also important for this function. Structural studies of AnnGh1 from cotton indicated that the presence of S_3 cluster, probably formed by intramolecular disulphide bonds (MCCY), forms the molecular basis for the redox reactions.[20] In a recent study, Arabidopsis plants showed drought tolerance by overexpression of *AnnAt1*, while the knock-out plants were drought-sensitive. Biochemical studies also revealed two cysteine residues present in AnnAt1 protein as the sites for post-translational modification. They undergo *S*-glutathionylation modification in vivo after treatment with ABA causing 50% reduction in calcium-binding,[12] promoting membrane association still retaining the peroxidase activity.[21]

The primary structure of all six deduced annexins from Brassica showed the presence of the two cys residues similar to those of AnnAt1 (**Fig. 2**). This has prompted us to speculate whether all these Brassica annexins have this redundant function in alleviating stress induced ROS

as observed in the case of Arabidopsis AnnAt1.[12] In our previous studies,[6] we demonstrated that constitutive expression of *AnnBj1* (a homolog of Arabidopsis *AnnAt1*) in tobacco protects the transgenic plants against various abiotic and biotic stresses. A low level of peroxidase activity was also observed with the recombinant AnnBj1. The membrane damage caused by peroxidation was also found to be reduced in annexin-overexpressing lines compared to that in wild-type during dehydration stress.

Here, we report the detoxification of ROS induced by dehydration stress caused by mannitol treatment (250 mM). It is known that 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a H_2O_2 sensitive dye and an indicator of oxidative stress. Epidermal peels of wild-type and transgenic tobacco plants with high expression of *AnnBj1* (lines 4 and 16) were loaded with H_2DCFDA and subsequently incubated in mannitol or buffer for 30 min. Using Confocal microscopy, the ROS was detected by fluorescence in wild-type plants upon incubation in buffer and mannitol. But, the intensity was more pronounced in mannitol treated in stomatal guard cells of the wild-type indicating the production of ROS. As expected, there was no fluorescence in stomatal guard cells of transgenic plants with either treatment indicating that AnnBj1 plays a role in detoxification of ROS produced by dehydration stress (**Fig. 3**). ABA induced ROS detoxification was also observed recently by *AnnAt1* overexpressed in Arabidopsis.[12]

The regulation of activity of annexins under stress conditions suggests that these genes may play a role in adaptation to environmental conditions and detailed studies on each member of the *Brassica juncea* annexin gene family via transgenic analysis would help in unraveling their functions in stress.

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                *
AnnBj1    (40) H-----MEVA (111) C-----IQ (239) CLTRPELY
AnnBj2    (40) H-----VEIA (111) C-----IT (238) CLTYPEKH
AnnBj3    (40) K-----LEIS (114) C-----IL (242) CIESPEKH
AnnBj4    (30) N-----VEVS (111) C-----LL (235) CLLKPSVY
AnnBj6    (40) H-----VEIA (111) C-----IK (240) CLTYPEKY
AnnBj7    (40) H-----VEIA (111) C-----IK (238) CLTYPEKY
AnnAt1    (40) H-----MEVA (111) C-----IQ (239) CLTRPELY
AnxGh1    (45) H-----MEIA (116) C-----VK (243) CLVYPEKY
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Figure 2. Multiple amino acid sequence alignment of partial sequences from Brassica annexins,[4] Arabidopsis, AnnAt1 and cotton, AnxGh1 (PDB 1N00A). Conservation of two cysteine residues in S_3 cluster which are involved in regulating redox conditions are represented by bold. His40 residue thought to be essential for peroxidase activity is represented as asterik (*).[17,18]

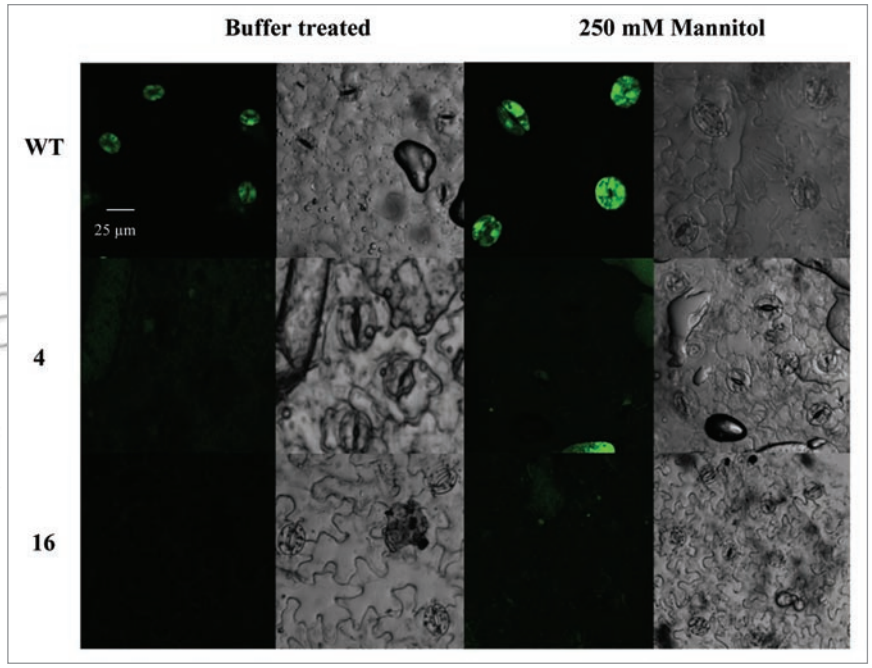

Figure 3. Confocal images showing guard cells from epidermal strips of WT and transgenic tobacco lines 4 and 16. WT plants show fluorescence in both buffer and mannitol treatments. Transgenic plants show detoxification of mannitol induced ROS as observed by the absence of fluorescence in the guard cells. Bright field images of WT and transgenic lines are also shown. Micrographs were taken from a representative lot of guard cells from epidermal strips loaded with H_2DCFDA and the method was followed as described by Murata et al.[22] *Bar* represents 25 µm.

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