

Characterization of genes of importance in stress resistance in mustard, *Brassica juncea*

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Abstract

We have been interested in cloning and characterization of genes expressed in mustard in various stress treatments. In this connection, we have characterized two genes, *NPR1* and *annexin*, that respond to various biotic and abiotic stress treatments. The full-length cDNAs of annexins, which encode proteins having molecular masses of 36.2, 36.0, 36.4 and 36.5 KDa (*AnnBj1*, *AnnBj2*, *AnnBj6*, *AnnBj7*) were isolated from mustard. A partial *AnnBj3* was also isolated along with the genomic clones of these annexins. Genomic DNA gel blot analysis indicated that annexins in mustard belonged to a small multigene family. RNA gel blot analysis showed regulation of annexin transcripts to various signaling molecules or abiotic stresses. Overexpression of *AnnBj1* in tobacco conferred tolerance to dehydration, salinity, heavy metal stress and oxidative stresses at various stages. The transgenic plants also showed enhanced resistance to the oomycete pathogen, *Phytophthora parasitica* var. *nicotianae*.

NPR1 is a gene that has been shown to have a regulatory role in the Systemic Acquired Resistance (SAR) pathway, acting down stream of salicylic acid. We have also cloned and characterized the differential expression of *NPR1* in mustard. The behavior of this followed the same pattern that has been elucidated in *Arabidopsis* showing conservation of SAR pathway in different species. Both these genes appeared to be good candidate genes, and can be deployed in transgenic plants for enhancing their stress tolerance.

Keywords: Abiotic stress, Annexins, Oxidative stress, *NPR1*, Systemic acquired resistance (SAR).

Introduction

Plants are always under threat from various biotic and abiotic stresses and they try to resist these attacks by activating various defense mechanisms. Three key plant hormones namely, jasmonic acid, salicylic acid and ethylene, play crucial role in triggering various signaling pathways in plants leading to defense response against various biotic stresses. Among the different signal transduction pathways, salicylic acid performs the most prominent role in disease resistance (White, 1979). *NPR1* (non-expressor of pathogenesis related proteins-1) gene is known to play a crucial role in salicylic acid-mediated signaling at the onset of systemic acquired resistance to fungal pathogens (Cao *et al.*, 1994; Shah *et al.*, 1997). *NPR1* is also known to be involved in SA-mediated suppression of jasmonic acid signaling pathway (Spoel *et al.*, 2003).

In order to characterize *NPR1* at molecular level in mustard, the 1.74kb full-length cDNA of *NPR1* was cloned and sequenced. The amino acid sequence deduced from the nucleotide sequence was 67% identical to the *NPR1* of *Arabidopsis* and revealed three ankyrin repeats, and a BTB/POZ protein-protein interaction domain. The amino acid sequence does not exhibit any DNA binding motifs. Therefore, *B. juncea* *NPR1* seems to participate in SA-mediated signal transduction via protein-protein interactions, as with *Arabidopsis* *NPR1*. Further, the promoter of *NPR1* gene revealed the presence of several inducible cis-acting elements recognized by different transcription factors like WRKY and TGA, which are major components of defense signaling. Southern blot analysis revealed that *NPR1* was a single copy inherited gene and devoid of any redundancy in all the diploid Brassica spp. Molecular characterization of *NPR1* was carried out with respect to transcriptional activation in mustard plants upon different treatments. It was observed that salicylic acid treatment could induce transcription of *NPR1*. Further, infection with biotrophic fungal pathogen *Erysiphe cruciferarum*, but not with necrotrophic fungal pathogen *Alternaria brassicicola*, induced a transcriptional up-regulation of *NPR1*. When the *NPR1* protein level was studied in mustard leaves, it was observed that *NPR1* was present constitutively as a high molecular weight oligomeric form with SA induced monomerization to 65kDa active form. In order to substantiate the fact that prominent nuclear localization signal was present in *NPR1* amino acid sequence, transient transformation of GFP-fused *NPR1* was carried out. In uninduced state *NPR1*-GFP, as well as GFP were present in the cytosol. However, SA treatment differentially translocated *NPR*-GFP to nucleus, suggesting that SA induced monomerization of *NPR1* was probably followed by translocation to nuclei. All these observations indicated that *NPR1* from mustard is functionally similar to *NPR1* reported from *Arabidopsis*. Further, its involvement in biotrophic fungal infection assigns *NPR1* an important status in defense signaling. *NPR1* would be an ideal gene for genetic engineering of crops to impart enhanced disease resistance (Meur *et al.* 2007).

In plant cells, calcium (Ca^{2+}) serves as a second messenger during abiotic stress signaling (Sanders *et al.*, 1999; Knight, 2000). The increase in calcium levels during abiotic stress or in response to ABA is transduced by certain calcium-binding proteins that include SOS3, Ca^{2+} -dependent protein kinases, calcineurin B-like proteins and calmodulins. Other classes of Ca^{2+} -binding proteins that participate more directly in Ca^{2+} signaling in response to the above stresses are annexins. Annexins

are structurally related calcium-dependent phospholipid-binding proteins found ubiquitously in animals and plants. They were identified in many plants (Clark and Roux, 1995). Plant annexins, like their animal counterparts, belong to a multigene family as in *Arabidopsis* and rice (Cantero et al., 2006; Clark et al., 2001) and are hypothesized to function in Golgi-mediated secretion (Clark et al., 2001, 2005). They also are reported to possess phosphodiesterase activity (Hofmann et al., 2000), peroxidase activity (Gidrol et al., 1996; Gorecka et al., 2005), F-actin binding activity and calcium channel activity (Hoshino et al., 2004), and regulation of callose synthase activity (Verma and Hong, 2001). Plant annexins are regulated developmentally and tissue-specifically. They are also regulated in response to various abiotic stress factors such as abscisic acid (ABA) and osmotic stress (Lee et al., 2004). Mammalian annexins A1, A5 and A6 as well as plant annexins from *Medicago sativa* and *Arabidopsis thaliana* have been implicated in oxidative stress response (Kush and Sabapathy, 2001; Sacre and Moss, 2002; Gidrol et al., 1996). Here we report on the isolation and characterization of annexin isoforms from Indian mustard. The expression of these annexins was upregulated in response to various abiotic stress treatments. To better understand its function in response to various stresses, we constitutively expressed *AnnBj1* in transgenic tobacco plants and conducted phenotypic analyses.

Plant annexins belong to a multigene family

Current complete genomic sequence data of *Arabidopsis thaliana* and *Oryza sativa japonica* indicated that there appears to be eight and ten different annexin cDNAs in *Arabidopsis* and rice respectively (Clark et al., 2001; Cantero et al., 2006). By utilizing the sequence data of *Arabidopsis*, we have cloned five cDNAs encoding annexins in Indian mustard, *AnnBj1*, *AnnBj2*, *AnnBj3* (partial), *AnnBj6* and *AnnBj7*, which are homologues to other plant annexins. Sequence analysis reveals that these cDNAs encode a single polypeptide each ranging from 36.0 to 36.5 KDa, while their iso-electric pH ranged between 5.44 and 6.48. At the nucleotide and protein levels, they shared significant similarity with each other. *In-silico* analysis of the 5'-upstream promoter regions of mustard annexins genes showed the presence of *cis*-elements involved in regulation under various abiotic stresses. *AnnBj1* promoter contains an antioxidant responsive element (ARE), which is present in the promoter of the genes of catalases, peroxidases, heme-oxygenases and animal glutathione-S-transferases.

Primary structure of annexins

The primary structure of these Brassica annexins comprises of a short N-terminal region, C-terminal core composed of four large repeats of 70-75 amino acids, which is a characteristic feature of plant annexins. The characteristic feature of all annexins is their ability to bind acidic phospholipids in a Ca^{2+} -dependent manner. In annexins, the type-II calcium-binding site is determined by the conserved glycine-X-glycine-threonine loop (GXGTD) found within the endonexin fold and is followed 42 amino acids downstream of the first glycine residue by acidic glutamic acid or aspartic acid residue. Similar to many plant annexins, mustard annexins also showed the type-II calcium-binding site in first and fourth repeats (E-in the first and D-in the fourth repeats) and are absent in second and third repeats which are implicated in phospholipid binding. The presence of several common putative phosphorylation sites suggests that these proteins are regulated and activated by similar phosphorylation and other post-translational modifications, which is characteristic of membrane association (Lee et al., 2004).

Expression of Annexins

Tissue-specific expression by Northern blot or RT-PCR analysis showed differential expression in tissues like stem, leaves, roots and flower. No expression was observed in stem with *AnnBj1*, *AnnBj2* and *AnnBj6*. To analyze the role of annexin family of genes in mustard, during abiotic stress and in plant defense, transcript regulation was studied in treatments of mustard leaves with several stress-related signaling compounds, oxidative stress inducers and upon wounding. In all these, annexins were upregulated in a time-dependent fashion suggesting their role in the regulation to diverse abiotic and biotic cues.

Development of transgenic tobacco and their functional studies

To analyze the biological role of annexins *in planta*, several independent transgenic tobacco plants expressing *AnnBj1* constitutively were generated. The integration and expression of the transgene were confirmed by PCR, Southern and Northern blot analysis in T_0 and in T_1 generation. The *AnnBj1* over expressing T_1 transgenic tobacco lines showed significant tolerance to dehydration (mannitol), salt (NaCl), heavy metal (CdCl_2) and oxidative stress caused by methyl viologen or hydrogen peroxide at the seedling stage. Detached leaf-disc senescence assay of the transgenic plants showed higher chlorophyll content and decreased accumulation of thiobarbituric acid reactive substances (TBARS). Lipid peroxidation and studies with hydrogen sensitive dye (H_2DCFDA) showed the detoxification property of *AnnBj1*. The T_1 transgenic tobacco plants also showed tolerance to dehydration under severe water-deficit conditions and able to produce viable seeds, while wild type plants produced aborted seeds. The T_1 transgenic plants showed enhanced resistance also to the oomycete pathogen *Phytophthora parasitica* var. *nicotianae*.

These studies indicate that annexin is a gene with a lot of commercial importance.

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