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Research article

Molecular cloning and characterization of five annexin genes from Indian mustard (*Brassica juncea* L. Czern and Coss)

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ABSTRACT

Plant annexins constitute a multigene family having suggested roles in a variety of cellular processes including stress responses. We have isolated and characterized five different cDNAs of mustard, *Brassica juncea* (*AnnBj1*, *AnnBj2*, *AnnBj3*, *AnnBj6* and *AnnBj7*) encoding annexin proteins using a RT-PCR/RACE-PCR based strategy. The predicted molecular masses of these annexins are ~36.0 kDa with acidic p*I*s. At the amino acid level, they share high sequence similarity with each other and with annexins from higher plants. Phylogenetic analysis revealed their evolutionary relationship with corresponding orthologous sequences in *Arabidopsis* and deduced proteins in various plant species. Expression analysis by semi-quantitative RT-PCR revealed that these genes are differentially expressed in various tissues. The expression patterns of these genes also showed regulation by various stress conditions such as exposure to signaling molecules, salinity and oxidative stress and wounding. Additionally, the *in silico* promoter analysis (of *AnnBj1*, *AnnBj2* and *AnnBj3*) showed the presence of different *cis*-responsive elements that could respond to various stress conditions. These results indicate that *AnnBj* genes may play important roles in adaptation of plants to various environmental stresses.

1. Introduction

Plants are continuously exposed to various abiotic stresses such as salinity, cold, drought, chemical toxicity, which are the primary causes of crop losses worldwide [7]. They adapt to these unfavorable conditions by perceiving and transducing the stress signal(s) through a cascade of molecular networks eventually leading to the expression of stress-related genes [59]. In plant cells, calcium ions (Ca^{2+}) serve as a second messenger during abiotic stress signaling [49]. The increase in calcium levels during abiotic stress is perceived and transduced by certain calcium-binding proteins such as calmodulin, calcium-dependent protein kinases (CDPKs) and calcineurin-B-like proteins (CBL) proteins [27]. There is increasing evidence that another class of proteins, annexins which also bind calcium and play important roles in abiotic stress responses in plants [10,26,29,31].

Annexins are calcium-dependent phospholipid-binding proteins. They are ubiquitous in animal and plant kingdoms. Plant annexins were first identified in tomato [5] and subsequently, isolated and characterized in a wide range of plant species [36]. In vertebrates, annexins are represented by at least thirteen distinct members [43]. DNA blot analyses in *Arabidopsis* [19], maize [3], bell pepper [41] and tobacco [42] have indicated that the annexin gene family in plants is relatively simple and possesses at least two different annexins. However, with the availability of complete genomic sequence data in *Arabidopsis* and rice, there appears to be eight and ten different annexin cDNA sequences, respectively [10,13]. The primary structure of mammalian and non-vertebrate metazoan annexins is characterized by a tetrad repeat of 70 amino acids containing calcium-binding endonexin sequence usually referred as G-X-G-T-{38}-(D/E) motif that binds calcium. Within the family of plant annexins, the endonexin sequence is only conserved within the first and fourth repeats with the presence of type-II calcium-binding sites (acidic residues). Recent studies on crystal structural analysis showed that calcium binds to cotton annexin in repeats first and fourth in the presence of acidic phospholipid vesicles [25].

Plant annexin gene expression is influenced by tissue/cell-specific developmental controls and environmental signals.

Abbreviations: ABA, abscisic acid; H_2O_2, hydrogen peroxide; mJA, methyl jasmonate; MV, methyl viologen; NUP, nested universal primer; ORF, open reading frame; PEG, polyethylene glycol; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-polymerase chain reaction; SA, salicylic acid; UPM, universal primer mix; UTR, untranslated region.

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Differential expression of annexins has been observed in various tissues of tomato, potato and barley [53], *Arabidopsis* [13], tobacco [42], alfalfa [29] and mustard [26]. Immunolocalization studies have shown annexin localization in tips of pollen tubes in *Lilium longiflorum* [4] and in the leaf and stem cells of pea [11]. Annexins are regulated developmentally during fruit ripening in bell pepper and strawberry [41,57], cell cycle progression of dividing cells of tobacco and alfalfa [29,42], cell-specific expression in developing seedlings of *Arabidopsis* [13], in cotton fiber development [51] and in cell volume control due to vacuole size regulation in celery and tobacco [50].

Annexin gene expression is regulated by treatment with hormones like abscisic acid (ABA) or salicylic acid (SA) as in alfalfa [29], *Arabidopsis* [28,19], and mustard [26]. The alfalfa *AnnMs2* was shown to be induced by osmotic stress induced by NaCl, mannitol and PEG [29]. Expression patterns of the *Arabidopsis* annexin gene family, *AnnAt1–AnnAt8*, in response to salinity, drought, cold and heat in seedlings have been recently characterized [10,28]. Additionally, studies have shown cellular redistribution by mechanical stimulation in *Bryonia dioica* [55], diurnal cycles in Mimosa [24], gravity in pea [12], nod factors and by symbiotic association in *Medicago truncatula* [39], and during low temperature treatment in wheat [8]. Annexin gene expression was also regulated in response to oxidative stress, H_2O_2 in *Arabidopsis* [19,28], *Medicago sativa* [29] and in mustard [26]. Genetic studies on T-DNA insertional mutants of *AnnAt1* and *AnnAt4* showed hypersensitivity to ABA and osmotic stresses in *Arabidopsis* [31]. Our previous report showed that tobacco plants ectopically expressing *AnnBj1* had enhanced tolerance to a variety of abiotic and biotic stresses [26]. A recent report evidenced more detailed information that *AnnAt1* provided drought stress in *Arabidopsis* [28].

Here we report on molecular cloning and characterization of five different annexin members (*AnnBj1*, *AnnBj2*, *AnnBj3*, *AnnBj6* and *AnnBj7*) from Indian mustard. Sequence analyses of the deduced forms of these proteins were analyzed and their phylogenetic relationships were compared with annexins from various plant species. The gene expression studies in various tissues and under stress conditions were analyzed by a semi-quantitative reverse transcription-PCR (RT-PCR). Furthermore, the upstream *cis*-elements of three annexins (*AnnBj1*, *AnnBj2* and *AnnBj3*) were analyzed and motifs were found that could be induced by various abiotic stress treatments.

2. Materials and methods

2.1. Plant material and stress treatments

Six-week-old mustard plants grown in the greenhouse were used in the present study. To investigate the effect of abiotic stress inducing compounds and different signaling compounds on the expression of annexin genes (*AnnBj1*, *AnnBj2*, *AnnBj3*, *AnnBj6* and *AnnBj7*), fully expanded leaves were fed through petiole separately with solutions containing 100 μM abscisic acid (ABA), 1% (v/v) ethephon, 100 μM salicylic acid (SA), 100 μM methyl jasmonate (mJA), 200 mM sodium chloride (NaCl), 10 μM methyl viologen (MV) or paraquat and 10 mM hydrogen peroxide (H_2O_2). Wounding stress was given by pressing the leaves with blunt ends of the forceps. The leaf treatments were carried out at different time periods up to 24 h and incubated in a growth room at 27 ± 1 °C under 16/8 light/dark photoperiod provided by light intensity of 30 μmol m^{-2} s^{-1}. Control plants were mock treated with water. After the treatment, leaves were collected, wrapped in aluminium foil, quick-frozen in liquid N_2 and stored at −70 °C until further use. For the expression at tissue-specific level, samples were collected from stems, roots, leaves and flowers from 2-month-old plants.

2.2. Identification of AnnBj genes by database search

To identify the members of *AnnBj* genes (*AnnBj1*, *AnnBj2*, *AnnBj3*, *AnnBj6* and *AnnBj7*), a BLAST search of the NCBI GenBank (http://www.ncbi.nlm.nih.gov) and *Brassica napus* DFCI-Plant Gene Index database (http://compbio.dfci.harvard.edu/tgi/plant.html) were used with nucleotide sequences of *Arabidopsis* gene family as query sequences. The genes identified were compared with the corresponding query sequences using ClustalW2 at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalW2) and numbered accordingly.

2.3. Sequence and phylogenetic analysis

The annotation of amino acid sequences, p*I* and the theoretical molecular mass of each of the AnnBj proteins were analyzed using the Expasy proteomic server (http://www.ca.expasy.org). Multiple sequence alignment of the deduced AnnBj proteins was carried out with the ClustalW2 at the European Bioinformatics Institute and further analyzed by the GenDoc program [38]. The exon–intron structures of *AnnBj* genes were confirmed by alignment of cDNA sequences with the corresponding genomic sequences using ClustalW2. The transcription start site (TSS) was predicted using a promoter prediction program, http://www.fruitfly.org/seq_tools/promoter.html [44]. The search for the putative potential *cis*-regulatory elements in the promoter sequences was performed with the PLACE database, http://www.dna.affrc.go.jp/htdocs/PLACE [21]. Phylogenetic analysis was performed to investigate the evolutionary relationships among deduced annexin sequences from mustard and from diverse plant species. The unrooted phylogenetic tree was generated by neighbor-joining method using MEGA4 from 1000 bootstrap replicates [54] and the evolutionary distances were calculated by Poisson correction method corresponding to the number of amino acid substitutions per site.

2.4. Total RNA extraction and cDNA synthesis

The plant material (0.2 g) from the stress-treated and unstressed leaves and the tissues such as stem, roots and flowers from the frozen samples were ground to a fine powder in liquid nitrogen and the total RNA was extracted using TRI reagent according to the manufacturer's instructions (Sigma–Aldrich, USA). Five μg of total RNA was taken to synthesize the first strand cDNA using oligo-$dT_{(18)}$ using M-MLV reverse transcriptase (Sigma–Aldrich, USA) following manufacturer's protocol.

2.5. Isolation of cDNA clones from Brassica juncea

The full-length cDNAs of *AnnBj* genes were isolated using gene specific or degenerate primers for reverse transcription (RT)-PCR or rapid amplification of cDNA ends (RACE)-PCR reactions.

The isolation of cDNA for *AnnBj1* was carried out previously [26]. For the isolation of other annexin cDNAs, total RNA from unstressed (*AnnBj2*, *AnnBj3* and *AnnBj7*) or ABA treated leaves (*AnnBj6*) were used in reverse transcription. For PCR amplification, 2 μL of the reaction mixture was used as a template using the set of gene specific or degenerate primers (Table 1S, Supplementary data). PCR amplifications were performed in a total volume of 50 μL using recombinant *Taq* DNA polymerase (Invitrogen, Brazil) at 94 °C for 4 min followed by 94 °C for 1 min (denaturation), 55 °C for 30 s (annealing), 72 °C for 1 min (elongation) for 30 cycles with a final extension of 20 min at 72 °C. The resultant PCR products were purified with Eppendorf Perfectprep Gel cleanup kit (Eppendorf, Germany) and cloned in pTZ57R/T vector (Fermentas, Germany).