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Research article

Ectopic expression of an annexin from *Brassica juncea* confers tolerance to abiotic and biotic stress treatments in transgenic tobacco

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ABSTRACT

Plant annexins belong to a multigene family and are suggested to play a role in stress responses. A full-length cDNA for a gene encoding an annexin protein was isolated and characterized from *Brassica juncea* (*AnnBj1*). *AnnBj1* message levels were regulated by abscisic acid, ethephon, salicylic acid, and methyl jasmonate as well as chemicals that induce osmotic stress (NaCl, Mannitol or PEG), heavy metal stress ($CdCl_2$) and oxidative stress (methyl viologen or H_2O_2). In order to determine if *AnnBj1* functions in protection against stress, we generated transgenic tobacco plants ectopically expressing *AnnBj1* under the control of constitutive CaMV 35S promoter. The transgenic tobacco plants showed significant tolerance to dehydration (mannitol), salt (NaCl), heavy metal ($CdCl_2$) and oxidative stress (H_2O_2) at the seedling stage and retained higher chlorophyll levels in response to the above stresses as determined in detached leaf senescence assays. The transgenic plants also showed decreased accumulation of thio-barbituric acid-reactive substances (TBARS) compared to wild-type plants in response to mannitol treatments in leaf disc assays. AnnBj1 recombinant protein exhibited low levels of peroxidase activity *in vitro* and transgenic plants showed increased total peroxidase activity. Additionally, the transgenic plants showed enhanced resistance to the oomycete pathogen, *Phytophthora parasitica* var. *nicotianae*, and increased message levels for several pathogenesis-related proteins. Our results demonstrate that ectopic expression of *AnnBj1* in tobacco provides tolerance to a variety of abiotic and biotic stresses.

1. Introduction

Plants are sessile organisms and are under continuous threat from abiotic and biotic stresses that limit their productivity. Under such situations, plants perceive stress signals through a network of signal transduction pathways leading to changes in gene expression [65]. In plants, atmospheric oxygen is partially reduced to superoxide ions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$), collectively called reactive oxygen species (ROS), through metabolic reactions that occur primarily in chloroplasts, mitochondria and peroxisomes [15].

Diverse stress conditions both biotic and abiotic, result in a rapid increase in ROS that can damage nucleic acids, proteins, and cause peroxidation of membrane lipids and other cellular components [20,35,39,54]. In chloroplasts, ROS produced in high light accumulates in excess under stresses such as drought and high temperatures, resulting in the degradation of D1 protein of the photosynthetic reaction center PS-II. This causes the inhibition of photosynthesis and hence the loss of chlorophyll pigment [2]. However, in spite of ROS toxicity at higher levels, low levels of ROS function in signaling during the activation of stress-response and defense pathways [2,39]. ROS signaling is very complex and is dependent on the type of ROS produced, the timing and location of ROS production, and the levels of ROS production [21]. Thus, ROS homeostasis is closely regulated and plant cells contain ROS scavengers to detoxify excess ROS [2,39,42].

Because early responses to stress in plants typically involve calcium signaling [62,64], calcium-binding proteins are important for transducing stress signals into adaptive responses. Included among these proteins are annexins, which are calcium-dependent phospholipid-binding proteins, found ubiquitously in animal and plant kingdoms. Plant annexins, like their animal counterparts,

Abbreviations: EDTA, ethylenediaminetetraacetic acid; ORF, open reading frame; PEG, polyethylene glycol; RACE, rapid amplification of cDNA ends; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TBA, thiobarbituric acid; TCA, trichloroacetic acid; UTR, untranslated region.

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belong to a multigene family, and have been identified in many plants (for review, see [12,16,28,40]). The earliest hypothesized function for plant annexins is their participation in Golgi-mediated secretion, a postulate based on immunolocalization studies, *in situ* hybridization results, and *in vitro* assays [5,9,11,13,14]. Different annexins are also reported to possess different enzyme or other protein activities, including phosphodiesterase activity [7,27,53], peroxidase activity [22,23], F-actin binding activity [7,30,31] and calcium channel activity [24,27,30]. They also may participate in the regulation of callose and cellulose synthase activity [1,28,61] and be involved in responses to low temperature [6] and mechanical stimulation [57].

Certain animal annexins have been shown to play a role in cells during their response to oxidative stress [46,48,56]. Plant annexins from *Medicago sativa* and *Arabidopsis thaliana* have also been implicated in oxidative stress responses [33,36]. In particular, an *Arabidopsis* annexin, AnnAt1, was able to rescue *Escherichia coli ΔoxyR* mutants from oxidative stress [22]. Two subsequent studies found that AnnAt1 can provide protection to mammalian cells from H_2O_2 stress and leads to a decrease in the production of superoxide ions [32,34].

The studies suggesting a potential role for annexins during oxidative stress raise the question of whether certain annexins are involved in defense against oxidative stress in plants. Here we report that the expression of an annexin from *Brassica juncea*, *AnnBj1*, which is a homologue of *Arabidopsis AnnAt1,* is up-regulated in response to ABA and various abiotic stress treatments. To better assess its importance in mediating responses to various stresses, we constitutively expressed *AnnBj1* in transgenic tobacco plants and conducted phenotypic analyses. We found that the ectopic expression of *AnnBj1* confers tolerance towards drought, salinity, heavy metal and oxidative stress and also resulted in enhanced resistance against the oomycetes pathogen, *Phytophthora parasitica* var. *nicotianae*. To our knowledge this is the first report on the effects of ectopic expression of an annexin in a transgenic plant.

2. Materials and methods

2.1. Plant material and stress treatments

Indian mustard plants (*Brassica juncea* L. Czern and Coss) grown in a greenhouse for 6 weeks were used in the present study. To investigate the effect of compounds that induce abiotic stress and different signaling compounds on the expression of *AnnBj1*, fully expanded leaves were fed through petioles with solutions containing one of the following compounds: 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 10 mM hydrogen peroxide (H_2O_2). Wounding stress was given by pressing the leaves with blunt ends of the forceps. The 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 h 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 aluminum foil, frozen in liquid N_2 and stored at −70 °C until further use. For treatments with 150 μM $CdCl_2$, 250 mM mannitol and 10% (w/v) PEG (MW 6000), 12-day-old *Brassica juncea* seedlings were transferred to liquid MS supplemented with treatment solutions and incubated in growth room conditions up to 8 h under the same photoperiod conditions. For the expression at tissue specific level, samples were collected from stems, roots, leaves and flowers from 2-month-old plants.

2.2. Cloning of annexin cDNA from Brassica juncea (AnnBj1)

Total RNA was extracted from flower buds using TRI reagent (Sigma, USA). Five μg of total RNA was used to synthesize first-strand cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA). This was then used as a template for the polymerase chain reaction (PCR) using forward primer 5′-GGA TCC ATG GCG ACT CTT AAG GTT TCT-3′ and reverse primer 5′-GGT CGA CTT AAG CAT CTT CAC CGA GA-3′ using Hot start *Taq* polymerase (Qiagen, Germany). The resultant PCR product was purified with Eppendorf perfectprep Gel cleanup kit (Eppendorf, Germany) and cloned in pTZ57R/T vector (Fermentas, Germany) designated as pTZ57R-*AnnBj1*. The 5′- and 3′-ends of the cDNA were cloned using SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). For 5′-RACE, first and nested PCR products were amplified using primers 5′-TAA GAA GAT CCT CTC CAA AGG ATT C-3′ and 5′-ATT GAT ATG ATC AAT TCC TCG TTG GT-3′ respectively. Similarly, the 3′-RACE products were obtained by using primers 5′-ACG AAG GAG CTC TCA CTA GAA T-3′ and 5′-TAC CAA AGA AGG AAC AGC ATT C-3′ as outer and nested gene primers, respectively.

2.3. Construction of plant expression vector and transformation of tobacco

To overexpress *AnnBj1* in tobacco (*Nicotiana tabacum* var. Petit Havana SR1), the plasmid pTZ57R-*AnnBj1* was digested with NcoI and XbaI restriction enzymes and cloned in the corresponding sites of plant expression vector pRT100 that was flanked by CaMV 35S promoter and the poly-adenylation signal to create pRT100-*AnnBj1*. This expression cassette (∼1.7 kb) was excised with PstI digestion and sub-cloned subsequently at PstI site in the binary vector pCAMBIA2300. The resultant recombinant vector pCAMBIA2300-*AnnBj1* carrying the annexin expression cassette and the marker gene for kanamycin resistance was transformed into *Agrobacterium tumefaciens* strain GV2260 by a freeze–thaw method. Single colonies of the *Agrobacterium* strain were grown at 28 °C in 50 ml of liquid LB medium containing 100 mg l^{-1} carbenicillin, 100 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin to post log phase. Then the bacterial suspension was pelleted at 5000 rpm for 10 min and the cells were resuspended in MSH medium at 1:2 (v/v) dilutions. This suspension was used for co-cultivation.

Fully expanded mature leaves from 6- to 8-week-old plants grown in a greenhouse were surface sterilized in 70% ethanol (v/v) for 1 min followed by 7 min with 0.1% (w/v) aqueous mercuric chloride and subsequent washes with sterile distilled water. Tobacco leaf discs were co-cultivated with *A. tumefaciens* (GV2260) containing pCAMBIA2300-*AnnBj1* according to the method of Horsch et al. [29]. The putative T_0 transformants were screened using genomic PCR, Southern and Northern blot analyses. The seeds from the primary transformants (T_0) were collected through self-pollination and were germinated on kanamycin-containing medium to select for all of the transgenic T_1 seedlings.

2.4. DNA and RNA gel blot analysis

For DNA blot analysis, 20 μg of genomic DNA from different independent T_0 tobacco plants that were PCR-positive was digested with PstI and EcoRI separately to detect the presence and copy number of *AnnBj1*. After restriction digestion, the DNA samples were electrophoresed on an 0.8% agarose gel and transferred onto Hybond-N^+ nylon membrane (Amersham Biosciences, UK) by capillary diffusion, using 20× SSC as a transfer buffer. The UV-cross linked membrane was pre-hybridized at 65 °C for 4 h in a phosphate buffer (0.5 M phosphate buffer, pH 7.2, 7% (w/v) SDS, 10 mM EDTA and 1% BSA) and hybridized for 16 h with a cDNA probe radiolabeled with [^{32}P] ATP. The probe was prepared from the *AnnBj1* PCR fragment with the same primers used for the isolation of *AnnBj1* cDNA following a hexalabeling method according to the manufacturer's instructions (Fermentas, Germany). After hybridization, the membranes were washed twice with 2× SSC, 0.1% SDS