

Cloning and Expression Characterization of Four Annexin Genes During Germination and Abiotic Stress in *Brassica rapa* subsp. *rapa* ‘Tsuda’

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Published online: 16 September 2015
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Abstract Annexins are a multigene family in most plant species and are suggested to play a role in a wide variety of essential cellular processes. They are well characterized in *Arabidopsis*; however, no such characterization of turnip annexin gene family has been reported thus far. So, four cDNAs of turnip, *BrANNEXIN1*, *BrANNEXIN2*, *BrANNEXIN3*, *BrANNEXIN4*, encoding annexin proteins using a RT-PCR/RACE-PCR-based strategy were isolated and characterized. The predicted molecular masses of these annexins are ~36.0 kDa. At the amino acid level, they share high sequence similarity with each other and with annexins from higher plants. Using quantitative real-time reverse transcription PCR to assess their differential expression in different tissues or after different stimuli, we found that these *BrANNEXIN* genes are differentially expressed in various tissues. The expression patterns of four annexin genes during germination in normal and dark-grown seedlings were determined. Our results indicate that transcripts for all four annexins are present in dark germinating seedlings, and *BrANNEXIN1*, *BrANNEXIN3*, and *BrANNEXIN4* are present in normal germinating seedlings. Only *BrANNEXIN4* was involved in UV-A-induced anthocyanin synthesis in the root epidermis of Tsuda turnip, which accumulates high levels of

anthocyanin. All four annexins differently expressed in the UV-A light-induced anthocyanin synthesis in the hypocotyl. When 4.0-day-old etiolated seedlings were treated with red or far-red light, transcript levels of the four annexins in hypocotyls and cotyledons significantly increased. Finally, we monitored annexin expression in response to various abiotic stresses. Expression of these genes also changed in response to abiotic stresses such as high and low temperatures, dehydration, and osmotic and salt stresses. These results indicate that *BrANNEXIN* genes may play important roles in adaptation of plants to various environmental stresses.

Keywords *Brassica rapa* · Annexins · Germination · Anthocyanin biosynthesis · Abiotic stress

Abbreviations

PEG	Polyethylene glycol
ABA	Abscisic acid
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
UTR	Untranslated regions
ORF	Open reading frame
GTP	Guanosinetriphosphate
ECM	Extracellular matrix
SUMO	Small ubiquitin-related modifier

Electronic supplementary material The online version of this article (doi:10.1007/s11105-015-0935-1) contains supplementary material, which is available to authorized users.

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Introduction

Annexins, an evolutionarily conserved group of Ca²⁺-dependent phospholipid-binding proteins, exist as multigene families of varying number depending on the organism (Moss and Morgan 2004). They are relatively abundant cellular proteins, mainly in the cytoplasm and in the membrane system

including the endoplasmic reticulum, secretory vesicles, plasma membrane, and intracellular sites of plants (Blackbourn and Battey 1993; Mortimer et al. 2008). Some annexins have also been detected in the nucleus, Golgi apparatus, and chloroplasts (Carter et al. 2004; Sun et al. 1992).

All annexins have an internally repetitive structure comprising four or eight repeats of a conserved 70–75 amino acid domain termed the annexin repeat. Each repeat contains a conserved 17-amino acid consensus sequence with the characteristic GXGTDE motif called the endonexin fold. 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; Hu et al. 2008). The C-terminal “core” is thought to be involved in Ca^{2+} binding, and it is distinct from the more common EF hand Ca^{2+} -binding motif (helix-loop-helix) (Kawasaki and Kretsinger 1994). The N-terminal regions of annexins are highly variable in length and amino acid composition. The N-terminal domains are almost certainly responsible for the distinct functions of annexins because they contain the major sites involved in phosphorylation, SUMOylation, proteolysis, or cellular interactions with other proteins (Raynal and Pollard 1994; Caron et al. 2013).

Plant annexins were first identified in tomato and, subsequently, isolated and characterized in a wide range of plant species (Boustead et al. 1989; Mortimer et al. 2008). With the availability of the complete genomic sequence data for *Arabidopsis* and rice, 8 and 10 different annexin complementary DNA (cDNA) sequences have been identified, respectively (Cantero et al. 2006; Jami et al. 2012; Clark et al. 2012). In Indian mustard, five different annexin cDNA sequences that were cloned and characterized (Jami et al. 2009) were found to be expressed in various tissues and at different developmental stages. Expression can also be tissue-specific as found for various tissues of *Arabidopsis*, mustard, and maize (Carroll et al. 1998; Cantero et al. 2006; Jami et al. 2009). The expression of *ANNEXINS* is regulated during cell-specific expression in developing seedlings of *Arabidopsis*, and the synthesis of annexins is involved in cell volume control by regulating the size of the vacuole in celery and tobacco (Seals and Randall 1997; Clark et al. 2001).

Now, it is clear that plant annexin genes are transcriptionally activated by a variety of abiotic stresses. The expression of *ANNMS2*, an *ANNEXIN-LIKE* gene in alfalfa, can be induced by osmotic stress using mannitol, polyethylene glycol (PEG), abscisic acid (ABA), and NaCl (Kovacs et al. 1998a, b). Other plant annexin genes are known to be regulated by other abiotic stresses such as dehydration, salinity, cold, heat, and oxidative stresses or by phytohormones such as ABA, jasmonic acid, ethylene, salicylic acid, and auxin in many species, including *Arabidopsis* (Lee et al. 2004; Cantero et al. 2006), Indian mustard (Jami et al. 2009), tobacco (Vandeputte et al. 2007; Baucher et al. 2011), and rice (Jami

et al. 2012). Moreover, another study showed that an interaction between AnnAt1 protein and AnnAt4 protein regulates responses to dehydration and salt stresses in a light-dependent manner in *Arabidopsis* (Huh et al. 2010).

Similarly, our laboratory also found a light-dependent increase in the expression of *ANNEXIN* in the epidermis of swollen hypocotyls (root) and in the upper hypocotyls of ‘Tsuda’ turnip (*Brassica rapa* subsp. *rapa*) seedlings using UV-A light and a cDNA microarray analysis (Zhou et al. 2007). Because this purple top cultivar accumulates high levels of anthocyanins, which are of great interest for their health benefits, we have been characterizing anthocyanin synthesis, accumulation, and regulation. After exposure to blue light and other monochromatic wavelengths, the seedlings fail to accumulate anthocyanins (Zhou et al. 2007; Wang et al. 2012). These results suggest the presence of a distinct UV-A photoreceptor and that *ANNEXIN* may be involved in this UV-A light signal transduction pathway and anthocyanin biosynthetic regulation pathway. To determine the number of annexin genes and characterize their expression at different growth states of Tsuda turnip and under different conditions in relation to anthocyanin production, here four annexin members (*BrANNEXIN1*, *BrANNEXIN2*, *BrANNEXIN3*, *BrANNEXIN4*) from Tsuda turnip were cloned and characterized. Sequences of the deduced forms of these proteins were analyzed, and their phylogenetic relationships were compared with annexins from various plant species. Quantitative real-time polymerase chain reaction (PCR) were used to profile transcript abundance of all four annexin genes in various tissues and any change in their abundance during seed germination, seedling growth and development, and anthocyanin accumulation after various light conditions and stress (cold, heat, dehydration, salinity) and phytohormone (ABA) treatments.

Materials and Methods

Plant Materials

For all experiments, seeds of *B. rapa* subsp. *rapa* ‘Tsuda’ were cold-stratified at 4 °C for 12 h. For use in gene cloning and tissue-specific expression analyses, seeds were sown in 20-cm pots of soil in a greenhouse. Plants were then grown at 26±3 °C with a 14-h light/10-h dark photoperiod. Samples of the epidermis from the roots grown in the dark (“white” roots) or in the sunlight (“red” roots), roots with the epidermis removed, and leaves from 2-month-old plants and buds (length 0.8 mm) and petals from 6-month-old plants were harvested, frozen in liquid nitrogen, and then stored at –80 °C.

For the analysis of annexin gene expression in the hypocotyls and cotyledons and the effects of UV-A, red, and far-red light on the expression in the hypocotyls and cotyledons,

seeds of the *Tsuda* turnip were sown in a row on wet filter paper. To avoid any influence of fluorescence from the irradiation of the filter paper, we covered the seeds with wet filter paper with aluminum foil that had a slit 1–2 mm wide to allow seedlings to emerge. These seedlings were then grown in the light or dark at 25 °C on wet filter paper and harvested and then frozen and stored in liquid nitrogen at –80 °C (Wang et al. 2012). Dark-grown seedlings were harvested on the same way but under a weak green light.

Light Treatments

UV-A Light Treatment of White Root

To study annexin gene expression in *Tsuda* roots that accumulated anthocyanin induced by UV-A light, we irradiated white roots of 2-month-old turnips for 0, 1, 3, 6, 12, and 24 h with a UV-A fluorescent lamp (FL10BLB, Toshiba), filtered through soda-lime glass plates (peak at 350 nm, 320–400 nm, 3.0 W m^{-2}) to induce anthocyanin accumulation. The pigmented epidermis, the tissue in which the anthocyanin preferentially accumulates, was harvested from the roots after UV-A irradiation under weak green light and frozen in liquid nitrogen before storage at –80 °C.

UV-A Light Treatment of 4-Day-Old Dark-Grown Seedlings

For the analysis of annexin gene expression in *Tsuda* seedling in relation to anthocyanin accumulation after UV-A light, 4-day-old dark-grown seedlings were irradiated with UV-A light for 0, 1, 3, 6, 12, or 24 h to induce anthocyanin accumulation. The cotyledons and hypocotyls (length 5 cm) were then harvested under weak green light and frozen in liquid nitrogen before storage at –80 °C. Under weak green light, the seedlings were then collected, trimmed of their roots, and separated into cotyledons and five sections of hypocotyls of equal length, designated as H1–H5 (H1–H3 for upper hypocotyls; H4–H5 for lower hypocotyls, Fig. 4a).

Red Light or Far-Red Light Treatment of 4-Day-Old Dark-Grown Seedlings

For testing the effects of red light or far-red light on annexin gene expression, 4-day-old, *Tsuda* seedlings grown in the dark were either exposed to 2 min of red light (660-nm LED, SLA-580-JT, Rohm, 13 W m^{-2}) followed by 30 min of dark or to 2 min of red light followed immediately by 3 min of far-red light (735-nm LED, 735 AU from Epitex, Kanagawa, Japan, 14 W m^{-2}) then 30 min dark, or to no light before harvesting upper hypocotyls (from H1 to H3) and lower hypocotyls (from H4 to H5) and cotyledons. All samples were harvested under weak green light and frozen in liquid nitrogen before storage at –80 °C.

Anthocyanin Measurement

The tissues ~1–2 mm thick were peeled from the red root epidermis, white root epidermis, root without epidermis, and leaf and trimmed to a 1-cm × 1-cm section. These sections, a bud, a petal, an upper hypocotyl section, and a lower hypocotyl, were soaked in 3 ml of methanol containing 1 % HCl for 12–24 h at 4 °C. The anthocyanin content was determined by absorbance at 535 nm and five or six replicates were prepared for each sample for anthocyanin analysis.

Stress Treatments

Accumulating evidence from various plant species, including *Arabidopsis*, has shown the upregulation of annexin genes in response to abiotic stress conditions (Laohavisit and Davies 2011). However, no such information is available on the response of annexin family genes in turnip plants. So, we test the effects of various abiotic stresses or phytohormone ABA on annexin gene expression in *Tsuda* seedling. We exposed 5-day-old seedlings to the following stress treatments for 2, 4, 8, or 24 h: heat stress (37 °C), cold stress (4 °C), osmotic stress (10 % PEG), salinity stress (100 mM NaCl), or phytohormone (150 μM ABA). For dehydration stress, 5-day-old seedlings were exposed on the bench 0.5, 1, and 2 h. Five-day-old seedlings without any stress treatments were used as controls, substituting normal growth conditions for the temperature stress, distilled water for the NaCl treatment, and normal growth temperature in water as the control for the dehydration stress. Harvested samples were frozen in liquid nitrogen before storage at –80 °C.

Total RNA Extraction and cDNA Synthesis

The plant material (100 mg) from different tissues and development stages and which were light-treated, stress-treated, and unstressed from the frozen samples was ground to a fine powder in liquid nitrogen, and the total RNA was extracted using TRNzol-A⁺ lysis solution (TIANGEN Biotech, Beijing, China), according to the manufacturer's instructions. cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Total RNA from the red root epidermis was used for gene cloning.

Isolation of cDNA Clones from *Tsuda* Turnip

Total RNA from the red root epidermis was used to synthesize full-length cDNAs of *Tsuda* turnip annexin genes (*BrANNEXIN1*, *BrANNEXIN2*, *BrANNEXIN3*, *BrANNEXIN4*) using gene-specific or degenerate primers for reverse transcription (RT)-PCR or rapid amplification of cDNA ends (RACE)-PCR reactions.

For PCR amplifications, 2 μ l of cDNA (from “Total RNA Extraction and cDNA Synthesis”) was used as a template using gene-specific or degenerate primers (Table 1Sa, Supplementary data). PCR amplifications were performed in a total volume of 50 μ l using recombinant *Taq* DNA polymerase (Invitrogen) at 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The resultant PCR products were purified with Eppendorf Perfectprep Gel cleanup kit (Eppendorf) and cloned in P^{EASY}-T5 vector using the manufacturer’s instructions (TransGen, Biotech, Beijing, China). Plasmids were purified using a Perfectprep plasmid mini Kit (Eppendorf) and sequenced.

The 3′- and 5′-untranslated regions (UTR) regions of annexins were amplified using Superscript III reverse transcriptase (Invitrogen) and using the sequence information of the corresponding cDNA isolated by RT-PCR. First-strand cDNA was synthesized using 1 mg total RNA from the red root epidermis as a template and an oligo(dT)₁₇ adapter primer. The cDNA was synthesized by Superscript III reverse transcriptase (Invitrogen) at 42 °C for 30 min followed by 94 °C for 5 min (Frohman et al. 1988).

3′-RACE-PCR was performed using first-strand cDNA from 3′- and 5′-UTR RT-PCR as a template and the respective forward primer (*BrANNEXIN1-3′F*, *BrANNEXIN2-3′F*, *BrANNEXIN3-3′F*, or *BrANNEXIN4-3′F*) and adapter primer (Table 1Sb, Supplementary data). PCR was performed using the following cycling conditions: 94 °C for 4 min; 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. 3′-RACE-PCR products were purified, cloned, and sequenced.

For the 5′-RACE-PCR template, the 5′-terminal of first-strand cDNA template was primed with an oligo(dT)₁₆ anchor primer. PCR was performed using the anchor primer and the respective primer (*BrANNEXIN1-3′R*, *BrANNEXIN2-3′R*, *BrANNEXIN3-3′R*, or *BrANNEXIN4-3′R*; Table 1Sb, Supplementary data) under the following conditions: 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The 5′-RACE-PCR products were purified, cloned, and sequenced.

Sequence and Phylogenetic Analysis

Annexin gene members in *Tsuda* were identified using a BLAST search of the NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) and nucleotide sequences of the *Arabidopsis* gene family as query sequences. The annotation of amino acid sequences, isoelectric point (pI), and the theoretical molecular mass of each of the annexin proteins were analyzed using the ExPasy proteomic server (<http://www.ca.expasy.org>). Protein motifs were functionally analyzed using

InterProScan (<http://www.ebi.ac.uk/Tools/pfa/ipscan>) and the SMART database (<http://smart.embl-heidelberg.de>).

Phylogenetic analysis was used to investigate the evolutionary relationships among deduced annexin sequences from turnip and from diverse plant species. An unrooted phylogenetic tree was generated by the neighbor-joining method using the program MEGA5 from 1000 bootstrap replicates, and the evolutionary distances were calculated using a Poisson correction method corresponding to the number of amino acid substitutions per site (Tamura et al. 2011).

Real-Time PCR

The transcript levels of turnip annexin genes were analyzed using real-time PCR. Total RNA was isolated from the samples using TRNzol-A⁺ lysis solution (TIANGEN Biotech, Beijing, China), and the concentration was adjusted to 1 μ g μ l⁻¹. The cDNA was reverse-transcribed with random primers using the MultiScribe reverse transcriptase (Applied Biosystems, USA) at 37 °C for 120 min. Quantitative real-time PCR (RT-PCR) assays for each gene target in each sample were performed in triplicate on cDNA samples. Quantitative real-time PCR was carried out on an ABI 7500 real-time system (Applied Biosystems) with the Power SYBR Green PCR Master Mix (ABI, USA). The PCR thermal cycling parameters were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The comparative CT ($\Delta\Delta C_T$) method was used to calculate relative amounts of the transcripts. Actin gene expression was chosen as an internal control, and white root epidermis, cotyledons, 0-day-old seedling, 0-h treatment, and dark-grown seedlings were used for calibration. The DNA sequences of PCR primers that come from the 3′-UTR of annexin genes we cloned are listed in Table 1. Means were calculated from values for three biological replications, each of which consisted of two or three independent amplification reactions using the same samples (technical replications).

Results

Isolation and Sequence Analysis of Turnip Annexin Genes

In this study, the cDNAs of four related genes encoding annexins in *B. rapa* were isolated and designated as *BrANNEXIN1*, *BrANNEXIN2*, *BrANNEXIN3*, and *BrANNEXIN4*. The numbering of these annexins was based on the similarity of their sequences with their corresponding homologous sequences from its crucifer relative, *Arabidopsis thaliana*. Then after amplification by RT-PCR and 3′- and 5′-RACE with specific primers, the sequences of the clones were aligned and converted to full-length cDNA sequences for *BrANNEXIN1* (GenBank accession no. KJ143523),

Table 1 The DNA sequences of PCR primers used for real-time PCR of *BrANNEXIN1*, *BrANNEXIN2*, *BrANNEXIN3*, and *BrANNEXIN4* genes

Primer name	Accession no.	Nucleotide sequence 5' to 3'
<i>BrANNEXIN1-F</i>	KJ143523	AAGCCATTACCAAAGACA
<i>BrANNEXIN1-R</i>	KJ143523	GAGAGATGAGAGAAAACAGA
<i>BrANNEXIN2-F</i>	KJ143524	TCGCTAAAGACACTTCTG
<i>BrANNEXIN2-R</i>	KJ143524	GGATGAACACGGAAACTA
<i>BrANNEXIN3-F</i>	KJ173682	GCATGGATAACGCTATCA
<i>BrANNEXIN3-R</i>	KJ173682	AAGCCAAGAATATTTAAAATTCAAA
<i>BrANNEXIN4-F</i>	KJ173683	GTGACATGAAAGAGATAGCAGAAG
<i>BrANNEXIN4-R</i>	KJ173683	GGGAATAAGTTCAGAAGTCAAATCA
<i>BrACTIN-F</i>	AF111812.1	GCTCAGTCCAAGAGAGGTATTC
<i>BrACTIN-R</i>	AF111812.1	GCTCGTTGTAGAAAGTGTGATG

Actin gene expression was chosen as an internal control. Transcript levels were quantified using the SYBR Green method

F forward primer, *R* reverse primer

BrANNEXIN2 (GenBank accession no. KJ143524), *BrANNEXIN3* (GenBank accession no. KJ173682), and *BrANNEXIN4* (GenBank accession no. KJ173683). The *BrANNEXIN1* cDNA consisted of an open reading frame (ORF) of 954 bp flanked by 43 and 188 bp as the 5'- and 3'-UTRs, respectively. The 3'-UTR had one putative polyadenylation signal at 142 bp after the stop codon with a 17-bp poly-A tail. The *BrANNEXIN2* had an ORF of 951 bp with the 5'- and 3'-UTRs of 40 and 91 bp, respectively. It also had a putative polyadenylation signal at 60 bp after the stop site and a poly-A tail of 17 bp.

BrANNEXIN3 contained an ORF of 960 bp with the 5'- and 3'-UTRs of 42 and 151 bp and showed a putative polyadenylation signal at 100 bp after the stop site and a poly-A tail of 17 bp. The cDNA of *BrANNEXIN4* had an ORF of 948 bp with 5'- and 3'-UTRs of 42 and 169 bp and a putative polyadenylation signal at 118 bp after the stop site and a poly-A tail of 17 bp (Fig. 1S). At the nucleotide level, the coding regions of these annexin genes shared 52.8–65.5 % identity among each other. The lowest similarity was found between *BrANNEXIN1* and *BrANNEXIN3* and the highest was found between *BrANNEXIN1* and *BrANNEXIN2* (data not shown).

Characterization of Deduced Annexin Proteins

These four turnip annexin genes, *BrANNEXIN1*, *BrANNEXIN2*, *BrANNEXIN3*, and *BrANNEXIN4*, encoded polypeptides consisting of 317, 316, 319, and 315 amino acids residues, respectively, with total predicted molecular masses of 36.1, 36.1, 35.8, and 35.5 kDa, and predicted *pI* values of 5.34, 5.76, 5.4, and 7.25, respectively. The deduced amino acid sequences of these *Brassica* annexins shared 32.6–64.5 % identity with each other. Among the four annexins, the lowest similarity was found between *BrANNEXIN2* and *BrANNEXIN3*, and the highest was found between

BrANNEXIN1 and *BrANNEXIN2* (Fig. 1S). In the comparative multiple alignment of these deduced turnip annexins with other annexins of dicot and monocot origin obtained in the tblastn search of the NCBI GenBank database, sequence similarity ranged from 38 to 99 % (data not shown).

When the core repeat regions for the four turnip annexins were compared with the annexin consensus sequences obtained from the many annexin core repeat regions analyzed by Barton et al. (1991), it was obvious that the structural repeats can aid in defining the gene family members that are present in turnip annexins. There were four core repeat regions in *BrANNEXIN1*, *BrANNEXIN2*, and *BrANNEXIN3*, and only the second and fourth repeat regions were present in *BrANNEXIN4*. The first and fourth repeats had the highest level of conservation; the second and third repeats were the most divergent. In annexins, the core repeat regions were type II calcium-binding sites. The type II calcium-binding site is determined by the conserved glycine-X-glycine-threonine loop that is followed by a glutamic acid or an aspartic acid residue 42 amino acids downstream of the first glycine residue (Hu et al. 2008). Many plant annexins have a fairly well-conserved type II calcium-binding site in either the first or fourth repeat (Fig. 1).

Similar to other plant annexins, the turnip annexins have deduced amino acid sequences that contain several conserved residues/motifs (Fig. 1). *BrANNEXIN1*, *BrANNEXIN2*, and *BrANNEXIN3* contain the predicted IRI motif involved in phosphodiesterase activity of F-actin- and GTP-binding regions (GXXXXGKT and DXXG) (Fig. 1). In addition, *BrANNEXIN1* contains one RGD motif in the fourth annexin repeat. The RGD sequence is found in human vitronectins and fibronectins present in the extracellular matrix (ECM) and is the minimal motif required for cell attachment via binding with the cell surface receptor integrin (Ruoslahti 1996).

When the deduced protein sequences were analyzed using ScanProsite (<http://ca.expasy.org/tools/scanprosite/>), sites for

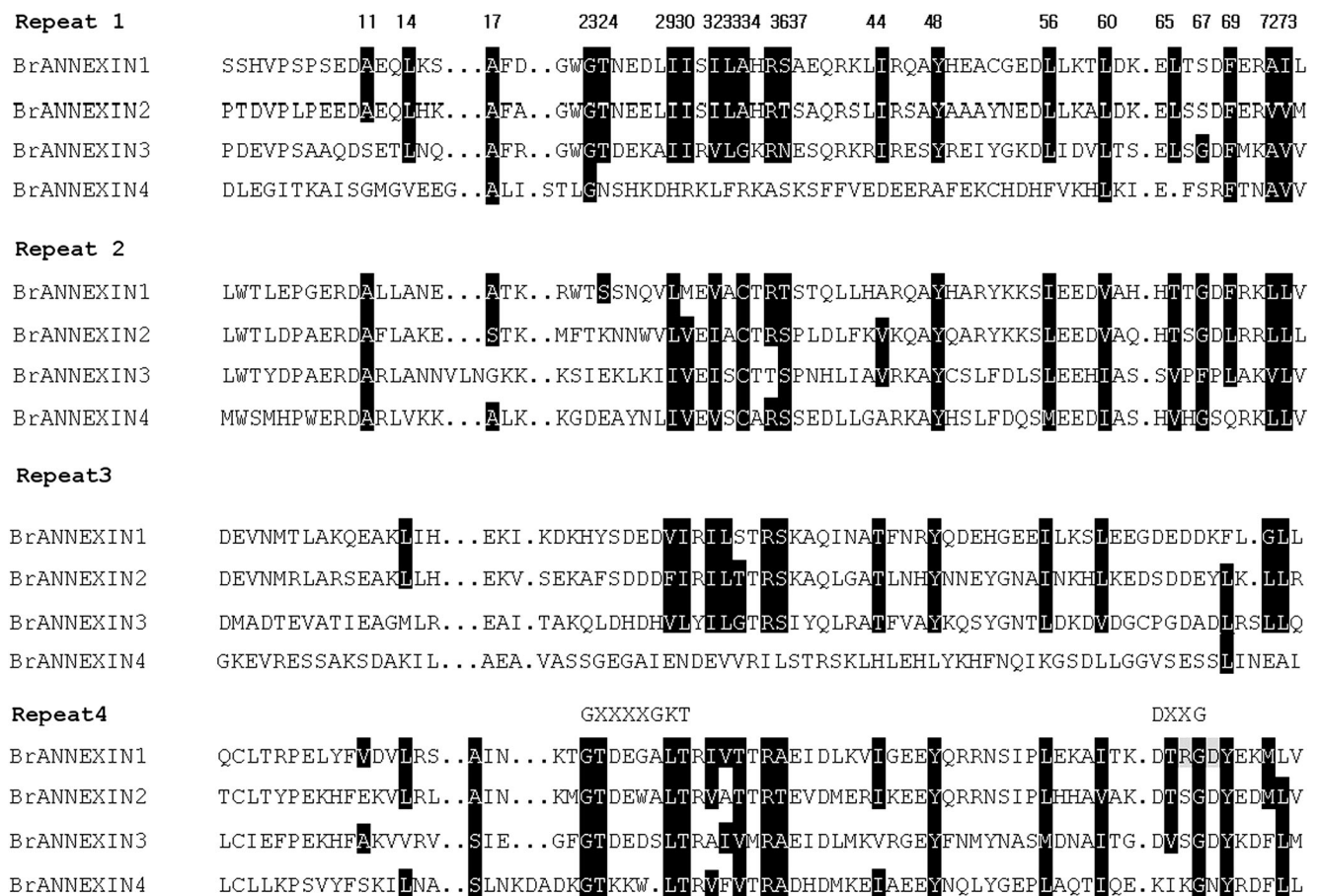


Fig. 1 Sequence alignment of turnip annexin repeats. The amino acids highlighted at the 21 numbered positions agree with the amino acid residues that are conserved in each of the four annexin repeats of 22 annexin sequences according to Barton et al. (1991)

various posttranslational modifications and other sequence-specific features were found (Table 2). For example, these protein sequences had multiple putative phosphorylation sites and, thus, may serve as substrates for kinases such as casein kinase II (8 each in BrANNEXIN1 and BrANNEXIN2, 7 in BrANNEXIN3, 6 in BrANNEXIN4) and protein kinase C (7 in BrANNEXIN1, 6 in BrANNEXIN2, 13 in BrANNEXIN3, 8 in BrANNEXIN4). In addition, N-glycosylation sites are also present in deduced amino acid sequences of BrANNEXIN1, BrANNEXIN3, and BrANNEXIN4. None of the four turnip annexins had N-myristoylation sites (Table 2).

Phylogenetic Analysis of Turnip Annexins

To investigate the evolutionary relationships of the turnip annexins, we created a phylogenetic tree using the deduced amino acid sequences of 34 annexins from various plant species. The phylogenetic tree was analyzed in MEGA5 using the neighbor-joining method with 1000 bootstrap replicates, and evolutionary distances were calculated by the Poisson correction method. The phylogenetic tree showed that annexins

were classified into four clades (Fig. 2). The first clade included turnip BrANNEXIN1, *Arabidopsis* ANNEXIN1, and annexins from tomato, tobacco, and cotton. The second clade contained turnip BrANNEXIN2 and annexins from other eudicots. Turnip BrANNEXIN3 and BrANNEXIN4 grouped into the third clade with an annexin from a conifer (a progymnosperm) and other eudicots. All the annexins from monocots grouped together in the fourth clade. Thus, the four annexins from turnip were primarily grouped with *Arabidopsis* and *Brassica juncea* orthologous sequences, suggesting similarity/identity in amino acid sequences.

Tissue-Specific Expression of *BrANNEXIN* Genes in Turnip

The expression patterns of turnip annexin gene family members in the white root epidermis, red root epidermis, root without epidermis, leaf, bud, and petal using quantitative real-time PCR were analyzed (Fig. 3). Four turnip annexins were expressed in most tissues but at different levels. Among the four genes, *BrANNEXIN1* and *BrANNEXIN4* were expressed in all tissues examined. Expression of *BrANNEXIN1* was 10-

Table 2 Features of predicted sequences for turnip annexin proteins

Protein	AA	MW/pI	Anx repeats	Ca ²⁺ sites	N-Myr	Casein	PKC	N-Glyc
BrANNEXIN1	317	36.1/5.34	4	1	–	8	7	3
BrANNEXIN2	316	36.1/5.76	4	0	–	8	6	–
BrANNEXIN3	319	35.8/5.54	4	1	–	7	13	2
BrANNEXIN4	315	35.5/7.25	2	0	–	6	8	1

Posttranslational modifications predicted using ScanProsite (<http://ca.expasy.org/tools/scanprosite/>)—casein phosphorylation sites for casein kinase II

AA number of amino acids, MW/pI molecular weight and isoelectric point, *Anx repeats* number of annexin repeat, PKC protein kinase C, *N-Myr* N-myristoylation site, *N-Glyc* N-glycosylation site

fold higher in the root without epidermis, leaf, bud, and petal than in the root epidermis. The expression of *BrANNEXIN4* was 10 times higher in the root without epidermis, bud, and petal than in the leaf and in the root epidermis. *BrANNEXIN2* and *BrANNEXIN3* were expressed in the root without epidermis and bud, but were barely detected in the petal. The anthocyanins were accumulated in the red root epidermis, but four annexins were not expressed in the red root epidermis especially in 2-month-old turnip (Fig. 3).

Because anthocyanin accumulates in the upper but not in the lower hypocotyls of the seedling when exposed to UV-A light (Wang et al. 2012), indicating that the gene expression pattern differs in the upper and lower hypocotyls, we also analyzed the expression of the four annexin genes in the two regions separately (upper H1–H3 vs lower H4–H5, Fig. 4a). Figure 4b shows the RNA levels of all four annexins in the various tissues in the steady-state condition of 4.0-day-old, dark-grown seedlings. Generally, expression of all four turnip annexins was higher in the lower hypocotyls than in the cotyledon (24.3-, 251.1-, 230.9-, and 42.9-fold increases for the four respective genes). RNA level for *BrANNEXIN4* was also much higher in the upper hypocotyls than in the cotyledon (41.7-fold). The expression of *BrANNEXIN1* and *BrANNEXIN2*, however, was lower in the upper hypocotyls than in the cotyledon (14.7- and 3.1-fold decrease, respectively).

Relative Quantitation of *BrANNEXIN* Gene Transcripts in Dark-Grown and Light-Grown Seedling

When we analyzed the expression of the four turnip annexin genes in dark-grown seedlings, the expression of all four annexins increased by different amounts after seed germination in the dark (Fig. 5a). Transcript levels of *BrANNEXIN2*, *BrANNEXIN3*, and *BrANNEXIN4* gradually increased after germination (0–2 days), increasing to 14.9-, 3.2-, and 9.4-fold on the second day compared with that in 0-day-old seedlings, respectively, and then dropped by day 3. Levels then decreased to a maintenance level that was 6.8-, 2.1-, and 4.6-fold, respectively, higher than in 0-day-

old seeds. The transcript level of *BrANNEXIN1* also increased, reaching 2-fold higher than that in 0-day-old seedlings by 3 days after germination, then decreased to the 0-day-old seed level.

Under normal growth conditions, the RNA levels of *BrANNEXIN1*, *BrANNEXIN3*, and *BrANNEXIN4* gradually increased in seedlings for 3 days after germination (0–3 days) and then decreased. Peak level for the respective genes was 10.3-, 1.7- and 4.1-folds than that in the 0-day-old seeds. The expression of *BrANNEXIN2* decreased (Fig. 5b).

Relative Levels of *BrANNEXIN* Genes in White Root Epidermis Induced by UV-A Light

In a previous study, the epidermis of swollen hypocotyls (roots) of the turnip *Tsuda* accumulates anthocyanins upon exposure to UV-A light (Zhou et al. 2007). The anthocyanins accumulated in the white root epidermis after 24-h UV-A irradiation (Fig. 6a). We thus analyzed the expression of the four turnip annexin genes in the white root epidermis after UV-A light irradiation (Fig. 6b). Expression of *BrANNEXIN4* in the white root epidermis was induced by UV-A light. After 1 h of irradiation, the messenger RNA (mRNA) level of *BrANNEXIN4* increased more than 15-fold over the level in the nonirradiated white root epidermis, gradually increasing to more than 60-fold after 24 h of irradiation. After 1 h of UV-A irradiation, the expression of *BrANNEXIN1*, *BrANNEXIN2*, and *BrANNEXIN3* was lower than that in the nonirradiated white root epidermis, then the expression of *BrANNEXIN2* increased 3.4-fold after 24 h of irradiation, but the levels of *BrANNEXIN1* and *BrANNEXIN3* were still lower than those in the control. The result showed that *BrANNEXIN4* may be involved in anthocyanin synthesis induced by UV-A in the root epidermis.

Relative Levels of *BrANNEXIN*s in Cotyledons and Hypocotyls Induced by UV-A Light

In *Tsuda* turnip, the upper hypocotyls of the seedling accumulate anthocyanins in response to UV-A light

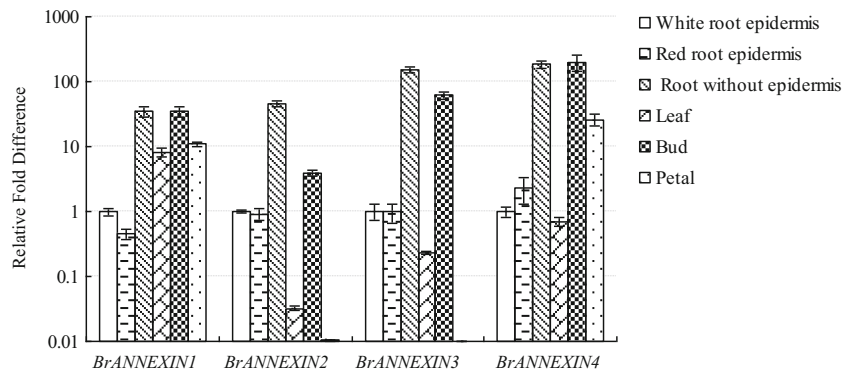


Fig. 3 Relative expression levels of *BrANNEXIN* genes among six types of turnip tissues: white root epidermis (grown in the dark), red root epidermis (grown in sunlight), root without epidermis, leaf from 2-month-old turnip and bud (0.8 mm long), and petal from 6-month-old

turnip. Results were normalized to *BrANNEXIN* levels in white root epidermis (value of 1). For keeping the increase or decrease in relative fold differences in RNA levels proportional, the y-axis is presented on a log scale. Error bars represent standard error of the mean

Red and Far-Red Light Effects on *BrANNEXIN* Expression

Most of the light effects on annexin expression in germinating seedlings are not significant (Tepperman et al. 2004). In our research, the general trends in the change of expression are consistent; annexin expression increased after light exposure in cotyledons and the upper and lower hypocotyls, except for a decrease in *BrANNEXIN2* in the lower hypocotyls (Fig. 8). In the cotyledons, *BrANNEXIN1*, *BrANNEXIN3*, and

BrANNEXIN4 were upregulated (4.6-, 5.1-, and 1.5-fold, respectively), and only the upregulation of *BrANNEXIN3* was reversed by far-red light (Fig. 8a). *BrANNEXIN2* was not significantly induced after red light treatment (slightly upregulated 1.1-fold; Fig. 8a). In the upper hypocotyls, all four turnip annexins were upregulated (21.7-, 4.9-, 30.1-, and 3.6-fold for *BrANNEXIN1*, *BrANNEXIN2*, *BrANNEXIN3*, and *BrANNEXIN4*, respectively) after red light exposure as compared with dark-grown seedling. This upregulation of *BrANNEXIN1*, *BrANNEXIN2*, and *BrANNEXIN3* was largely

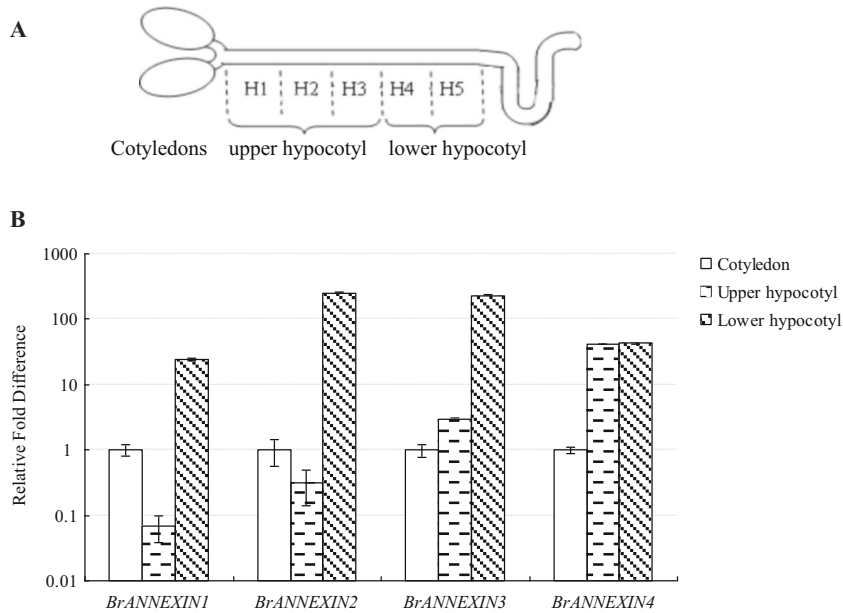
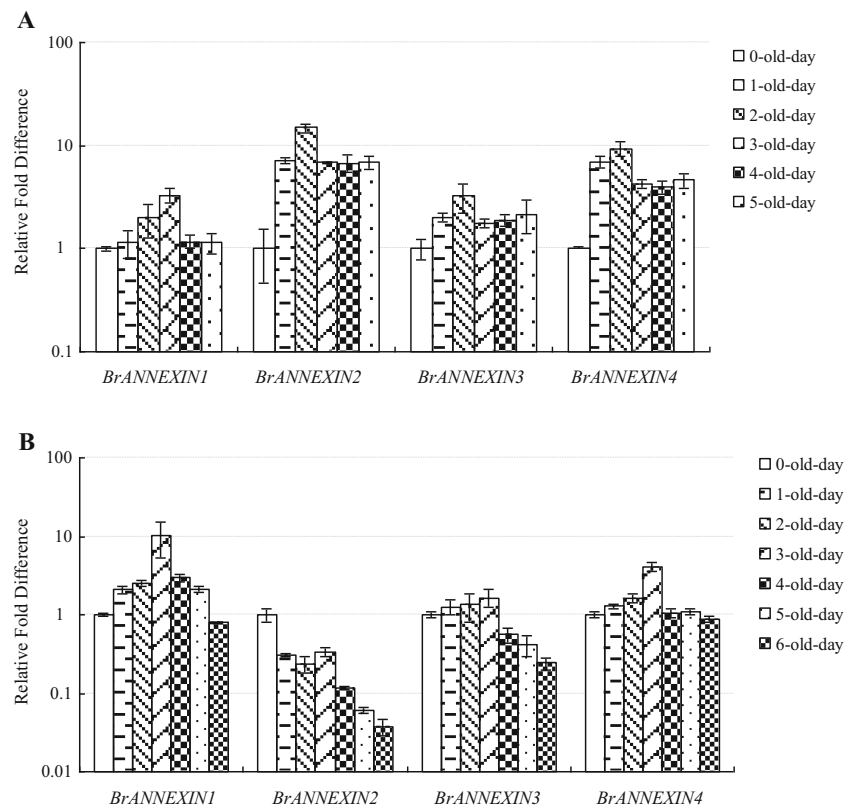


Fig. 4 a Preparations of sample sections for anthocyanin measurements and RT-PCR analyses. The seedlings were divided into cotyledons, upper hypocotyl, and lower hypocotyl. The hypocotyls were divided into five sections of equal length. The hypocotyl sections were numbered from the uppermost sections and designated as H1–H5. The sections of H1, H2, and H3 were designated as upper hypocotyl and H4 and H5 were designated as lower hypocotyl. b Relative expression levels of *BrANNEXIN*

genes among three types of tissues from 4-day-old etiolated seedlings: upper hypocotyls, lower hypocotyls, and cotyledons. Results were normalized to *BrANNEXIN* levels in cotyledons (value of 1). For keeping the increase and decrease in relative fold differences in RNA levels proportional, the y-axis is presented on a log scale. Error bars represent standard error of the mean

Fig. 5 Relative expression levels of *BrANNEXIN* genes in whole seedling grown in the dark (a) and light (b) conditions. Results were normalized to *BrANNEXIN* levels in 0-old-day seedlings (value of 1). For keeping the increase and decrease in relative fold differences in RNA levels proportional, the *y*-axis is presented on a log scale. Error bars represent standard error of the mean. **a** Seedling grown in dark condition. **b** Seedling grown in light condition



reversed and, in the case of *BrANNEXIN4*, totally reversed by far-red light treatment (3.0-fold, Fig. 8b). In the lower hypocotyls, *BrANNEXIN1*, *BrANNEXIN2*, and *BrANNEXIN4* were upregulated 1.7-, 4.6-, and 3.7-fold after red light exposure, respectively, and only the upregulation of *BrANNEXIN1* was reversed by far-red light (Fig. 8c).

Differential Expression of Annexin Genes in Whole Seedlings Under Various Abiotic Stresses

Because evidence for various plant species, including *Arabidopsis*, has shown that annexin genes are upregulated in response to abiotic stresses (Laohavisit and Davies 2011), we examined the expression patterns of the turnip annexin genes in whole 5-day-old turnip seedlings after exposure to cold (4 °C), heat (37 °C), dehydration, osmotic stress (PEG), salinity (NaCl), and a phytohormone (ABA). RNA was extracted from the treated seedlings after various durations of exposure and subjected to real-time PCR analyses for four annexin genes (Fig. 9). A mock treatment with water served as the control. We found that annexin gene members were differentially regulated during the stress conditions.

The expression of *BrANNEXIN3* (3.2-fold) and *BrANNEXIN4* (2.6-fold) between 1 and 2 h of exposure to 4 °C, then declined (Fig. 9a). RNA levels of *BrANNEXIN1* (1.2-fold) and *BrANNEXIN2* (1.6-fold) were slightly induced after the 1-h cold treatment and then declined (Fig. 9a). In the

case of heat treatment, transcript levels increased to a maximum at 1, 4, and 8 h, respectively, for *BrANNEXIN1* (1.8-fold), *BrANNEXIN2* (2.5-fold), and *BrANNEXIN4* (4.7-fold) and then declined (Fig. 9b). *BrANNEXIN3* transcripts decreased during this treatment (Fig. 9b). With the dehydration treatment, the level of induction for *BrANNEXIN2* increased from 0.5 to 2 h (reached 4.2-fold), and the transcript levels of *BrANNEXIN3* and *BrANNEXIN4* increased to a maximum at 0.5 h (2.6- and 3.9-fold, respectively) and then declined (Fig. 9c). *BrANNEXIN1* transcripts decreased until 2 h, when the level rose slightly but to a level that was still lower than that in the control (Fig. 9c).

Osmotic stress induced by PEG treatment caused a significant increase after 8 h of treatment in the transcript abundance of *BrANNEXIN2* (15.1-fold) and *BrANNEXIN3* (8.0-fold) relative to the untreated control (Fig. 9d). The transcript levels reached a maximum at 0.5 h for *BrANNEXIN4* (2.6-fold) and then declined (Fig. 9d). The expression of the *BrANNEXIN1*, however, was not altered (Fig. 9d).

In the case of salt stress by NaCl, transcript levels of *BrANNEXIN2* and *BrANNEXIN4* were induced to peak level as early as 2 h (32.7-fold and 19.5-fold) and declined thereafter (Fig. 9e). Expression of *BrANNEXIN1* or *BrANNEXIN3* was not induced by NaCl (Fig. 9e). Treatment with ABA caused an increase in transcript level in all turnip annexin genes. The transcript levels of *BrANNEXIN1*, *BrANNEXIN2*, *BrANNEXIN3*, and *BrANNEXIN4* increased to a maximum at 2 h and then declined (Fig. 9f).

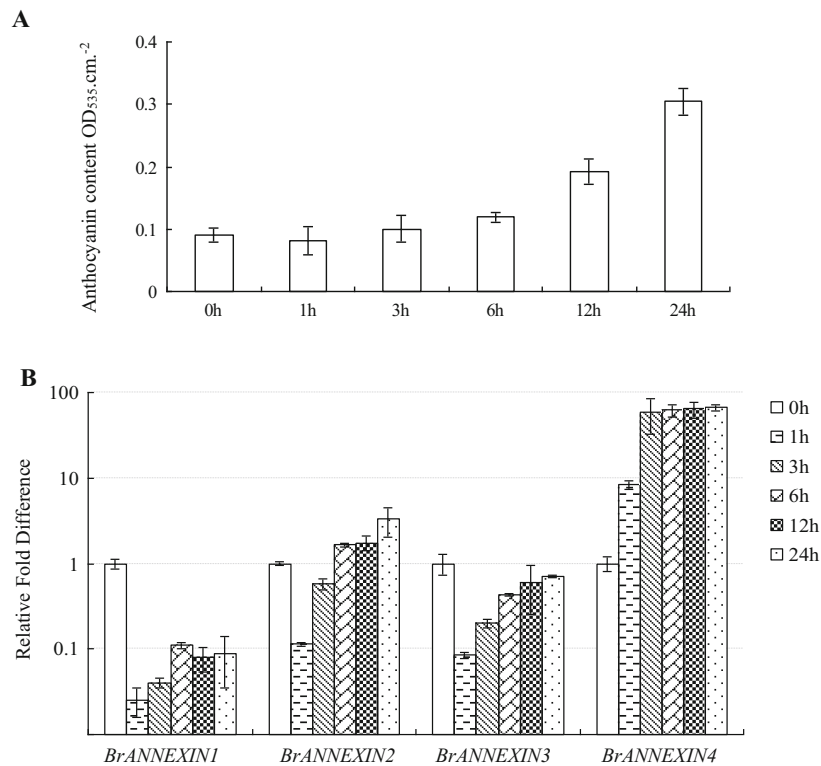


Fig. 6 **a** Anthocyanin accumulation in white root epidermis induced by UV-A light. The 2-month-old white root was exposed to UV-A (320–400 nm, 3.0 W m^{-2}) for 1, 3, 6, 12, and 24 h or collected without UV-A exposure (0 h). Anthocyanin was extracted from the UV-A-exposed part of the epidermis of root and then the concentration was determined. Plots indicate the means \pm SE of five to six replications. **b** Relative expression

levels of *BrANNEXIN* genes in white root epidermis induced by UV-A light. Results were normalized to *BrANNEXIN* levels in white root epidermis without UV-A light irradiation (0 h; value of 1). For keeping the increase and decrease in relative fold differences in RNA levels proportional, the y -axis is presented on a log scale. Error bars represent standard error of the mean

Discussion

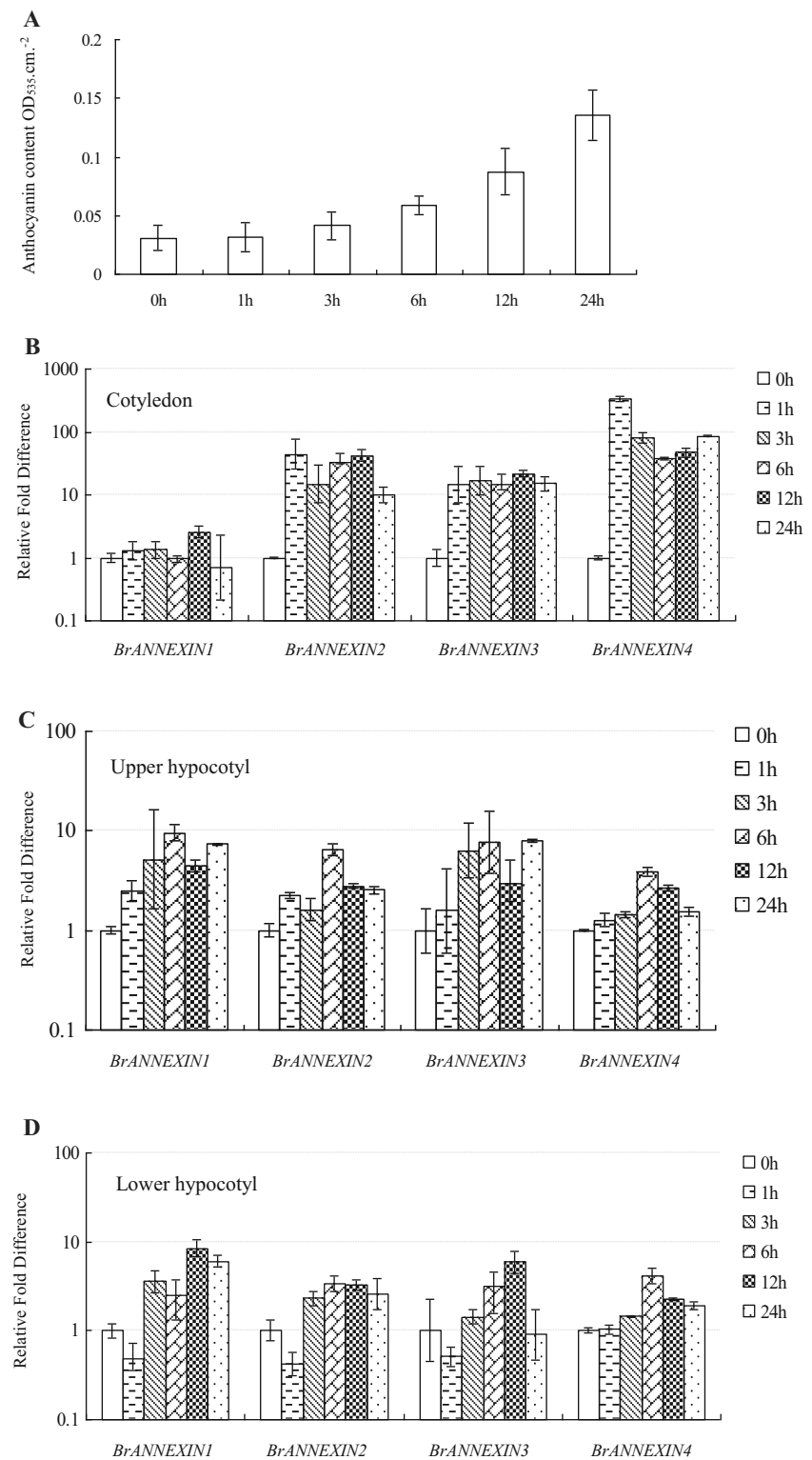
In this study, the cDNAs of four related genes encoding annexins in *B. rapa* were isolated and designated as *BrANNEXIN1*, *BrANNEXIN2*, *BrANNEXIN3*, and *BrANNEXIN4*. Our sequence analysis suggests that each of the annexins encodes single polypeptides with molecular masses ranging from 35.5 to 36.1 kDa and with isoelectric pH ranging between 5.34 and 7.25. Annexins in plants appear to be a smaller, less diverse gene family than in vertebrates (Jami et al. 2009). DNA blot analysis showed several hybridization bands upon probing with *BrANNEXIN1* and demonstrated the presence of other similar annexin sequences in the turnip genome (Fig. 2S). This DNA blot result is consistent with the available genome sequence data of *Arabidopsis*, rice, cotton, and mustard, which contain 8, 10, 14, and 7 distinct annexin genes, respectively, indicating that annexins belong to a multigene family in plants (Clark et al. 2001; Cantero et al. 2006; Jami et al. 2012; Tang et al. 2014), while in the human genome, there are 13 members (Raynal and Pollard 1994).

All four annexins in turnip also had the conserved amino acids in the repeat sequences that define the gene annexin family (Barton et al. 1991). The repeat in the four annexins in

Tsuda turnip indicates the presence of type II calcium-binding sites (acidic residues) implicated in phospholipid binding in the first and fourth repeats. The primary structure of *BrANNEXIN1*, *BrANNEXIN2*, and *BrANNEXIN3* contains the four characteristic tandem repeats, and *BrANNEXIN4* contains two characteristic tandem repeats of the second and the fourth repeats. The first repeat indicates the presence of glutamic acid (E-68) in *BrANNEXIN1*, *BrANNEXIN2*, and *BrANNEXIN3*, while Asp in the fourth repeat is positioned at D-299, D-298, D-302, and D-305 in their corresponding members *BrANNEXIN1*, *BrANNEXIN2*, *BrANNEXIN3*, and *BrANNEXIN4*, respectively. Our research predicted that each *BrANNEXIN1* and *BrANNEXIN3* has calcium-binding residues. Calcium residues in the endonexin sequence are important in binding membrane lipids on the convex side; thus, the variable presence or absence of calcium residues in their corresponding annexin repeats might result in different protein conformations and different specificities for binding phospholipids (Jami et al. 2012).

Like the annexin proteins in rice, the turnip annexin proteins also had phosphodiesterase activity (Jami et al. 2012). This activity may be involved in the formation of salt bridges that have been hypothesized to be involved in the formation of

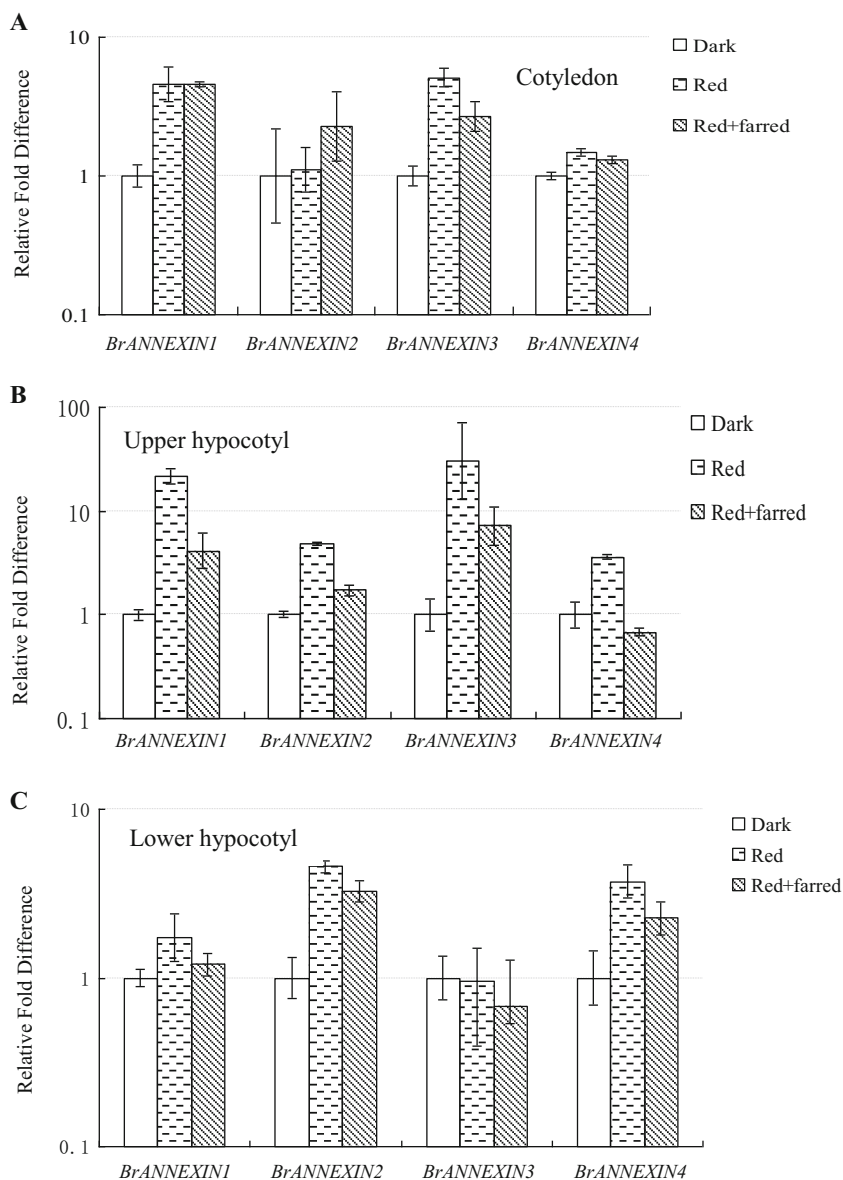
Fig. 7 a Anthocyanin accumulation in 4-day-old upper hypocotyl induced by UV-A light. The upper hypocotyls were exposed to UV-A (320–400 nm, 3.0 W m^{-2}) for 1, 3, 6, 12, and 24 h or collected without UV-A exposure (0 h). Anthocyanin was extracted from the UV-A-exposed upper hypocotyls and then the concentration was determined. Plots indicate the means \pm SE of five to six replications. **b–d** Relative expression levels of *BrANNEXIN* genes in cotyledons (**b**), upper hypocotyls (**c**), and lower hypocotyls (**d**) induced by 1, 3, 6, 12, or 24 h of UV-A light. Results were normalized to *BrANNEXIN* levels in cotyledons without UV-A light irradiation (0 h; value of 1). For keeping the increase and decrease in relative fold differences in RNA levels proportional, the y-axis is presented on a log scale. Error bars represent standard error of the mean. **b** Four-day-old cotyledons. **c** Upper hypocotyl and **d** lower hypocotyl tissues



ion channels (Laohavisit and Davies 2011). In addition, *BrANNEXIN1* contains one RGD motif in the fourth annexin repeat. The RGD sequence is found in human vitronectins and fibronectins in the ECM and is the minimal motif for cell attachment due to binding of the cell surface receptor integrin

(Ruoslahti 1996). Vertebrate annexin-V binds to the integrin receptor through the RGD motif (Anderson et al. 2002). Rice annexin Os05g31760 contains two RGD motifs: one in the third and the other in the fourth annexin repeat also (Jami et al. 2012), but the function of the RGD motif in plant annexins is

Fig. 8 Relative expression levels of *BrANNEXIN* genes after red or far-red light in cotyledons and upper and lower hypocotyls. Results were normalized to *BrANNEXIN* levels in the cotyledons without red and far-red light irradiation (0 h; value of 1). For keeping the increase and decrease in relative fold differences in RNA levels proportional, the y-axis is presented on a log scale. Error bars represent standard error of the mean. **a** Four-day-old cotyledons. **b** Upper and **c** lower hypocotyl tissues



unknown. The turnip annexins also had catalytic properties of casein kinase, protein kinase C, and N-glycosylation activity. Further experimental studies are required to support these predictions. Based on these motif analyses, however, it is clear that the activity of many turnip annexins may be regulated by posttranslational modifications.

Our results indicate that the expression of all four turnip annexins is tissue-specific in 2-month-old and 4-day-old plants. *BrANNEXIN1* transcripts accumulated more in the root tissue, which agrees with the high level of expression of annexin in the roots of *Arabidopsis* and mustard (Gidrol et al. 1996; Jami et al. 2009). The higher transcript level for all four turnip annexins in the lower hypocotyls in comparison with the level in the cotyledons is similar to the findings of Cantero et al. (2006) for *Arabidopsis* annexins. These results indicate variable expression of annexin transcripts in tissues across

diverse plant species. We found that *BrANNEXIN2* and *BrANNEXIN3* were expressed strongly in the roots without the epidermis and in the buds, but almost never in petals. The differences in these tissue patterns of annexin expression seem to indicate spatial functional specificity of the turnip annexin genes.

All four turnip annexin transcripts apparently increase when plants are grown in the dark, but on the basis of their relative increases, *BrANNEXIN2* and *BrANNEXIN4* are the most important contributors for seedling growth in the dark. Under normal conditions, *BrANNEXIN1*, *BrANNEXIN3*, and *BrANNEXIN4* were present at the highest levels during early growth. Growth regulation by the annexin family genes was also reported for the young seedlings of *Arabidopsis* (Cantero et al. 2006). The trends that we found in the various tissues are also congruent with the general concept that

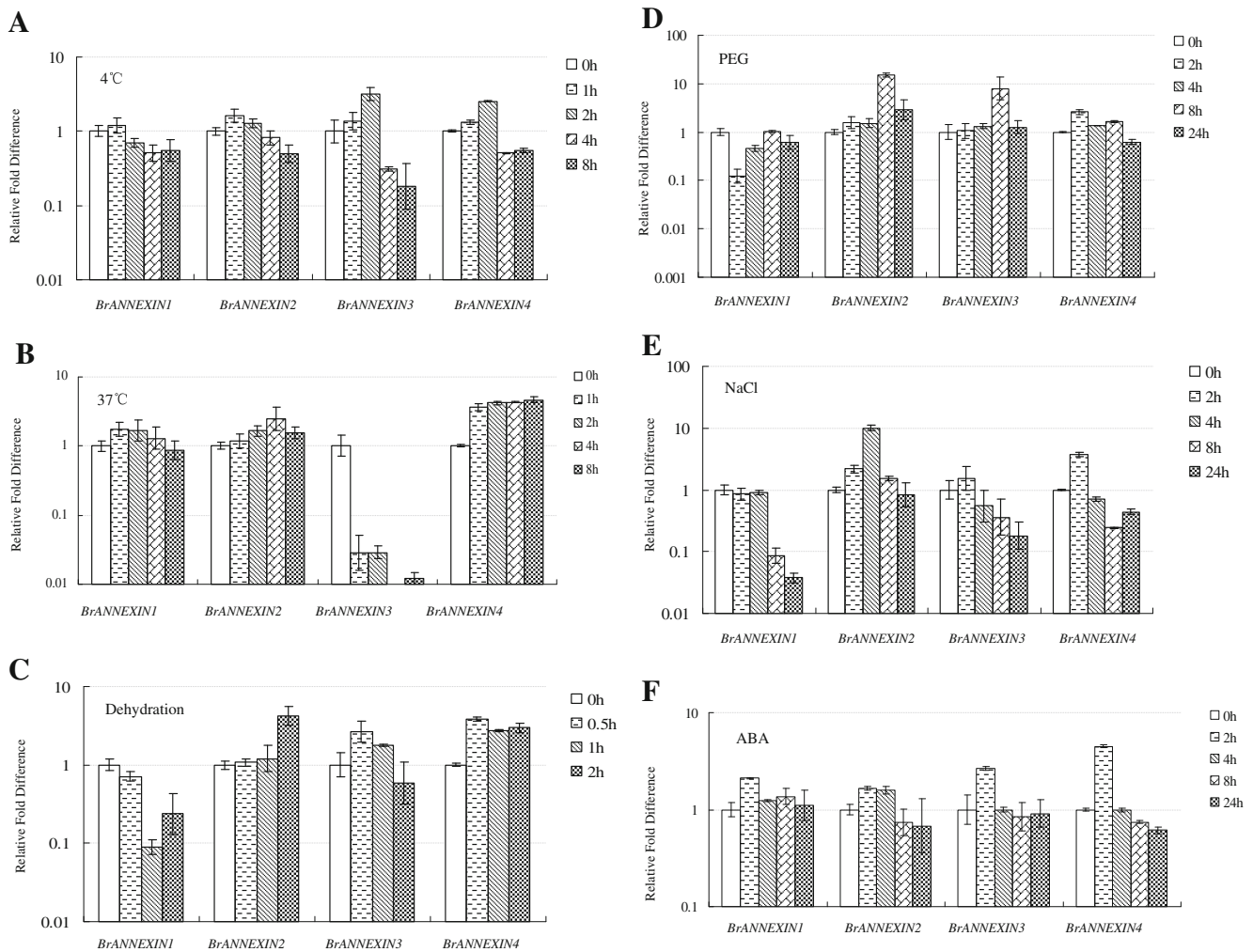


Fig. 9 Relative expression levels of *BrANNEXIN* genes in 5-day-old whole seedlings under various abiotic stresses. Results were normalized to *BrANNEXIN* levels in cotyledons from untreated 5-day-old whole seedlings (0 h; value of 1). For keeping the increase and decrease in

relative fold differences in RNA levels proportional, the y-axis is presented on a log scale. *Error bars* represent standard error of the mean. **a** 4 °C. **b** 37 °C. **c** Dehydration. **d** PEG8000. **e** NaCl. **f** ABA

annexins are involved in growth, since in an etiolated plant, the majority of cell expansion occurs in the roots and hypocotyls, not in the cotyledons (Cantero et al. 2006; Tang et al. 2014).

In higher plants, cryptochrome is the photoreceptor for blue and UV-A light signals (Ahmad and Cashmore 1993). In *Tsuda* turnip, however, the root epidermis and the upper hypocotyls accumulate anthocyanin after exposure to UV-A light but not blue light, demonstrating that the UV-A-induced anthocyanin biosynthesis is distinct from cryptochrome regulation (Zhou et al. 2007). In our study, the expression of *BrANNEXIN4* in the root epidermis was induced by UV-A light, which may indicate that *BrANNEXIN4* is involved in anthocyanin synthesis induced by UV-A in the root epidermis. In the upper hypocotyls, where anthocyanins accumulate, mRNA transcripts for all four turnip annexins were induced by UV-A light irradiation. In these two conditions of anthocyanin synthesis, the pattern of expression for the four annexin proteins differed,

demonstrating that they were involved in different regulatory pathways induced by UV-A light in *Tsuda*.

Light signals provide important environmental cues for plants to alter their growth patterns. For example, as shown by microarray studies, some of the specific transcript changes are regulated by phytochrome during seedling de-etiolation (Tepperman et al. 2004). Because annexins are involved in mediating growth and development of seedlings, we examined the changes of the expression of annexin genes in cotyledons and hypocotyls, two tissues with different growth responses to various light conditions to elucidate any involvement of the phytochrome. Because growth changes induced by red light are very rapid, we examined very early changes in gene expression, that is, after only 30 min, on the assumption that these changes would be the most influential in mediating the light effects. In cotyledons, red light induces cell division and cell expansion; red light induced a 4.6- and 5.1-fold increase within 30 min in transcript abundance for *BrANNEXIN1* and *BrANNEXIN3*. Far-red light reversed this

effect for *BrANNEXIN3*; thus, this annexin gene is rapidly regulated by phytochrome in seedlings. In the upper hypocotyls, RNA levels for *BrANNEXIN1* and *BrANNEXIN3* increased 21.7- and 30.1-fold after red light, and this increase was mostly reversed by far-red light, indicating that *BrANNEXIN1* and *BrANNEXIN3* are among the “early-response” genes rapidly regulated by phytochrome in the hypocotyl tissue that accumulates anthocyanins. *BrANNEXIN1* and *BrANNEXIN3* may be participating in cellular changes leading to growth inhibition. Such growth regulation of *AnnAt6* and *AnnAt5* was also reported for *Arabidopsis* (Cantero et al. 2006). Both red light and far-red light treatments have only minor effects on the transcript levels of *BrANNEXIN2* and *BrANNEXIN4* in cotyledons and hypocotyls, suggesting that these two annexins are not among the genes rapidly regulated by phytochrome. Whether any of these other annexins are among the “late-response” genes that are regulated by phytochrome remains to be tested.

Previous reports implicated plant annexins in responses to abiotic and biotic stimuli (Laohavisit and Davies 2011). Transcripts for all four turnip annexins increased in the cold treatment similar to the increased expression of *AnnAt2* and *AnnAt6* in the cold response of *Arabidopsis* seedlings (Cantero et al. 2006) and wheat shoots (Breton et al. 2000). In our study, heat treatment resulted in the increased expression of *BrANNEXIN1*, *BrANNEXIN2*, and *BrANNEXIN4* but a decrease of *BrANNEXIN3*. Similarly, some *Arabidopsis* annexin gene family members (*AnnAt1*, *AnnAt2*, *AnnAt5*, *AnnAt6*, *AnnAt7*, and *AnnAt8*) are also expressed in response to heat stress, whereas the expression of two (*AnnAt3* and *AnnAt4*) decreases (Cantero et al. 2006). In rice also, exposure to heat has a differential effect on annexin gene family members; transcript levels of *Os08g32970* and *Os02g51750* increased significantly, but levels of *Os09g20330* transcripts decreased (Jami et al. 2012).

A similar pattern of differential gene expression pattern was found in *Tsuda* seedlings after treatments with dehydration and PEG. For each of these stresses, the transcript levels of *BrANNEXIN2*, *BrANNEXIN3*, and *BrANNEXIN4* increased, and the levels of *BrANNEXIN1* decreased. Annexin genes in other plants have also been differentially expressed after these treatments. *Arabidopsis* responded to dehydration by increasing the expression of *AnnAt2* and *AnnAt4*; *AnnAt1*, *AnnAt3*, *AnnAt5*, *AnnAt6*, *AnnAt7*, and *AnnAt8* decreased (Cantero et al. 2006). In rice, PEG treatment caused a significant increase in the transcript abundance of *Os08g32970* relative to the untreated control but a decrease for *Os01g31270* (Jami et al. 2012). Upregulation of annexin gene expression in response to drought stress has also been reported in the seedlings of *Medicago* and *Triticum aestivum* (Kovacs et al. 1998a, b; Peng et al. 2009).

Relative to the untreated control, tissues exposed to NaCl expressed higher levels of *BrANNEXIN2* and *BrANNEXIN4*, commensurate with the reported increase in annexin gene

transcript levels in response to NaCl for *Brassica* (*AnnBj3* and *AnnBj7*), *Arabidopsis* (*AnnAt4* through *AnnAt8*), tobacco (*NtAnn12*), and rice (*Os08g32970* and *Os09g32160*) (Cantero et al. 2006; Vandeputte et al. 2007; Jami et al. 2009, 2012). Treatment with ABA also resulted in an increase in transcript level for all annexin genes in *Tsuda* turnip as what also occurred for the *Brassica* annexins (Jami et al. 2009).

Although we tested the effects of each stress separately on annexin expression, each of these abiotic stresses can occur at the same time, and there is a cross-talk among the various stress pathways. Drought and salinity can both result in osmotic stress, and both involve calcium and gene expression changes in their signal transduction pathways. *BrANNEXIN2* and *BrANNEXIN4* were all upregulated by all the treatments we tested, although their respective levels of expression differed in response to the various treatments. Overall, our results indicate that the expression of annexin genes in turnip is differentially regulated by various abiotic stressors.

Acknowledgments We are grateful to Dr. Beth Eva Hazen for improving the English of this manuscript. This work was supported by the Fundamental Research Funds for the Central Universities (DL12CA10, 2572014EA03-01, 2572014EA03-02), the State Key Laboratory of Tree Genetics and Breeding (Northeast Forestry University k2013205), and the Fund for Fostering Talents in Basic Science of the National Natural Science Foundation of China (J1210053).

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