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ORIGINAL RESEARCH

Cloning and Characterization of an Annexin Gene from *Cynanchum komarovii* that Enhances Tolerance to Drought and *Fusarium oxysporum* in Transgenic Cotton

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Abstract A novel plant annexin, *CkANN*, was isolated from *Cynanchum komarovii*. Sequence analysis showed that CkANN contained four conserved exdonexin fold regions, a putative peroxidase heme binding motif and two S3 clusters. Real-time PCR analysis indicated that the *CkANN* mRNA was abundant in leaf, flower, root and stem and that its level is highest in leaf and flower. The transcription level of *CkANN* was increased significantly following stress by salicylic acid (SA), methyl jasmonate (MeJA), abscisic acid (ABA), NaCl, PEG 6000 (polyethylene glycol) and H_2O_2. Over-expression of *CkANN* in transgenic cotton caused a significant increase in tolerance to drought and led to higher levels of proline and soluble carbohydrates compared to wild-type (WT) plants. In response to drought, the transgenic plants also displayed higher total chlorophyll levels and reduced accumulation of thiobarbituric acid-reactive substances (TBARS) compared to WT plants. Furthermore, the transgenic cotton showed enhanced resistance to *Fusarium oxysporum* via increased total peroxidase activity and induction of the expression of some pathogenesis-related proteins. In conclusion, this study has demonstrated that *C. komarovii* CkANN is involved in drought tolerance and disease resistance and therefore may contribute significantly to the development of drought- or disease-resistant crops.

Keywords Annexin · CkANN · Drought · *Fusarium oxysporum* · *Cynanchum komarovii* · Transgenic cotton

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Introduction

Annexins are a multigene family of multifunctional lipid-binding proteins that have been isolated and cloned from more than 65 species to date, including fungi, protists, plants, higher vertebrates and a prokaryote (Talukdar et al. 2009). Various aspects of animal annexins have been studied extensively, including the establishment of membrane domains and maintenance of their stability, the regulation of trans-membrane ion transportation and intracellular Ca^{2+} concentration, endocytosis and exocytosis, and programmed cell apoptosis (Rescher and Gerke 2004). In contrast, the study of plant annexins has lagged behind, although researchers have recently become more interested in plant annexin features and functions. Plant annexins represent a smaller and less diverse family of proteins that constitute 0.1% of total plant protein (Delmer and Potikha 1997; Clark et al. 2001). Boustead et al. (1989) first identified plant annexins (p34 and p35) that can bind Ca^{2+} and liposomes of phosphatidylserine from suspension cultures of tomato cells. Sequentially, annexins were purified from maize coleoptiles and tip-growing pollen tubers of *Lilium longiflorum* using similar methods (Clark et al. 1992). Proust et al. (1996) cloned an annexin gene regulating fruit development of *Capsicum annuum*. Seigneurin-Berny et al. (2000) identified an annexin in the chloroplast envelope membrane of spinach. So far, annexins isolated from various plants are capable of binding to F-actin, hydrolyzing ATP and GTP and acting as peroxidases or cation channels (Talukdar et al. 2009).

Due to the lag in research into plant annexins, the first three-dimensional structure of a member of the plant annexin family (Ann24Ca), which came from *C. annuum*

(Hofmann et al. 2000), was not reported until 2000. The crystal structure of annexin from *Gossypium hirsutum* was then identified (Hofmann et al. 2003). Based on the structural information thus obtained, plant annexins were seen to have common conserved regions. The size of plant annexins falls in the range of 32–42 kDa, and typically only the first and fourth repeated domains contain calcium (Ca^{2+}) binding sites—provided by the characteristic endonexin fold sequence (K-G-X-G-T-[38]-D/E) (Geisow et al. 1986). Plant annexins exhibit high α-helical folding of the C-terminal (core) domain, forming a slightly curved disk, and have a short N-terminal region. The crystal structure of AnnCa24 indicated that the short N-terminal region interacts with the annexin core, suggesting that some regulatory function of this region is conserved in plant annexins (Hofmann et al. 2000).

Plant annexins play an important role in the response to various stimuli (Laohavisit and Davies 2011). In alfalfa (*Medicago sativa*), annexin is activated in cells or tissues exposed to osmotic stress, abscisic acid (ABA) or water deficiency (Kovacs et al. 1998). Annexin expression in Arabidopsis changes in response to various abiotic stresses, such as salt, osmotic stress and cold (Kreps et al. 2002), and ABA is considered as a general regulator in response to osmotic stress (Lee et al. 2004). Cantero et al. (2006) assessed eight annexin gene expression patterns in Arabidopsis and established their differential regulation by salt, drought and other abiotic stresses. Arabidopsis T-DNA mutants indicated that AnnAt1 and AnnAt4 responded to osmotic stress during germination (Lee et al. 2004). In *Brassica juncea*, over-expression of AnnBj1 enhanced drought- and salt-tolerance in transgenic tobacco and cotton, respectively (Jami et al. 2008; Divya et al. 2010). Overexpressing *AnnAt*1 in Arabidopsis resulted in increased drought tolerance, and knockout plants were more drought-sensitive than ecotype Columbia plants (Konopka-Postupolska et al. 2009). In addition, annexins have a protective role in defense responses against pathogens. For example, infection with *Pseudomonas fluorescens* or cucumber mosaic virus down-regulates *AtANN4* expression as assessed by microarray analysis (Marathe et al. 2004; Wang et al. 2005), but *Pseudomonas syringae* infection up-regulates *AtANN4* (Truman et al. 2007). Transgenic tobacco *AnnBj1* enhanced resistance to the oomycete pathogen *Phytophthora patasitica* var. nicotianae and increased the mRNA levels of several pathogenesis-related proteins (Jami et al. 2008). The experimental evidence indicates that annexins play an important role in plant stress responses; however, the exact physiological and biological functions of annexins from different plants remain unknown.

Cynanchum komarovii Al Iljinski, belonging to Cynanchum in the Asclepiadaceae family, is a desert plant adapted to the dry and barren environment encountered during desertification processes. *C. komarovii* has been used as a sand-fixing plant to prevent soil and water runoff and for its resistance to drought, salinity and sandstorm (Zhang et al. 2007). In addition, an antifungal protein from *C. komarovii* is able to inhibit fungal growth of *Verticillium dahliae*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Botrytis cinerea* and *Valsa mali* (Wang et al. 2011). Thus, we hope to obtain a novel annexin gene from *C. komarovii* that could be used to improve crop resistance to drought and fungal infection via transgenic engineering.

Here, we report a novel annexin-like gene from *C. komarovii*. Over-expression of CkANN in transgenic cotton confers tolerance to drought and resistance against *F. oxysporum*, which causes widespread disease, specifically Fusarium wilt of cotton (Kim et al. 2005).

Materials and Methods

Plant, Fungi, and Abiotic Stress Treatment

Cynanchum komarovii Al Iljinski seeds were provided by Ningxia Academy of Agricultural Sciences. Seeds were sterilized in 4% NaClO solution and 75% ethanol, then planted in a mixture of soil and vermiculite (2:1, w/w) and cultured under a photoperiod of 16 h light/8 h dark, day and night temperature of 25°C and 22°C in the greenhouse of China Agricultural University.

Cotton seeds (CCRI 45) were provided by the Cotton Research Institute, Chinese Academy of Agriculture Science. Seeds were sterilized with 4% NaClO solution, 75% ethanol and sterilized water in turn, then planted in jars in MS medium (1×MS salts, 1×MS vitamins, 3% sucrose, 1% agar, pH 5.8) under a photoperiod of 16 h/8 h with a light intensity of 150 μmol m^{-2} s^{-1} and day/night temperature of 25°C/22°C.

For the abiotic treatment experiments, 3-week-old seedlings of *C. komarovii* were removed to solutions containing 1 mM salicylic acid (SA), 100 μM methyl jasmonate (MeJA), 100 μM abscisic acid (ABA), 300 mM NaCl , 10% (w/v) polyethylene glycol (PEG 6000) and 10 mM H_2O_2, over three repeated trials. Control samples were treated with the culture solution. Samples were taken at 0, 1, 3, 6, 12 and 24 h after the stress treatments.

Strains of *F. oxysporum* f. sp. Vasinfectum (AYF-1) were isolated from diseased plants in fields around Anyang, Henan, China. Conidial suspensions of *F. oxysporum* was prepared from a culture grown for 5 days at 25°C in Czapek liquid medium ($NaNO_3$ 2 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4$ 0.01 g, sucrose 30 g, distilled water 1 L, pH 7.2). The analysis of resistance in transgenic cotton was performed on the conidial suspensions (10^6 conidia /mL) of *F. oxysporum*.

Cloning of *CkANN*

Total RNA from *C. komarovii* was isolated using an extraction kit (Promega, Madison, WI). To obtain cDNA encoding *CkANN*, we designed two degenerate primers as follows: 5′-CATATCCATCTTGHCTCN-3′ (forward) and 5′-CTCAGCCCTTYTAGWCN-3′ (reverse), which was based on the homologous sequences from *Arabidopsis thaliana* (AF083913), *Zea mays* (X98244); *Nicotiana tabacum* (AF113545) and *Gossypium hirsutum* (U89609). First-strand cDNA was obtained from total RNA using a reverse transcription (RT) kit (Takara, Shiga, Japan). RT-PCR conditions were as follows: 94°C for 30 s, 48°C for 30 s, 72°C for 1 min, for 33 cycles, and a final extension at 72°C for 10 min. The amplified product was purified and cloned in pGEM-T vector (Promega) for sequencing. 3′ RACE was performed using the RT-PCR product as template with two nested gene-specific sense primers, GPS1 (5′-CGAGTTGT GACTACAAGGGCTG-3′) and GPS2 (5′-TCGATCAA CAAGCGTGGAACAG-3′), following the specification of 3′RACE kit (Takara). To obtain the full-length cDNA encoding the *CkANN*, 5′RACE amplification was performed according to the manufacturer's instructions (Invitrogen, La Jolla, CA) and primers were designed based on the sequence of RT-PCR product. First-strand cDNA synthesis was performed with the antisense primer GPS3 (5′-CCCCTGTT GTGTGATGTGCAACA-3′). The tailed cDNA was used as template for amplification with the adapter primer and two nested primers GPS4 (5′-GGGCATGATAAGCTTCCCTAAC-3′) and GPS5 (5′-CTTGTTTGCTTGATGTCCACCTC-3′). The RT-PCR reaction was started at 94°C for 5 min, and the following cycle was repeated 33 times: 94°C for 30s, 52–55°C for 30s, 72°C for 1 min, and a final extension at 72°C for 10 min. The reaction products were purified and cloned into the pGEM-T vector for sequencing.

Sequence and Phylogenetic Analysis

The amino acid sequence was deduced using the DNAMAN tool. Sequence features, such as signal peptide, p*I* and molecular mass were evaluated using protein analysis tools (http://expasy.org/tools). Annexin sequences were selected from NCBI and aligned with Cluster X version 2.0 and gaps were removed from the alignment. The phylogenetic tree of those alignments was calculated by the neighbor-joining method using the program MEGA 4, and bootstrap values from 1,000 replicates were indicated at the branches.

Real-Time PCR

An aliquot of 2 μg total RNA was reverse transcribed to first-strand cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) according to the manufacturer's specifications. Primers unique to *CkANN*, ANN-F (5′-ATCCTCATGAACGGGACGCA-3′) and ANN-R (5′-CCTGTTGTGTGATGTGCAAC-3′) were designed. The endogenous control was *EF-1-α* (HQ849463) from *C. komarovii* and was identified with the sense primer EF1α-F (5′-TGCATCCAACTCGAAGGATG-3′) and antisense primer EF1α-R (5′-CCTTACCAGATCGTCTGTCT-3′). Real-time PCR was performed in a 20-μL reaction mixture in a 96-well plate with an ABI 7500 thermocycler (Applied Biosystems), using Power SYBR Green PCR Master Mix (Applied Biosystems). The PCR solution included 2 μL diluted cDNA, 10 μL SYBR Green Master Mix, 0.1 μmol forward and reverse primers in a final volume of 20 μL. The following condition was used for real-time PCR: 95°C denaturation for 10 min, 40 cycles of 95°C for 15 s, 58°C for 20 s, and 72°C for 30 s. Before proceeding with real-time PCR, we routinely verified that the primers of *CkANN* and the *EF-1-α* gene had a similar slope with high correlation coefficients by constructing standard curve (R^2=0.94 and R^2= 0.95 respectively). The threshold cycle (CT) values of the triplicate real-time PCRs were averaged and the fold changes of transcription levels of target gene (*CkANN*) relative to the reference gene (*CkANN*) was analyzed by the comparative CT ($2^{-\Delta\Delta CT}$) method. Statistical analysis of real-time PCR date and standard deviation (SD) values were performed as previously described by Livak and Schmittgen (2001).

Vector Construction and Plant Transformation

The coding region of *CkANN* was amplified by RT-PCR, using specific primers (5′GCCGTCTAGAATGGCTTC TCTTGTCGTG3′ and 5′ATAACCCGGGTGCATCCTCTG CCCCAAT 3′) to generate *Xba*I and *Sma*I restriction sites (underlined) at the 5′ end and 3′ end, respectively. The resulting DNA fragment was sub-cloned into the vector pBI 121 containing a neomycin phosphotransferaseII (NPTII) gene and a β-glucuronidase reporter gene (GUS), both of which are under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The plasmid was transformed into competent cells of *Agrobacterium* strains (LBA4404) by the freeze-thaw method (An et al. 1988). *Agrobacterium* suspension cells (OD_{600} =0.6) were used for inoculation of hypocotyl segments.

Agrobacterium-mediated transformation experiments were carried out according to the protocols described by Sunikumar and Rathore (2001). Transgenic cotton seeds were selected on MS medium with kanamycin (50 mg L^{-1}) and surviving seedlings were confirmed using PCR. PCR using genomic DNA of transgenic cotton leaves as template was carried out with CkANN vector specific primers: 121-F (5′-CCCACTATCCTTCGCAAG-3′) and 121-R (5′-CCAGACT GAATGCCCACA-3′). A total of 16 positive transgenic seeds

were obtained and grown under the same conditions. Three (T-1, T-2 and T-3) stable heritable T1 progeny (T2) transgenic cotton plants were chosen for subsequent experiments.

Drought Tolerance in Transgenic Cotton

For drought tolerance experiments, 75 seedlings from T2 three transgenic lines (T-1, T-2 and T-3) and wild-type (WT) plants were grown in soil as described above. After 4 weeks, watering was withdrawn for 10 days and phenotypes were observed. The relative water content (RWC) of cotton leaves was calculated as described by Lv et al. (2007).

Determination of Chlorophyll and Lipid Peroxidation

Total chlorophyll was extracted with 80% acetone from 4-week-old transgenic and WT plant seedling leaves after drought treatment, and total chlorophyll was measured spectrophotometrically as described previously by Arnon (1949). Thiobarbituric acid reactive substances (TBARS) were assayed to estimate the degree of lipid peroxidation of transgenic and wild cotton using the method of Herth and Packer (1968).

Proline and Carbohydrate Content

The leaves of 4-week-old WT and transgenic seedlings were collected, and the proline and soluble carbohydrate content was estimated under drought stress. The proline content was determined as described (Bates et al. 1973), and the soluble carbohydrate, sucrose, glucose and fructose measurements were performed as described previously (Sekin 1978).

Antifungal Assay in Transgenic Cotton

The bioassay for resistance against infection by *F. oxysporum* was performed on T2 transgenic cotton. The roots of 2-week-old seedlings (2 cm) were cut off and immersed in 100 mL conidial suspensions (10^6 conidia mL^{-1}) for 15 min. The ratio of infected plants was recorded as the number of diseased plants divided by the total number of plants at 14 days and 28 days after inoculation, respectively. The disease severity and disease index were detected as described (Emani et al. 2003).

Analysis of Total Peroxidase Activity after Inoculation

The total peroxidase activity was assayed 1 min after guaiacol oxidation (micromoles of tetraguaiacol formed, min^{-1} g^{-1}FW) according to Srivastava et al. (2004). After inoculation with *F. oxysporum*, transgenic and WT cotton leaves were collected at 5, 10, 15, 20, 25 and 30 days, respectively

Analysis of PR Genes

Total RNA of mature leaves was extracted from 2-month-old WT and T2 transgenic lines (T-1, T-2, T-3). Real-time PCR was performed as above to analyze transcript levels of the PR genes chitinase, glucanase and PR1 in WT and transgenic lines under *F. oxysporum*-challenged conditions. The endogenous control was the *UBQ7* gene (DQ116441) from cotton, which was detected using the sense primer UBQ-F (5′-GAAGGCATTC CACCTGACCAAC-3′) and antisense primer UBQ-R (5′-CTTGACCTTCTTCTTCTTGTGCTTG-3′). The PR gene-specific primers were as follow: chitinase (GHU60197), F: 5′-TCACCATGACTTTCTCCCACCATATATTT-3′ R: 5′-AAGC TACATTGAGTCCACCG-3′; β-1,3 glucanase (Z68154), F: 5′-GCATTCAACTATGCTTCTTCTGCTTTCG-3′, R: 5′-CCCCAATGTTTCTCATATGCCGGTTC-3′; PR1 (AF305066), F: 5′-GTCACTTATGACTATGAGAA TACCTCCC-3′, R: 5′-TAGTTGCAGGCTTCGGGAT TAGCCA-3′.

Results

Cloning and Characterization of *CkANN*

The target cDNA *CkANN* (GenBank accession no. GU067483) was obtained via RT-PCR and RACE-PCR. The cDNA consisted of 1,242 bp with a single open reading frame (ORF) of 948 bp, encoding a protein of 316 amino acid residues and a calculated molecular mass of 36 kDa with p*I* 5.9 (Fig. 1a). The CkANN protein has an N-terminal tail and a type-II Ca^{2+}-binding site (G-X-GT-[38]-E, from position 25) in the first repeat of the C-terminal core in which the W residue (position 27) may be important for phospholipid binding (Mortimer et al. 2008). The salt bridges D88–R113 and E128–R257, deduced from human and plant annexins, may be involved in ion conduction and selectivity in the plant annexin family. In addition, CkANN also contained a heme-binding site at aa 11–40 with a highly conserved H residue at aa 40, two S3 clusters formed by two C residues (C-111 and C-238) similar to annexin Gh1 from *G. histutum*. These data suggest that CkANN from *C. komarovii* belongs to the plant annexin family.

According to a BLAST search, CkANN shares high identity with annexins from *G. hirsutum* (72%), *C. annuum* (71%), *A. thaliana* (71%), *Z. mays* (62%) and *B. juncea* (69%) (Andrawis et al. 1993; Proust et al. 1996; Gidrol et al. 1996; Battey et al. 1996; Jami et al. 2008). Phylogenetic analysis of plant annexins revealed four main branches (Fig. 1b), in which CkANN was grouped with *C. annuum*32 (X93308) and *G. hirsutum* (U89609), which suggests they may have similar features and functions.

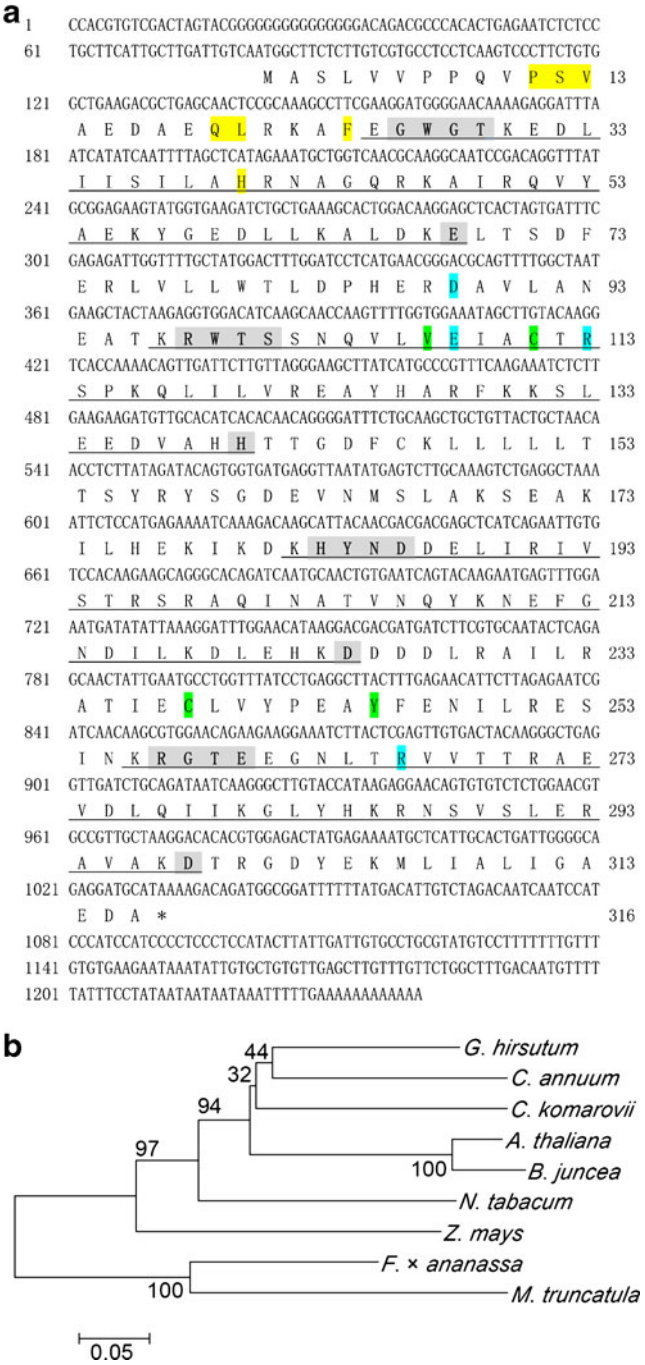

Fig. 1 **a**,**b** Sequence and phylogenetic tree of a plant annexin, CkANN, isolated from *Cynanchum komarovii*. **a** Full-length *CkANN* cDNA and putative amino acid sequence. Nucleotides are numbered on the right and amino acids on the left. * Termination codon. The putative annexin repeats (I–IV) are *underlined*, and the corresponding exdonexin (typeII Ca^{2+} binding) sequences are shown *shaded* and in *bold*. The putative peroxidase heme binding motif and conserved H residue are shown in *yellow*. Conserved salt bridges are shown in *blue*. The S3 clusters putatively involved in redox reactions are shown in *green*. **b** Phylogenetic tree of annexins constructed by the neighbor-joining method; bootstrap values are indicated at the branches. Amino acid sequences of annexins come from *Cynanchum komarovii* (GU067483), *Arabidopsis thaliana* (AF083913); *Brassica juncea* (AY356355); *Zea mays* (X98244); *Fragaria×ananassa* (AF188832); *Medicago truncatula* (Y15036); *Nicotiana tabacum* (AF113545); *Gossypium hirsutum* (U89609); and *Capsicum annuum* (X93308)

Transcript Levels of *CkANN*

A tissue-specific expression pattern of *CkANN* was detected in roots, stems, leaves and flowers (Fig. 2a). *CkANN* mRNA was most abundant in leaves (5.47±0.54-fold than in stem), with lower levels in flowers (2.63±0.22-fold), followed by still lower levels in root (2.58±0.37-fold); levels were lowest in stems.

To study the expression of *CkANN* in response to abiotic stress, *C. komarovii* seedlings were treated with different chemical inductions. Following SA treatment, the expression of *CkANN* was up-regulated to a maximum of 20.21±1.57-fold over basal activity at 3 h post-treatment (Fig. 2b). After MeJA treatment, the transcript level reached 15.49±0.62-fold over basal level at the 1-h time point, and declined gradually thereafter until 24 h (Fig. 2c). ABA treatment resulted in mRNA accumulation at 3 h and a further increase to 23.70±0.63-fold of basal level until 6 h (Fig. 2d). *CkANN* mRNA accumulation in NaCl (300 mM) increased rapidly to 14.09±1.77-fold at the 3 h time point and continue to climb to a maximum of 20.32±1.35-fold high levels at 6 h before declining gradually during 12–24 h (Fig. 2e). After treatment with PEG 6000-induced drought, the *CkANN* transcript levels were up-regulated up to 6 h and then decreased slightly during 12–24 h (Fig. 2f). The accumulation of *CkANN* mRNA in response to H_2O_2 was up-regulated to 3.05±0.48-fold at 1 h, and the trend continued until 6 h (Fig. 2g).

Drought Tolerance of CkANN in Transgenic Cotton

Dehydration experiments were performed on 4-week-old WT and T2 transgenic cotton seedlings (T-1, T-2 and T-3). Wilt symptoms appeared in WT plants as early as 10 days after water withdrawl but were not present in transgenic lines (Fig. 3a). Under drought stress, the transgenic plants developed 1.5-fold longer primary roots (data not shown) and more lateral roots than WT plants (Fig. 3b). The RWC reduction in transgenic cotton was significantly less than in WT (Table 1). During drought stress, the transgenic plants were able to retain more chlorophyll content when compared to WT (Fig. 3c). In addition, the transgenic plants showed a reduction in TBARS compared to WT in response to drought (Fig. 3d).

Over-Expression of CkANN Improves Accumulation of Proline and Carbohydrate

Under drought stress, the proline concentrations in both WT and transgenic plants increased (Table 2). After 10 days,

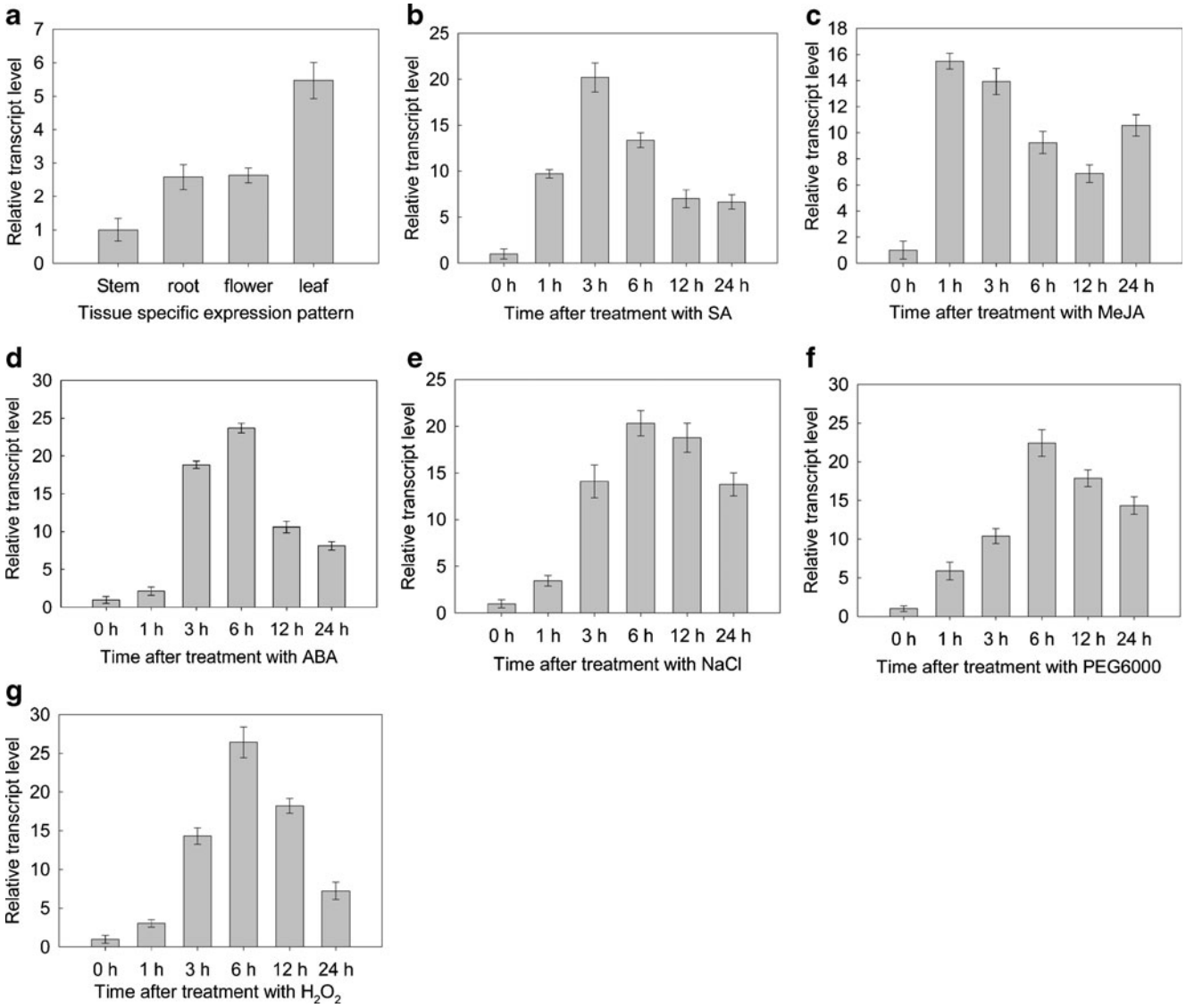

Fig. 2 **a**–**g** Relative *CkANN* transcript levels. **a** Tissue-specific expression pattern of CkANN in cotton. **b**–**g** Transcript levels after various treatments: **b** salicylic acid (SA), **c** methyl jasmonate (MeJA), **d** abscisic acid (ABA), **e** NaCl, **f** drought, **g** H_2O_2. Results are expressed as means±SD (n=3)

the proline content in transgenic lines has increased up to 12-fold, which was significantly higher compared to the 3-fold increase in controls($P<0.05$). Similarly, transgenic leaves displayed markedly higher levels of carbohydrate content under drought (Table 3). The sucrose content of transgenic line T-2 reached a maximum level of 1.3-fold over WT, and T-1 and T-3 increased 1.2-fold. The glucose and fructose content also increased significantly in all transgenic lines.

Disease Resistance of CkANN in Transgenic Cotton

Two-week-old WT and transgenic cotton seedlings were inoculated with *F. oxysporum*, and the differences in disease severity were significant (Table 4, Fig. 4a). The WT plants showed more prominent symptoms than transgenic plants, and the infection rate was 64.12±2.82 % at 14 dpi, whereas transgenic lines (T-1, T-2, T-3) was significantly less. At 28 dpi, WT cotton showed severe symptoms (84.89±2.75% infection rate) while transgenic cotton showed only 42% (on average). The disease index value for WT was 34.69± 2.75 at 14 dpi and 49.03±3.33 at 28 dpi. There was a significant reduction in disease index values for all transgenic lines at 14 dpi and 28 dpi. These results suggested the CkANN protein is capable of protecting cotton against *F. oxysporum*.

The peroxidase activities of transgenic plants displayed significantly higher activity compared to WT plants when

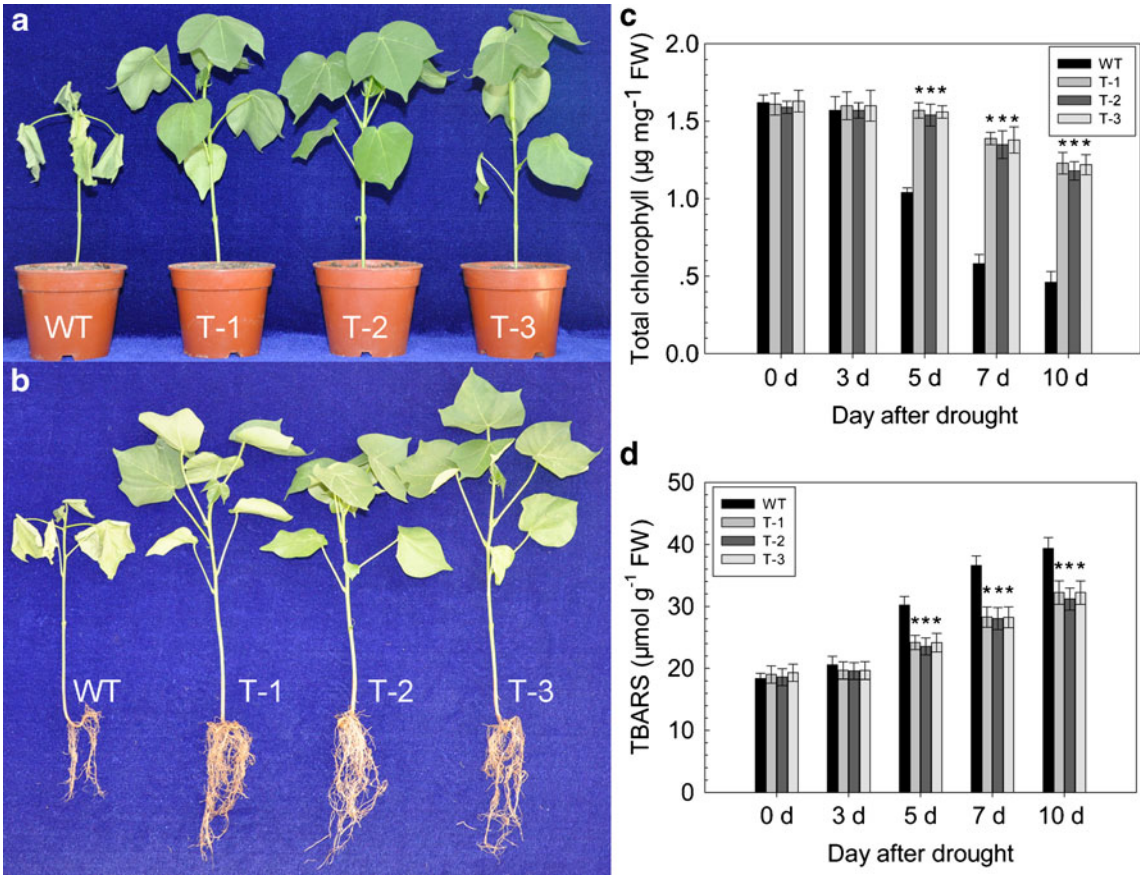

Fig. 3 **a**–**d** Effect of drought stress on transgenic and WT plants. **a** Phenotypes of transgenic and WT seedlings after 10 days of drought. **b** Root phenotypes. **c** Changes in total leaf chlorophyll content after drought treatment. * Significant difference from WT plants values at $P<0.05$. Data represents mean±SD of three individual experiments. **d** Thiobarbituric acid reactive substances (TBARS) content in leaves from drought seedlings

inoculated with *F. oxysporum* (Fig. 4b). The peroxidase activity of both increased gradually from the time of inoculation with *F. oxysporum*, but no significant difference between WT and transgenic lines was observed under control conditions.

The real-time PCR results for PR gene expression patterns indicated that the transcript levels of PR-1, chitinase and glucanase increased in transgenic cotton. Transgenic line T-1 showed 9.33±0.41-fold higher PR-1 mRNA levels compared to WT, T-2 and T-3 lines were 10.24±0.85-fold and 8.55±0.66-fold higher, respectively (Fig. 4c). For chitinase transcript levels, T-1 showed 6.79±0.57-fold higher compared to WT, T-2 and T-3 showed 7.32±0.39-fold and 6.33±0.72-fold higher levels, respectively (Fig. 4d). The transcript level of glucanase mRNA was about 13–16 times higher in transgenic lines than in WT cotton (Fig. 4e).

Table 1 Relative water content (RWC) in cotton leaves under drought treatment. Values were determined at 10 days after drought. Values followed by different lower case letters show significant differences at $P<0.05$ probability levels between WT and transgenic lines

Line	RWC(%)	
	H_2O (unstressed)	Drought (10 days)
WT	85.2±2.1 a	56.3±1.9 c
T-1	84.7±1.7 a	68.2±3.1 b
T-2	85.3±1.5 a	69.4±2.6 b
T-3	84.9±2.3 a	68.8±2.7 b

Table 2 Effect of drought stress on proline contents of WT and transgenic plants. Values were determined at 10 days after drought. Values followed by different lower case letters show significant differences at $P<0.05$ probability levels between WT and transgenic lines

Line	Proline content (mg g-1 FW)	
	Non-stressed	Drought (10 days)
WT	0.18±0.03 a	0.83±0.05 c
T-1	0.20±0.02 a	2.49±0.04 b
T-2	0.17±0.04 a	2.41±0.02 b
T-3	0.19±0.02 a	2.45±0.04 b

Table 3 Effect of water stress on carbohydrate contents of WT and transgenic plants. Values were determined at 10 days after drought. Values followed by different lower case letters show significant differences at $P<0.05$ probability levels between WT and transgenic lines.

Line	Sucrose (mg g^{-1} FW)	Glucose (mg g^{-1} FW)	Fructose (mg g^{-1} FW)	Total soluble sugars (mg g^{-1} FW)
WT	1.32±0.03 a	3.01±0.04 a	3.83±0.04 a	8.19±0.07 a
T-1	1.65±0.04 b	3.43±0.06 b	4.04±0.05 b	9.13±0.08 b
T-2	1.73±0.03 b	3.47±0.03 b	4.07±0.03 b	9.21±0.07 b
T-3	1.62±0.06 b	3.41±0.02 b	4.01±0.06 b	9.09±0.09 b

Discussion

This paper describes the cloning and characterization of the plant annexin *CkANN* (GU067483) from *C. komarovii*. Based on alignment with sequences from Arabidopsis and other plants, several functional consensus motifs identified in various eukaryotic organisms were found in the deduced full-length CkANN sequence. CkANN from *C. komarovii* can be divided into four repeat annexins domains and contains type II Ca^{2+} binding sites in the first and fourth repeat regions (Fig. 1a). The plant annexin motif S3 cluster is responsible for the possible peroxidase activity and may play a role in transferring electrons to an oxidizing molecule, which could be a reactive oxygen species (ROS; Talukdar et al. 2009). According to the crystal structure, the S3 cluster of AnnGh1 from cotton is formed by two adjacent C residues and a nearby M (Hofmann et al. 2003). Similar S3 clusters were observed in Arabidopsis AnnAt1 and *B. juncea* AnnBj1 (Divya et al. 2010). The CkANN from *C. komarovii* has two potential S3 clusters formed by two C residues at C-111 and C-238. In addition, some plant annexin sequences display strong sequence similarity with the heme binding motif of plant peroxidases and contain a conserved H residue H40 (Gidrol et al. 1996; Gorecka et al. 2005). Gorecka et al. (2005) proved that the H40 residue of AnnAt1 from Arabidopsis was involved in peroxidase activity by showing that a mutation in H40. AnnAt1 can be rescued from H_2O_2 and oxidative stresses by the ΔoxyR mutant of *Escherichia coli* (Gidrol et al. 1996; Kush and Sabapathy 2001). These results demonstrated that plant annexins could play a role as peroxidases. Consistently, CkANN has heme-binding motif and conserved H40 with a relatively consistent position in AnnAt1. Furthermore, plant annexins contain two conserved salt bridges, which is similar to human AnxA5 (D92-R117 and E112-R271) and which play a role as a channel for Ca^{2+} entry into the cell (Hofmann et al. 2003; Gorecka et al. 2007). These two salt bridges are conserved at D88-R113 and E107-R265, respectively in CkANN, which suggests that CkANN might act as plasma membrane Ca^{2+}-permeable channel, like annexins from bell pepper, maize and Arabidopsis.

Our results revealed no significant differences in morphology between WT and transgenic cotton. At the seedling stage, T2 transgenic cotton over-expressing *CkANN* showed enhanced drought tolerance (Fig. 3a). Similarly, in tobacco and cotton, *AnnBj*1 from *B. juncea* conferred tolerance to various stresses such as dehydration, salinity, heavy metal stress and oxidative stress during seedling growth and development (Jami et al. 2008; Divya et al. 2010). In Arabidopsis, over-expressing *AnnAt*1 and loss-of-function (Δ*annAt1*) plants studies suggested that *AnnAt*1 plays an important role in sustaining plant growth and productively under severe drought (Konopka-Postupolska et al. 2009). In the drought stress experiment, we found that transgenic *CkANN* seedlings grew taller than WT plants and recovered quickly after water withdrawal. The significantly higher chloroplast content in transgenic plants supported the idea that some annexins can protect plants against the chlorophyll loss caused by excess ROS (Jami et al. 2008) (Fig. 3c). Furthermore, higher peroxidase activity has found in leaves from *AnnBj*1 transgenic plants and may account for ROS detoxification under various stresses (Jami et al. 2008; Divya et al. 2010). Studies on the over-expression of *AnnAt*1 in Arabidopsis showed ABA-induced ROS was significantly lower in transgenic lines compared to WT, but mutant lines displayed higher fluorescence, as identified by the fluorescent dye H_2DCRDA (Konopka-Postupolska et al. 2009). This also helps explain the role of annexins in plant ROS detoxifica-

Table 4 Resistance of transgenic cotton to *Fusarium oxysporum* compared to WT plants. Values are mean (±SE) calculated from 15 plants per line. *dpi* Days post-inoculation

Line	Infection rate (%)		Disease index	
	14 dpi	28 dpi	14 dpi	28 dpi
WT	64.12±2.82	84.89±2.75	34.69±1.47	49.03±3.33
T-1	28.37±1.52*	41.61±3.27*	13.29±2.08*	17.57±1.51*
T-2	25.49±1.74*	40.74±1.99*	11.74±1.84*	16.73±2.99*
T-3	29.68±2.15*	43.69±2.54*	14.21±1.93*	18.26±3.04*

*Significant difference from control values at $\alpha=0.10$

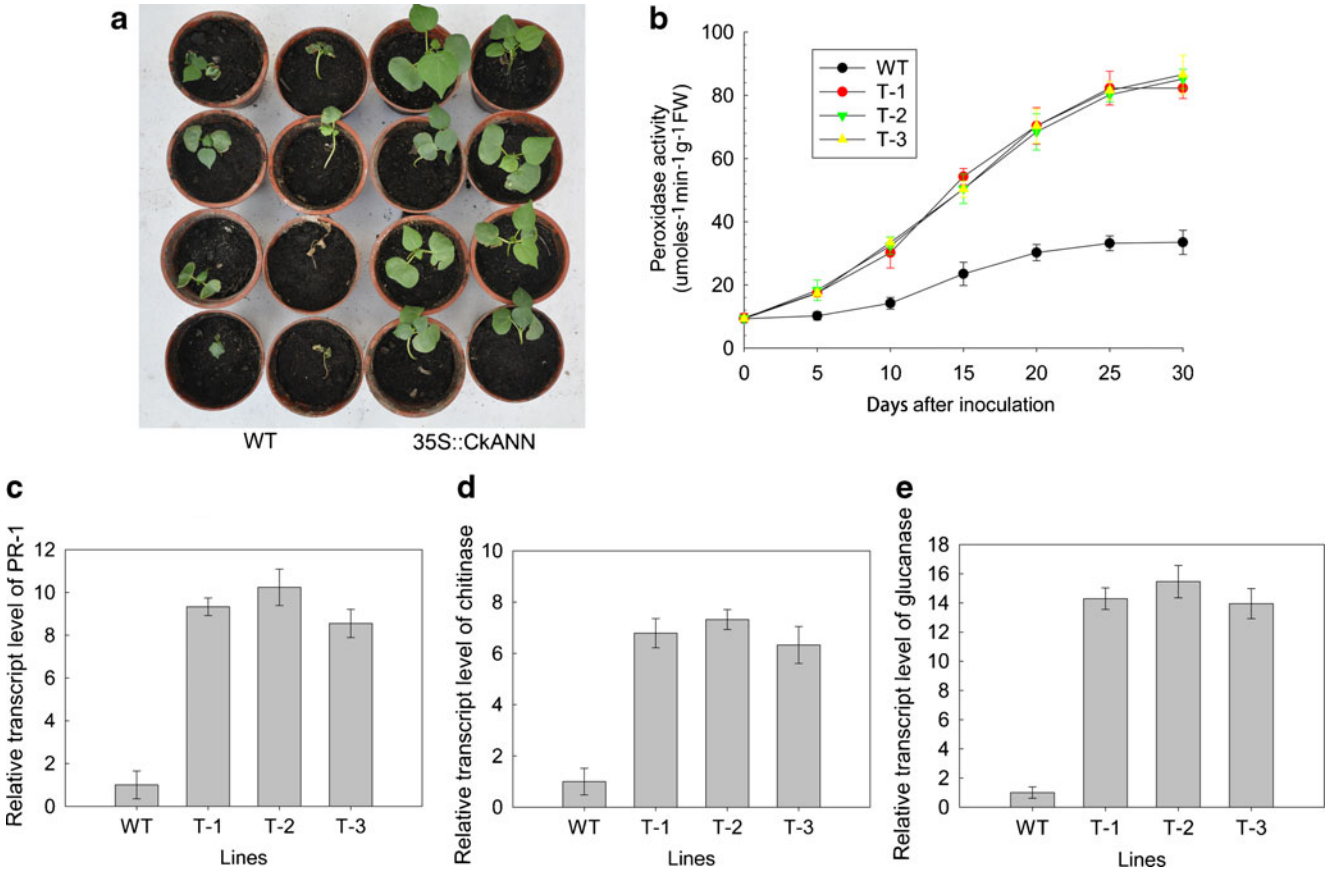

Fig. 4 **a**–**e** Effect of fungal disease on transgenic and WT plants. **a** *Fusarium oxysporum* disease resistance assay in transgenic and WT cotton. The image shows disease severity after the 2-week inoculation. **b** Changes in activity of total peroxidase in seedlings after inoculation with *F. oxysporum*. Mean (±SE) were calculated from three plants per line. All transgenic lines show significant differences from control values at $P<0.05$. Real-time PCR analysis in WT and transgenic cotton for the relative transcript levels of PR-1 (**c**), chitinase (**d**) and glucanase (**e**). The results are expressed as means±SD ($n=3$)

tion. In addition, lipid peroxidation levels, which directly indicate the extent of membrane damage, were significantly reduced in transgenic plants compared to WT plants in response to drought stress (Fig. 3d). This suggests that the presence of CkANN protein in transgenic cotton can also protect cellular membranes by inhibiting lipid peroxidation levels during drought stress. This is in line with the transgenic tobacco and cotton over-expressing *AnnBj*1 under mannitol and salinity stresses (Jami et al. 2008; Divya et al. 2010). To better elucidate the role of annexin in drought, the levels of free proline and soluble carbohydrates as osmotic regulators in the WT and transgenic cotton were estimated under drought stress. Over-expression of *CkANN* in transgenic cotton enhanced the levels of proline and soluble sugars compared to WT plants under drought conditions (Tables 2, 3). Similar results also reported that transgenic cotton overexpression *AnnBj*1 improved the contents of proline and sucrose under conditions of high salinity (Divya et al. 2010). Calcium signaling is essential for proline accumulation in response to abiotic stress (Parre et al. 2007). Sucrose accumulation can create an intracellular calcium uptake (Martinez-Noel et al. 2006). Taken together, these findings indicate that annexin could participate in calcium channeling under stress conditions.

T2 transgenic cotton seedlings had enhanced disease resistance to *F. oxysporum* compared to WT (Fig. 4, Table 4), which suggested that CkANN protein has a similar function to AnnBj1 in plant defense responses (Jami et al. 2008). Plant annexins displayed resistance by reducing the spread of the disease symptoms, and rapidly generating ROS during the interaction of plant and pathogens (Gidrol et al. 1996; Hofmann et al. 2000; Gorecka et al. 2005, 2007; Jami et al. 2008). In the transgenic cotton, AnnBj1 showed higher transcription levels of several PR proteins prior to fungal attack, as well as the production of H_2O_2 signals (Jami et al. 2008). In our study, *CkANN* transgenic cotton also induced transcription of PR proteins (PR-1, chitinase, glucanase) under challenge inoculation (Fig. 4c–e). This suggests that resistance induced by PR proteins and other responses might be regulated by the ROS signaling pathway. However, annexin proteins have been found to effect cytosolic free calcium ($[Ca^{2+}]_{cyt}$) and form Ca^{2+} channels by creating a Ca^{2+}-

permeable transport pathway (Hofmann et al. 2000). Ca^{2+} is used as an intracellular messenger to regulate the response to external stimuli in both animal and plant cells, and is therefore capable of influencing multiple aspects of cellular function including growth, division and cytoplasmic streaming (Hepler and Wayne 1985). Therefore, annexins, as novel Ca^{2+}- and charged phospholipid-binding proteins, also might function via Ca^{2+} signaling to increase plant disease resistance.

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