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ORIGINAL PAPER

Two NAC transcription factors from *Citrullus colocynthis*, CcNAC1, CcNAC2 implicated in multiple stress responses

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Abstract NAC (no apical meristem, *Arabidopsis* transcription activation factor 1 and 2, cup-shaped cotyledon 2) transcription factors (TFs) play important roles in plant growth, development, and responses to abiotic and biotic stress. Two novel NAC TFs were isolated from *Citrullus colocynthis*, a highly drought-tolerant cucurbit species: CcNAC1 and CcNAC2 each with conserved A–E NAC domains. Subcellular location of CcNAC1 and CcNAC2 investigated via transient expression of 35S::*CcNAC1*::*GFP* and 35S::*CcNAC2*::*GFP* fusion constructs in *Arabidopsis* protoplasts, revealed nuclear localization. The transactivation ability of CcNACs was examined in the GAL4 yeast assay system, and showed that only the C-terminal domain of CcNAC1 has the ability to activate reporter genes *LacZ* and *His3*. The CcNAC genes accumulated in a tissue-specific manner with expression levels in male flowers of *C. colocynthis* higher than leaves, hypocotyls or roots. Genome walking was used to isolate the CcNAC1 and CcNAC2-promoter regions. A high number of stress-related sequence motifs were detected, especially in the CcNAC1 promoter. *C. colocynthis* seedlings were treated with PEG, abscisic acid, salicylic acid (SA), jasmonic acid (JA), H_2O_2, ethylene, gibberellic acid (GA), wounding or salt. High CcNAC1 expression levels were detected following JA application, and wounding, while high CcNAC2 levels followed treatment with GA, JA, SA, and wounding, indicative of differential regulation of these stress responsive TFs in this cucurbit species.

Keywords *Citrullus colocynthis* · NAC transcription factor · CcNAC1 · CcNAC2 · *Arabidopsis* · Stresses

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Introduction

Plant responses to abiotic and biotic stresses involve changes at the transcriptome, cellular and physiological levels. The interaction between biotic and abiotic stress is orchestrated by hormone signaling pathways (Atkinson and Urwin 2012). Responses to stress require the production of important functional proteins, such as those involved in the synthesis of osmoprotectants, and regulatory proteins, kinases, and transcription factors (TFs), operating in the signal transduction pathways (Saibo et al. 2009). Several different techniques can be used to study the transcriptome during multiple stress responses. Affymetric ATH1 microarrays are commonly used to investigate universal components of the plant's response to different stress conditions (Mongkolsiriwatana et al. 2009; Swindell 2006). Quantitative trait analysis can be used for the identification of useful regions of genomes (Ashraf 2010), and next-generation high-throughput sequencing (Quail et al. 2012) offers whole plant transcriptome surveys (Wang et al. 2010), but gene functional analyses are still needed to study plant development and gain an understanding of responses to biotic and abiotic stress conditions.

Transcription factors are DNA-binding proteins that activate or repress transcription of downstream genes by binding to a consensus sequence in their promoters. The

NAC TF family is one of the largest TF families in plants, with more than 100 members identified in both *Arabidopsis* and rice (Wang and Dane 2013) and 80 in *Citrullus lanatus* (Guo et al. 2013). The acronym NAC originates from the no apical meristem (NAM), *Arabidopsis* transcription activation factor 1 and 2 (ATAF), and cup-shaped cotyledon (CUC) genes. NAC proteins typically share a well conserved N-terminal NAC domain, which is divided into five conserved subdomains (A–E), and a diversified C-terminal transcription regulatory domain (Puranik et al. 2012). NAC TFs are specific to plants and associated with many biological functions during embryonic, floral and vegetative development, and stress-related processes (Olsen et al. 2005; Atkinson and Urwin 2012). A number of NAC proteins interact with pathogens, the hormones abscisic acid (ABA), JA, and salicylic acid (SA) and exhibit interactions with both biotic and abiotic stress responses. Signaling crosstalk among phytohormones in NAC-associated pathways regulate the protective responses in plants via synergistic or antagonistic actions (Tuteja and Sopory 2008). Different phytohormones, which can bind to the NAC recognition sequence, can regulate different NACs and further regulate stress-related genes. Many *NAC* genes are associated with stress, and some of the *NAC* genes have multiple functions. *OsNAC6* in rice, for example, is involved in both abiotic and biotic stresses (Nakashima et al. 2007). *CsNAC1* is induced by salt stress, cold and ABA (Oliveira et al. 2011). Overexpression of ONAC045 results in enhanced drought and salt tolerance (Zheng et al. 2009), while RD26 was induced by drought, ABA, and high salinity (Fujita et al. 2004). NACs have long been associated with stress signaling, and recent discoveries suggest that they may make excellent targets for improving broad-spectrum tolerance in crops through genetic engineering (Nakashima et al. 2007; Xu et al. 2011). Although quite a few NACs have been functionally characterized primarily in model plants like *Arabidopsis*, the functions of the majority of the members of the large NAC gene family remain unknown (Hu et al. 2010).

Citrullus colocynthis (L.) Schrad, closely related to domesticated watermelon (*C. lanatus* var. *lanatus*), is a non-hardy drought-tolerant perennial herbaceous species in the *Cucurbitaceae* family (Jeffrey 2008; Al-Zahrani and Al-Amer 2006). It can survive arid environments by maintaining its water content without wilting of the leaves or desiccation under severe stress conditions. *C. colocynthis* has a rich history as an important medicinal plant and as a source of valuable oil (Dane et al. 2006). Its seeds appear in several early Egyptian, Libyan, and Near Eastern sites from about 4000 BC (Zohary and Hopf 2000). The species grows in sandy areas throughout northern Africa, southwestern Asia, and the Mediterranean region (Zamir et al. 1984; Burkill 1985; Jarret et al. 1997). Drought-tolerance studies in *C. colocynthis* pointed to several drought-inducible genes, including a partial NAC transcript (GenBank accession number GH626169), with complex adaptive transcriptional regulation (Si et al. 2009, 2010a, b). To further characterize the function of stress-tolerant genes in *C. colocynthis*, two novel plant-specific TFs, CcNAC1 (KC814686), and CcNAC2 (KC814687) and their promoter regions, were cloned using 5′RACE and the genome walker kit. Here, we report the characterization of the *CcNAC1* and *CcNAC2* genes to gain an understanding of their function under stress conditions. Manipulation of TFs represents a potential strategy for development of transgenic stress-tolerant plants. Results indicate that CcNAC1 and CcNAC2 may have multiple functions to regulate the plant's defense responses to abiotic stress.

Materials and methods

Plant materials

Citrullus colocynthis seeds (No. 34256) from Israel with high tolerance to drought were sown in potting mix in the greenhouse with a 14 h photoperiod and temperatures ranging from 22 to 33 °C and ambient relative humidity and light conditions (600–720 μmol m^{-2} s^{-1}). *Arabidopsis* seeds were planted for leaf protoplast isolation. Wild-type *Arabidopsis* (*A. thaliana*, ecotype Columbia) was grown in growth chambers for 5–6 weeks, as previously described (Jensen et al. 2007).

NAC isolation and phylogenetic analysis

NAC domain proteins from more than ten different species were used as query sequences for Blastx searches of the GenBank database (http://www.ncbi.nlm.nih.gov/) for analysis of the conserved domains of NAC proteins. Primers were designed based on conserved domain sequences for cloning of the *CcNAC* genes into T-easy-vector (Promega, Madison, WI), followed by sequence analysis and re-blasting into the NCBI database. The 5′ RACE cloning technique (Clontech Lab Inc, Mountain View, CA) was used to obtain full-length *NAC* sequences from *C. colocynthis*. Vector sequences and low-quality sequences were manually removed following sequencing. The non-redundant *C. colocynthis* sequences with the highest similarity to the query sequences were investigated as putative NAC domain genes. Evaluation of putative open reading frames (ORFs) of the identified sequences was based on (1) the sequences near the translation start site corresponding to the eukaryotic consensus sequence GCC(AG)CCATGG, (2) the sequence length and homology to the in vitro translated product, (3) the conserved

Table 1 Oligonucleotide primer sequences for *CcNAC1* cDNA cloning and *CcNAC2* cDNA cloning

Primer	T_m (°C)	Length	Aim	Sequence (5′–3′)
Con-F	60	27	Conserved region	TTCCATCCAACGGATGAGGAGCTCCGT
Con-R	60	27	Conserved region	TCAAAACGGCTTCTGCAGGTGCATAAA
GSP1	64	29	5′ region	TGGCTTCTCCTCCTCCTCCTCATTTTCAA
GSP2	65	28	3′ region	AACGCCTTTGATTTTGCAGCTGGACGAT
ccNAC1 F	58	20	NAC1 CDS	ATGGCCGCCGATTTGCAGTT
ccNAC1 R	56	30	NAC1 CDS	TCAAAAAGGCTTGTGAATATACATGAACAT
2.1-GSP2	64	24	5′ region	TTTTACAGAACTGGCCTTGCACGGAGAGA
2.1-GSP1	63	23	3′ region	TTCGGCTTCTCGTCTTCTTCCTCTTCGTAG
ccNAC2F	56	24	NAC2 CDS	ATGACCACCGAGTTGACTCAGCTG
ccNAC2R	58	21	NAC2 CDS	TCAGAACGGCTTCGGCAGGTG

eukaryotic polyadenylation signal 50-AATAAA-30 following the stop codon. The ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to detect ORFs and predict amino acid sequences. Primers described in Table 1 were used for cloning of the full length *CcNAC1* and *CcNAC2* genes. Con-F and Con-R were used for obtaining the conserved regions of *CcNAC1* and *CcNAC2*; GSP1 and GSP2 for the 5′ region of *CcNAC1*; 2.1-GSP1 and 2.1-GSP2 for 5′ region of CcNAC2; CcNAC1R/F and CcNAC2 F/R were used separately to clone the ORF of *CcNAC1* and ORF of *CcNAC2*.

Previously published plant NAC-like gene sequences were retrieved from the GenBank database: ATAF1 (X74755), AtNAC2 (AB049071), AtNAM (AF123311), CUC3 (AF54194), AtNAC3 (AB049070), ANAC (AY11722), ANAC019 (At1g52890), NAC1 (AF198054), CUC1 (AB049069), CUC2 (AB002560), TIP (AF281062),NAP (At1g69490), BnNAC5-11 (AY245884), BnNAC14 (AY245886), OsNAC19 (AY596808), OsNAC5 (AB028184), OsNAC4 (AB028183), OsNAC6 (AB028185), GmNAC8 (EU661911). Multiple sequence alignment of NAC proteins was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic analysis was based on neighbor-joining (NJ) using MEGA5 (Tamura et al. 2011) with 1,000 bootstrap replications. Jones Taylor Thornton (JTT) model was used as substitution model, while Gamma distribution with invariant sites (G + I) as range substitution pattern. Amino acid sequences of NAC genes with high homology to CcNAC1 and CcNAC2 (AEF80001, XP_004161162, AFY26893, ACS94038, XP_004149802, XP_004172335, ACS94038) were aligned to investigate conserved NAC domains.

Green fluorescent protein-conjugated plasmid construction

The cDNA was amplified with primers CcNAC1F/R and CcNAC2F/R (Table 1), and the resulting PCR product was fused into the pCR8/GW/TOPO entry vector. After a sequencing check, the insert was transferred into pMDC43 via the LR reaction (Gateway^R Entry vector, Life Technologies). The resulting plasmids containing Pro35S: CcNAC-green fluorescent protein (GFP) insert were used in electroporation experiments to determine the subcellular localization of *CcNAC*.

Protoplasts of *Arabidopsis* were isolated and transformed essentially as previously described in Sheen et al. (1999) with minor modifications. The tissues for protoplasts isolation were *Arabidopsis* leaves. The leaves were collected before flowering, excised and cut into 1 mm strips and immediately placed into an enzyme solution for overnight digestion in the dark. The enzyme solution which contained 2 % cellulose R10, 0.5 % macerozyme R10, 0.5 % driselase, 2.5 % KCl, 0.2 % $CaCl_2$, pH 5.7, was filter sterilized. After overnight incubation, leaf tissue was gently shaken for 30 min at 40 rpm to release protoplasts, followed by filtration through a 40 μm cell sifter to remove debris and centrifugation at 150 g to pellet the protoplasts. Protoplasts were washed twice with a washing solution (0.5 M mannitol, 4 mM MES pH 5.7, and 20 mM KCl) and re-centrifuged at 150*g*. The protoplasts were suspended in washing solution on ice for electroporation.

Protoplasts were transformed in a manner essentially as previously described (Sheen et al. 1999; Rashotte et al. 2006). Electroporation was typically carried out with 1–2 × 10^5 protoplasts in 200 μl of wash solution and about 1–2 μl 400 ng/μl of plasmid DNA. Protoplasts were electroporated at 300 V in a 0.1 mm cuvette using an Eppendorf Electroporator 2510 (Hauppauge, NY). After overnight incubation in the dark, protoplasts were examined under Accu-scope 3025 phase fluorescence microscope (New York Microscope Company, Inc.). A GFP filter was used to block the chlorophyll autofluorescence and a UV filter was used to detect Hoechst 33342 fluorescence under UV light. All photographs were taken with a Qimaging Fast 1394 digital camera (imaging).

Table 2 Primers used for transcription activation assay of CcNAC1 and CcNAC2

Primer	Sequence (5′–3′)	Product length (bp)	T_m (°C)
ccNAC1-N-F	CCGGAATCCATGGCCGCCGATTTGCAG	478	55
ccNAC1-N-R	ACGCGTCGACCGCCCTTCTTGTTGTATATACGG	478	
ccNAC1-C-F	CCGGAATCC GTAATCGAGAAACAGCAACAGC	421	50
ccNAC1-C-R	ACGCGTCGACATGACCACCGAGTTGACTCAGCTG	421	
ccNAC2-N-F	ACGCGTCGACATGACCACCGAGTTGACTCAGCTG	481	56
ccNAC2-N-R	AAAACTGCAG CGCCTTTCTTGTTGTAAATCCGG	481	
ccNAC2-C-F	ACGCGTCGACGTAATCGAGAAGCGAAATCAGATAGC	408	55.8
ccNAC2-C-R	AAAACTGCAG GAACGGCTTCTGCAGGTGCAT	408	

Transcriptional activation activity

The yeast strain YPG-2 containing *His3* and *LacZ* reporter genes was used as an assay system (Stratagene, La Jolla, CA, USA). The coding sequences of *ccNAC1* and *ccNAC2* and the *ccNAC1* and *ccNAC2* N-terminal and C-terminal fragments were obtained by PCR using primers described in Table 2. The PCR products were cloned into the vector containing the GAL4 DNA binding domain to obtain pBD-ccNAC1, pBD-ccNAC2, pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC2-N, pBD-ccNAC2-C. According to the protocol of the manufacturer (Stratagene), pBD-ccNAC1, pBD-ccNAC2, pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC2-N, pBD-ccNAC2-C and the positive control pGAL4 and the negative control pBD vector were all transformed into the yeast YPG-2 competent cells. PCR products were inserted into the *Sal*I-*Pst*I site of pDB vector containing CcNAC1/CcNAC2-F, -N, -C, respectively.

The transformed strains were confirmed by PCR and streaked on YPAD or SD/His-plates. The transcription activation activities of each protein were evaluated according to their growth status. The underlined nucleotide bases in Table 2 indicate restriction enzyme digestion sites.

Isolation of CcNAC1 and CcNAC2 promoters and in silico promoter analysis of CcNAC1 and CcNAC2 promoters

The promoters (1,585 and 1,299 bp) of CcNAC1 (KC814688) and CcNAC2 (KC814689) were obtained using the Genome walker universal kit (Clontech Cat NO.638904). Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), a database of plant *cis*-acting regulatory elements and a portal of tools for in silico analysis of promoter sequences (Lescot et al. 2002), was used to identify consensus motifs in the promoter sequences of *CcNAC1* and *CcNAC2*.

Abiotic treatments

Treatments were conducted on seedlings at the 5–6 leaf stage. Seedlings were placed in 20 % PEG8000 solution (−0.5 MPa osmotic potential) or 50 mM NaCl to induce drought or salt treatment or water as the control treatment. Hormone and hydrogen peroxide treatments were conducted on 14-day-old seedlings. Seedlings were treated with 100 μM MeJA, 200 μM ABA, 100 μM SA, 100 μm ethephon (ET), 20 mM H_2O_2, or 40 μM gibberellic acid (GA). The leaves were also wounded using a hemostat (wounding). Leaves were harvested following each treatment at specific time points: 0, 1, 2, 6, 12, and 24 h.

cDNA synthesis and relative quantitative (RQ) real-time RT-PCR

RNA was extracted from leaf material using the Trizol (Invitrogen Life Technologies, Grand Island, NY) method. To eliminate the remaining genomic DNA, RNA was treated with Dnase I (Ambion Life Technologies) according to the manufacturer's instruction. cDNA was synthesized using RETROscript™ (Ambion).

qRT-PCR was carried out using an Bio-Rad, iCycler Real Time PCR (Hercules, CA) system and iCycler detection system software. The *C. colocynthis*-specific actin gene (ccActin154F/R), used as the reference gene, was amplified in parallel with the target gene, allowing normalization of gene expression and providing quantification. Primers were designed based on specific regions. Primers sequences of the *CcNAC1* (Q-NAC1F/R), *CcNAC2* (Q-NAC2F/R) and Actin (ccActin154F/R) are listed in Table 3. Detection of RQ real-time RT-PCR products was conducted using the SYBR® Green PCR Master mix kit (Applied Biosystems, Life Technologies) following the manufacturer's recommendations. Quantification of the relative transcript levels was performed using the comparative C_T method. The induction ratio was calculated as recommended by the manufacturer and corresponds to $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (C_{T,\ \text{target gene}}, -C_{T,\ \text{actin}})_{\ \text{treatment}} - (_{CT,\ \text{Target}} -$

Table 3 Q-RT PCR primers for detection of relative expression levels of *CcNAC1* and *CcNAC2*

Primers	Sequence (5′–3′)	T_m (°C)	Product length (bp)
ccActin 154F	CACCATCACCAGAATCCAGCACGA	59	140
ccActin 154R	GGCTCCACTCAACCCAAAGGCTAAC	59	140
Q-NAC1F	GTCAACCGAGAATGAAAGAAGAGTA	59	132
Q-NAC1R	TATACATGAACATATCCTGCAATGG	59	132
Q-NAC2F	GTGCCGGATTTACAACAAGAA	59	106
Q-NAC2R	AATCTTCGGCTTCTCGCTTC	59	106

$C_{T,\ actin})_{control}$. Relative quantification relies on the comparison between expression of a target gene versus a reference gene and the expression of same gene in the target sample versus the reference sample (Pfaffl 2001).

Results

Cloning and sequence analysis of the CcNAC1 and CcNAC2 genes

Earlier research on gene expression changes in response to drought in *C. colocynthis* had identified a partial NAC transcript (GH626169), which showed high expression levels in shoots following drought (PEG) treatment and in response to different hormones such as ABA, JA, and SA (Si et al. 2009, 2010a). Following primer design based on conserved regions of NAC genes, two NAC genes from *C. colocynthis* were sequenced and cloned and designated as *CcNAC1* and *CcNAC2*. *CcNAC1* encodes a 900 bp (300 amino acids) long sequence. *CcNAC2* encodes an 888 bp (296 amino acids) long sequence. Amino acid alignment of CcNAC1, CcNAC2, and other NAC proteins was used to construct a phylogenetic tree (Fig. 1a). Phylogenetic analysis indicated that NAC proteins can be classified into several subgroups based on similarities to published NACs (Ooka et al. 2003). Major subgroups are shown in Fig. 1. Both CcNAC1 and CcNAC2 align with proteins in the ATAF subgroup composed of ATAF1, BnNAC5-11, GmNAC8, OsNAC19, OsNAC5, OsNAC4, OsNAC6, and BnNAC14. Amino acid alignment analysis (Fig. 1b) indicated that CcNAC1 and CcNAC2 show high homology to NACs with conserved A–E domains (five N-terminal subdomains). Even though the *CcNACs* are variable, especially at the C-terminal region, several conserved amino acid domains were detected.

CcNAC1 and CcNAC2 are localized to the nucleus

To identify the subcellular localization of the CcNACs, the following constructs were made: 35S::*CcNAC1*-GFP and 35S::*CcNAC2*-GFP. The constructs were used to transform *Arabidopsis* leaf protoplasts. Analysis of more than 20 protoplasts showed nuclear localization of the fusion protein of *CcNAC1* and *CcNAC2* with GFP, as illustrated in Fig. 2, whereas the GFP protein was distributed ubiquitously in protoplasts transformed with vector plasmid control, PMDC43 (Fig. 2a2). These results indicated that both *CcNAC1* and *CcNAC2* are nuclear proteins.

CcNAC1-C terminal has transcription activation function

CcNAC1, CcNAC2, their N-terminal domain and C-terminal domain, and full length coding sequence were fused to the GAL4 DNA binding domain to investigate their transcription activation activity. The yeast strain YRG-2 was transformed with the fusion plasmids pBD-ccNAC1, pBD-ccNAC2, pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC2-N, pBD-ccNAC2-C, the positive control pGAL4 and the negative control pBD. As shown in Fig. 3, all transformed cells can grow well on YPAD medium, but only pBD-ccNAC1-C can grow on SD medium without histidine. The filter lift assay showed that the yeast cells that grew on the SD medium without histidine turned blue in the presence of 5 bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) due to the activation of another reporter gene *LacZ*. The results indicate that only the C terminal region of CcNAC1 has transcription activation activity, while the full length and N-terminal region of CcNAC1 do not have this activity. This phenomenon was also observed in GmNAC20 where the C-terminal has transcriptional activation ability (Hao et al. 2010). *CcNAC2* did not show transcriptional activation activity using yeast assay, since blue color was not observed using the X-Gal assay (data not shown).

CcNAC1 and CcNAC2 expression patterns

Expression analysis of CcNAC1 and CcNAC2 was conducted using semi-quantitative real-time PCR. Actin, used as an internal control for constitutive expression, was

uniformly expressed in all organs. As shown in Fig. 4, *CcNAC1* and *CcNAC2* are expressed in every tissue of *C. colocynthis*. *CcNAC1* and *CcNAC2* showed the highest level of expression in male flowers, and low expression levels were detected in fruits. Comparisons between the two genes also indicated that *CcNAC2* is highly expressed in the hypocotyl of *C. colocynthis*.

In silico identification of stress-related promoter motifs

CcNAC1 and CcNAC2 promoters were isolated using the genome walking method. A 1,585 bp region upstream of the *CcNAC1* gene and a 1,298 bp region upstream of the *CcNAC2* gene were cloned, which should contain most of the regulatory domains. For further analysis of stress-related motifs in the two promoters, Plant CARE was used. Table 4 shows the details of stress-related motifs detected in the two promoters, with in the attachment the promoter sequences and motifs in color (Fig. 5). The *CcNAC1* promoter contains the ABA-response element (ABRE) (Yamaguchi-Shinozaka et al. 1989; Mundy et al. 1990; Michel et al. 1993; Giraudat et al. 1994; Barker et al. 1994), ARE motif (Manjunath and Sachs 2005), CE-3 (coupling element 3) (Hubo et al. 1999), CGTCA motif, TC-rich motif, TGA-box, and TGACG-motif, which are correlated with ABA response, anaerobic induction, JA response, and auxin response. The *CcNAC2* promoter contains an ABRE motif, ARE motif, HSE (heat stress responsiveness), MBS (MYB-binding site), Box-W1, and TC-rich repeats, which are correlated with ABA response, drought stress, anaerobic stress, JA response, and auxin response. Promoter motifs provide evidence for the involvement of *CcNAC1* and *CcNAC2* in biotic and abiotic stresses. The two promoters contain several identical motifs, such as ABRE, ARE, TC-rich repeats, which indicate that both promoters might have similar functions. However, some key differences in the composition or distribution of putative stress-related *cis*-acting elements (Fig. 5) were observed. Five ABRE motifs are in the *CcNAC1* promoter, but only two ABRE in the *CcNAC2* promoter. *CcNAC2* promoter contains three TC-rich repeats, while *CcNAC1* promoter contains only one TC-rich repeats. Both contain some special motifs, for example, *CcNAC1* contains CE3, CGTCA-motif and TGA-motif, whereas the *CcNAC2* promoter contains Box-W1, HSE, and MBS motifs. These special characteristics indicate that although both promoters have similar regulatory domains, they might be regulated by different factors. It can also be deduced that the CcNAC1 and CcNAC2 TFs might have differential regulation.

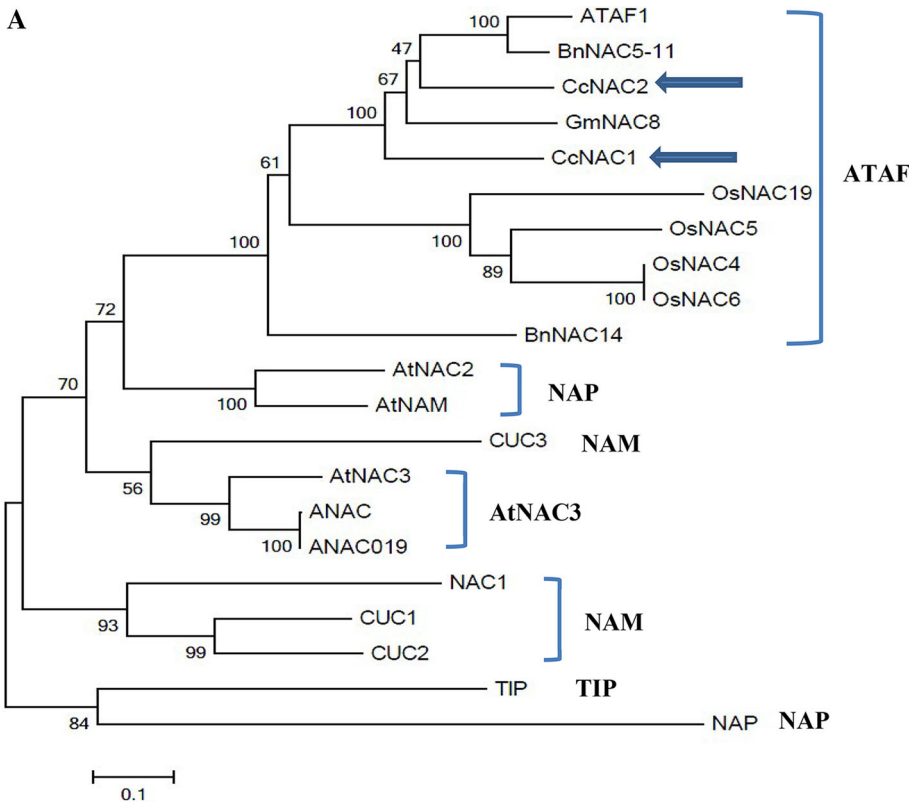

Fig. 1 **a** Phylogenetic tree of CcNAC1, CcNAC2 with other NACs proteins in plants. Numbers at the nodes of the trees represent the bootstrap vales for the node (100 replicates). CcNAC1and CcNAC2 proteins are indicated by *arrows*. **b** Analyses of amino acid sequences of *Cc NAC1* and *CcNAC2*. The five sub-domains (A–E) are *underlined by dashes*. A–E domains are conservative domains of NAC transcription factors

Fig. 1 continued

B

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 cNAC1          MAADL-QLPPGFRFHPTDDELVTHYLCRKCASQPISVPIIAEIDLYKYNP 49
CcNAC2          MTTELTQLPPGFRFHPTDEELVMHYLCRRCASQPIAVPIIAEIDLYKFDP 50
XP_004161162    MASDL-QLPPGFRFHPTDDELVTHYLCRKCASQPISVPIIAEIDLYKYNP 49
AEF80001        MTAEL-QLPPGFRFHPTDDELVMHYLCRKCASQSIAVPIIAEIDLYKYDP 49
AFY26893        MTAEL-PLPPGFRFHPTDEELVMHYLCRKCASQPIAVPIIAEIDLYKYDP 49
ACS94038        MASEL-QLPPGFRFHPTDEELVMHYLCRKCTSQPISVPIIAEIDLYKYDP 49
XP_004149802    MTTDLTQLPPGFRFHPTDEELVMHYLCRRCASQPIAVPIIAEIDLYKFDP 50
XP_004172335    MTTDLTQLPPGFRFHPTDEELVIHYLCRRCASQPIAVPIIAEIDLYKFDP 50
                *::::*  ***********:*** *****:*:**.*:***********::*
                                  A                 B
CcNAC1          WDLPEKALYGEKEWHFFSPRDRKYPNGSRPNRSAGSGYWKATGADKPIGR 99
CcNAC2          WDLPELALHGEKEWYFFSPRERKYPNGSRPNRAAGSGYWKATGADKPIGH 100
XP_004161162    WDLPERALYGEKEWYFFSPRDRKYPNGSRPNRSAGSGYWKATGADKPIGR 99
AEF80001        WELPRMALYGEKEWYFFSPRDRKYPNGSRPNRAAGTGYWKATGADKPIGH 99
AFY26893        WELPGMALYGEKEWYFFSPRDRKYPNGSRPNRAAGTGYWKATGADKPIGH 99
ACS94038        WDLPGMASYGEKEWYFFSPRDRKYPNGSRPNRAAGSGYWKATGADKPIGH 99
XP_004149802    WDLPELALHGEKEWYFFSPRERKYPNGSRPNRAAGSGYWKATGADKPIGH 100
XP_004172335    WDLPELALHGEKEWYFFSPRERKYPNGSRPNRAAGSGYWKATGADKPIGH 100
                *:**  * :*****:*****:***********:**:*************:
                                                C
CcNAC1          PKPVGIKKALVFYSGKAPKGEKTNWIMHEYRLADVDRSARKKNSLRLDDW 149
CcNAC2          PKPVGIKKALVFYAGKAPRGEKTNWIMHEYRLADVDRSARNKNNLKLDDW 150
XP_004161162    PKAVGIKKALXFYSGKAPKGEKTNWIMHEYRLADVDRSARKKNSLRLDDW 149
AEF80001        PKAMGIKKALVFYAGKAPKGEKTNWIMHEYRLADVDRSARKKNSLRLDDW 149
AFY26893        PKAVGIKKALVFYAGKAPRGEKTNWIMHEYRLADVDRSARKKNSLRLDDW 149
ACS94038        PKPVGIKKALVFYAGKAPKGDKTNWIMHEYRLADVDRSIRRKNSLRLDDW 149
XP_004149802    PKPVGIKKALVFYAGKAPRGEKTNWIMHEYRLADVDRSARNKNNLKLDDW 150
XP_004172335    PKPVGIKKALVFYAGKAPRGEKTNWIMHEYRLADVDRSARNKNNLKLDDW 150
                **.:****** **:****:*:***************** *.**.*:****
                                  D
CcNAC1          VLCRIYNKKGVIEKQQQLQNVSINRPEMNTIGFVENEEEEEKPEILNERA 199
CcNAC2          VLCRIYNKKGVIEKRNQIA---VNR-----NTNVYEEEEDEKPKIFTSRG 192
XP_004161162    VLCRIYNKKGAIEKQNPP---EMNT-----IGFFENEEQEEKPEILNDRA 191
AEF80001        VLCRIYNKKGTTEKQQQ-G---INL-----QKMSDSEMEDKKPENLTIGG 190
AFY26893        VLCRIYNKKGTIEKQQQQI---INR------RMNSFETEEKKPEILTNGG 190
ACS94038        VLCRIYNKKGTIEKQPNSG--VINR------KTDPSEIEDKKPEILTRGS 191
XP_004149802    VLCRIYNKKGVIEKRYQMA---MNR-----NTKGLEEVEEEKPKIFISGG 192
XP_004172335    VLCRIYNKKGVIEKRYQMA---MNR-----NTKGLEEVEEEKPKIFISGG 192
                **********. **:       :*            * :::**: :   .
                           E
CcNAC1          VSGR--IPSPS----PSQ--APPVNDYMYFDPSDSIPRLHADSSCSEHVV 241
CcNAC2          GADTL--IPAV----AAA--PASVDDYIHYDTSDSIPRLHTDSSCSEHVV 234
XP_004161162    ISGR--IPSASPLQGPPS--SGVVNDYVYFDPSDSIPRLHADSSCSEHVV 237
AEF80001        ATS-AILSGPA----ATR--TAAGDDYVYFDTSDSVPRLHTDSSCSEHVV 233
AFY26893        TTPAPLLPAPP----PSAVPKAAVNDYTYFDTSDSVPRLHTDSSCSEHVV 236
ACS94038        G--L--PPHPP----PQA--TAGMRDYMYFDTSDSIPKLHTDSSCSEHVV 231
XP_004149802    GDGGG--DVLM----PSA--TASVGDFVHFETSDSIPRLHTDSSGSEQVV 234
XP_004172335    GGGGG--DVLM----PSA--TASVGDFVHFETSDSIPRLHTDSSGSEQVV 234
                              .         *: :::.***:*:**:*** **:**

CcNAC1          SSEFT-SEVQSQPRMKEEY--SGFPYNYMDT-SLENAFCAQFPTLNQMSP 287
CcNAC2          SPEFTTGEVQSEPKWKELENAFNFQYNYIDNNSLDHEFATQFQSGNQMSP 284
XP_004161162    SSEFT-SEVQSEPRLKEEYCGLGFQYNYTDS-SLESAFCAQFPSLHQMSP 285
AEF80001        SPEFT-CEVQSEPKCW--EKTIDYHFNYMDA-TLDNGFGAQFQSSNQMSP 279
AFY26893        SPEFT-CEVQSEPKYW--EKPMDFSFNYMDA-TFDNGFGSQFQSNNQLSP 282
ACS94038        SPEFA-SEVQSEPKWNEWEKHLEFPYNYVDT-TLNSGFGSQFQSNNQMSP 279
XP_004149802    SPEFTTGEVESERKWKEMENAFSYRYNYIDDSLLDHEFATQFQNGNQMSP 284
XP_004172335    SPEFTTGEVESERKWKEMENAFSYRYNYIDDSLLDHEFATQFQNGNQMSP 284
                *.**:  **:*: :         : :** *   ::  * :** . :*:**

CcNAC1          LQDMFMYIHKPF 299
CcNAC2          LQDMFMHLQKPF 296
XP_004161162    LQDMFMY--KPF 295
AEF80001        LQDMFMYMQKPF 291
AFY26893        LQDMFMYLQKPF 294
ACS94038        LQDMFMYLPKTF 291
XP_004149802    LQDMFMYLQKPF 296
XP_004172335    LQDMFMYLQKPF 296
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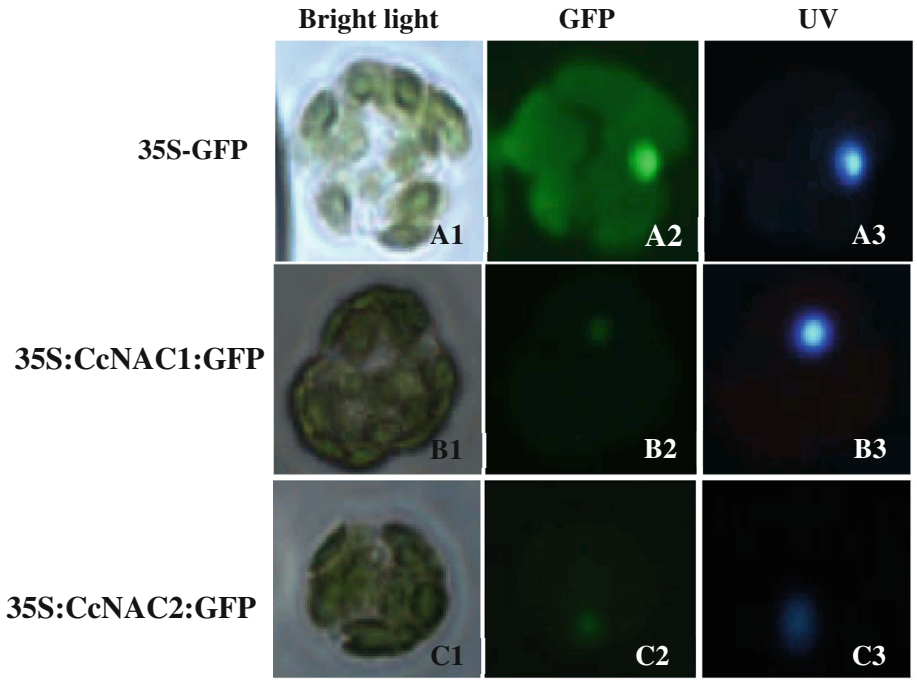


Fig. 2 Subcellular localization of the CcNAC1 and CcNAC2 protein in *Arabidopsis* protoplasts. A representative example of 35S:CcNAC1:GFP, and 35S: CcNAC2:GFP fusion in a leaf mesophyll protoplast of *Arabidopsis* are (*B1–B3* and *C1–C3*). The control (*A1*, *A2*, *A3*) protoplast was transformed with vector pMDC43. Protoplasts were visualized under white light (*A1*, *B1*, *C1*), GFP excitation (*A2*, *B2*, *C2* with filter blocking UV and other wavelengths) and UV light (*A3*, *B3*, *C3*)

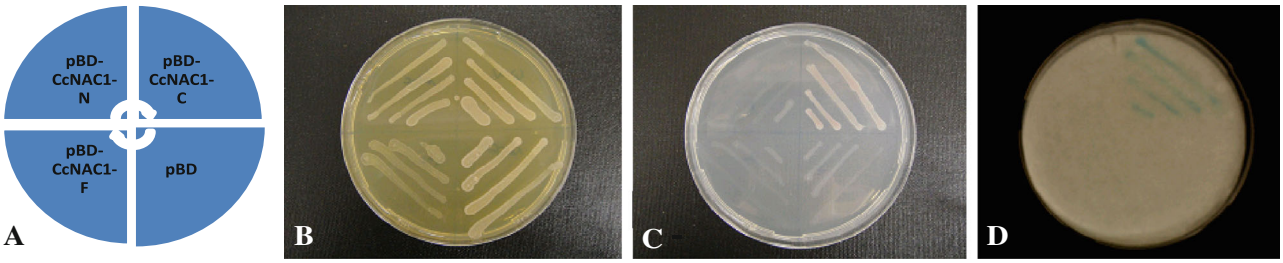


Fig. 3 Transactivation activity of CcNAC1. **a** Diagram of arrangement of transformants. **b** pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC1-F, and pBD transformants were streaked on YPAD medium. **c** pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC1-F, and pBD transformants were streaked on SD-His$^-$ medium. **d** Filter lift assay results of pBD-ccNAC1-N, pBD-ccNAC1-C, pBD-ccNAC1-F, and pBD transformants

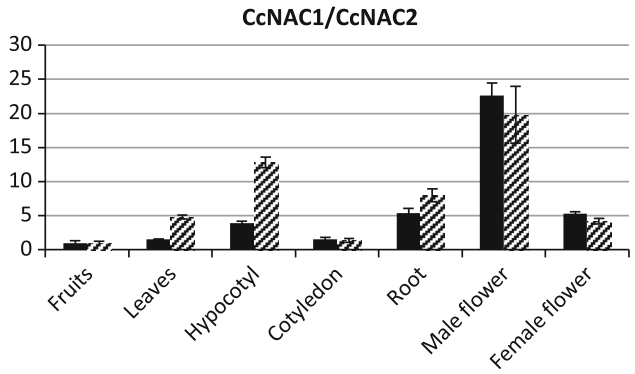


Fig. 4 *CcNAC1* and *CcNAC2* relative expression in different plant tissues and different stages as determined by qRT-PCR. Results are relative to expression in fruits. *Arrow bars* show SE ($n = 3$).CcNAC1 expression was shown as *solid black*, and CcNAC2 was shown as *gray dashed lines*

CcNAC gene expression during stress and plant hormone treatments

NAC TFs are known to play important roles in plant growth and development, and can be induced by multiple biotic and abiotic stresses (Wang and Dane 2013). The complex regulatory and interaction network occurring between hormone-signaling pathways allows the plant to activate responses to different types of stimuli (Bari and Jones 2009). *CcNAC1* and *CcNAC2* gene expression was studied under different stress (drought, wounding, and salt) and phytohormone (ABA, JA, SA, GA, ET) treatments as shown in Fig. 6. Results indicated that both genes, *CcNAC1* and *CcNAC2,* show similar expression patterns under hormonal and stress treatments. Both genes are induced by stress factors and hormones, even though dif-

Table 4 Details of stress-related elements in CcNAC1 promoter and CcNAC2 promoter

Name of *cis* element	Sequence	Number of *cis* elements	Function	Reference
CcNAC1 promoter				
ABRE	CCTACGTGGC/CGCACGTGTC/ GACACGTGGC/CACGTG/	5	ABA responsiveness	Barker et al. (1994)
ARE	TGGTTT	4	Anaerobic induction	Walker et al. (1987)
CE-3	GACGCGTGTC	1	ABA and VP1 (seed-specific transcription factor) responsiveness	Hubo et al. (1999)
CGTCA motifs	CGTCA	3	MeJA-responsiveness	Wang, et al. (2011)
TC-rich repeats	ATTTTCTCCA	1	Defense and stress responsiveness	Diaz-De Leon et al. (1993)
TGA-box	TGACGTAA	1	Auxin-responsive element	
TGACG-motif	TGACG	3	MeJA-responsiveness	Rouster et al. (1997)
CcNAC2 promoter				
ABRE	CACGTG/ACGTGGC	2	ABA responsiveness	Barker et al. (1994)
ARE	TGGTTT	3	Anaerobic induction	Walker et al. (1987)
Box-W1	TTGACC	1	Fungal elicitor responsive element	Shi et al. (2011)
HSE	AAAAAATTTC	1	Heat stress responsiveness	Schramm et al. (2006)
MBS	CAACTG	1	MYB binding site involved in drought-inducibility	Mongkolsiriwatana et al. (2009)
TC-rich repeats	GTTTTCTTAC/ATTTTCTTCA	3	Defense and stress responsiveness	Diaz-De Leon et al. (1993)

ferences in the timing of induction and level of expression were observed. Hormones can thus regulate CcNACs expression levels. For example, treatment with GA resulted in a 10-fold up-regulation of *CcNAC1* at 2 h, while much higher levels of *CcNAC2,* 40-fold increases, were observed at a later time point, 24 h. Similarly treatment with JA resulted in 30-fold up-regulation of *CcNAC1* at 24 h, while the highest levels of *CcNAC2* (40-fold) were detected following JA treatment at 12 h. Treatment with ET resulted in higher expression levels of *CcNAC2* than *CcNAC1*, although both showed the highest level at 6 h. The highest levels of *CcNAC1* were detected 10 h following ABA treatment, while *CcNAC2* showed the highest levels already at 2 h (40-fold). Treatment with SA resulted in 6–8-fold up-regulation of *CcNAC1* from 6 to 24 h, while *CcNAC2* was expressed from 1 to 12 h, and reached very high levels (40×).

Abiotic stresses do regulate *CcNAC*s expression. For example, H_2O_2 treatment did result in high expression of *CcNAC1,* more than 15-fold at 24 h, while it did result in up-regulation of *CcNAC2* more than 40-fold at 40 h. PEG treatment did not induce changes in expression of *CcNAC1*, but did cause high *CcNAC2* expression, 15–25-fold. The effect of salt treatment did effect the expression of *CcNAC2* more than *CcNAC1*; both showed similar patterns. Wounding resulted in high (40×) up-regulation of *CcNAC1* and *CcNAC2* after 2 h, and 24 h and similar expression patterns.

Discussion

In this study, two members of the NAC gene family in *C. colocynthis* were identified and NAC gene expression in different tissues and under different treatments was examined. This is the first report of molecular characterization of NAC genes in *C. colocynthis* The Cucurbitaceae is a large and diverse family containing several domesticated species such as watermelon, melon and cucumber (*Cucumis* species), squashes, pumpkins, and gourds (*Cucurbita* species). *C. colocynthis* is a source of genetic improvement for drought resistance, since this species is widely distributed in the Sahara-Arabian desert areas and well adapted to drought stress (Dane et al. 2006). Tolerance to drought stress is a complex phenomenon, comprising a number of physio-biochemical processes at both the cellular and whole plant level which are activated during different stages of plant development. Molecular mechanisms involved in different stresses have been revealed in other plant species, and TFs are one of the promising players in stress signaling pathways (Fujita et al. 2006).

The plant-specific NAC proteins constitute a major TF family implicated in many developmental processes (Puranik et al. 2012). Like most NACs, CcNAC1, and CcNAC2 contain conserved NAC domains. NAC domains are N-terminal regions of NACs that can bind both DNA and other proteins (Ernst et al. 2004). N-terminal amino

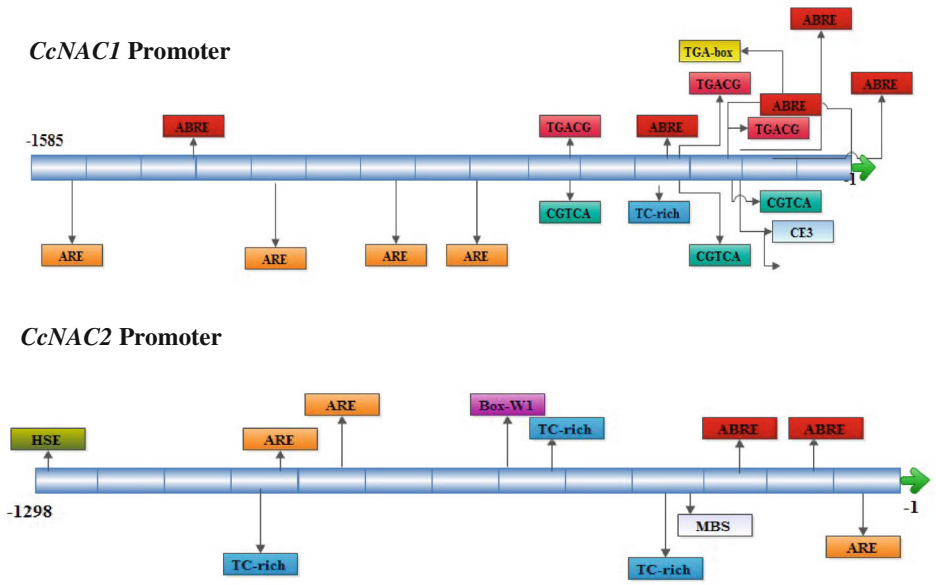


Fig. 5 Location of stress-related *cis*-regulatory elements in *CcNAC1* and *CcNAC2* promoters

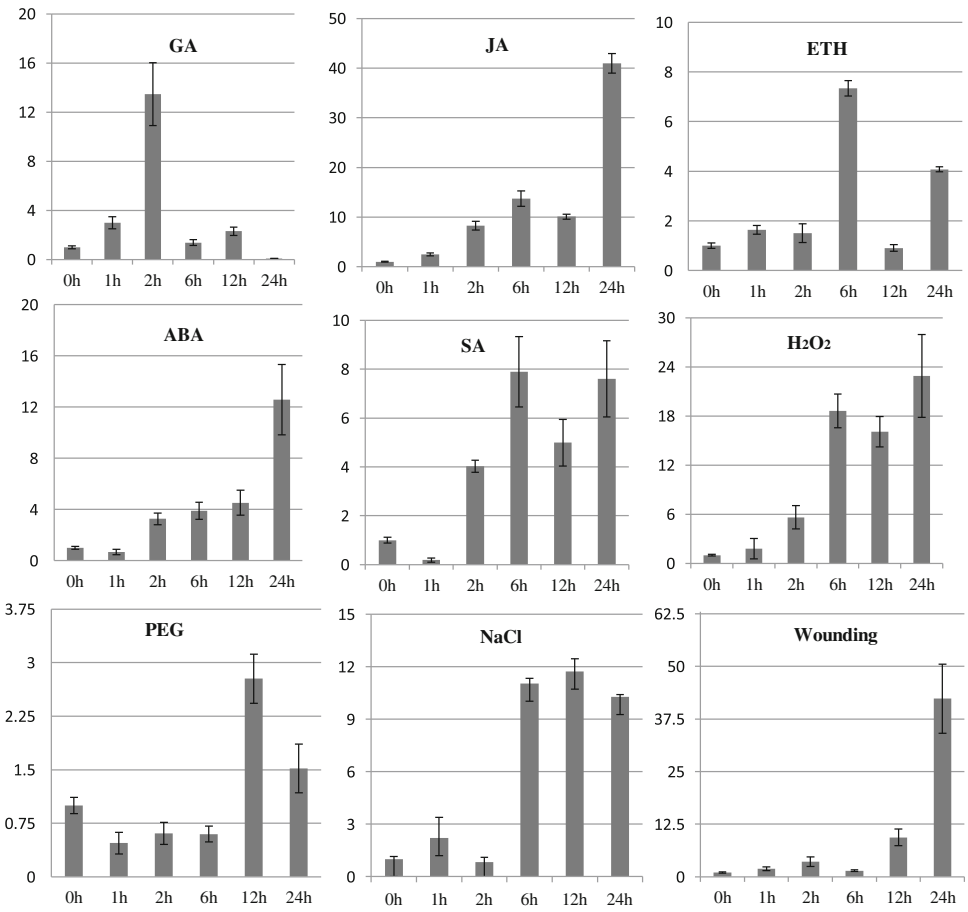
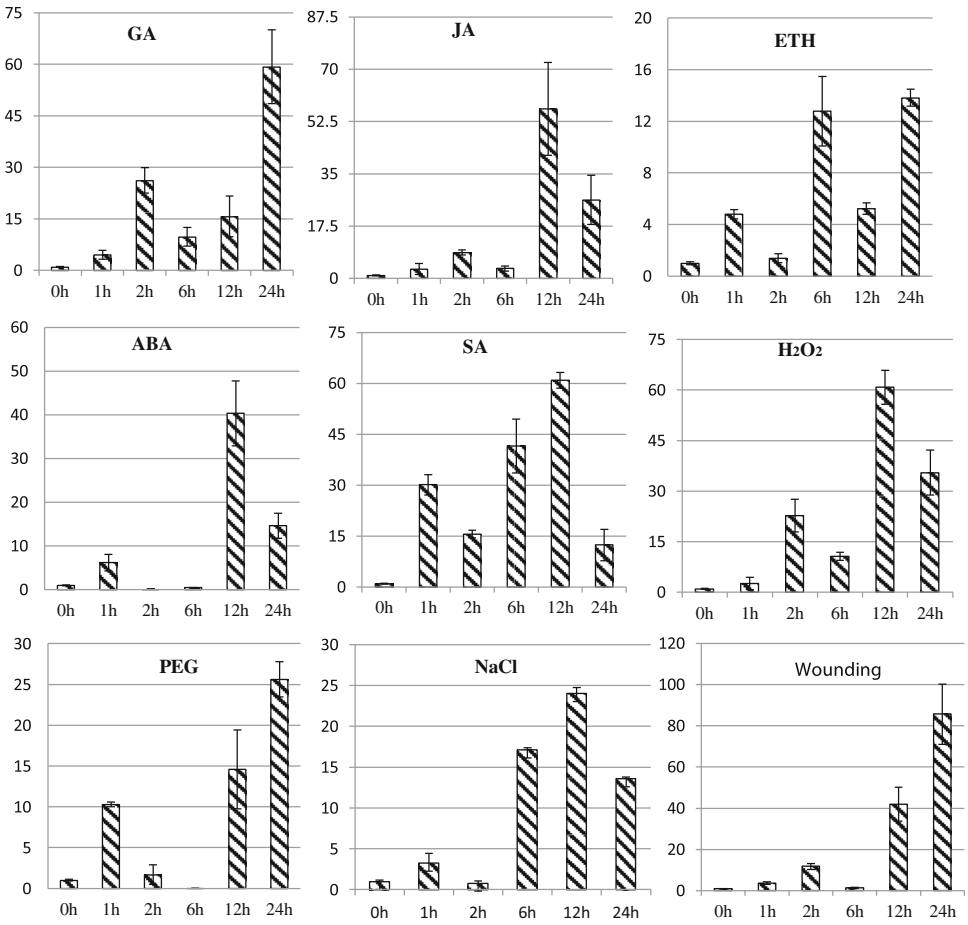


Fig. 6 *CcNAC1* and *CcNAC2* expression profiles under different treatments. The *gray* figures are expression patterns of *CcNAC1*, while the figures with *slanted lines* show expression patterns of *CcNAC2*. Gene expression was normalized by comparing ΔΔCT to control (0 h) ($n = 3$). *Y*-axis shows the expression level of *CcNAC1* and *CcNAC2*

Fig. 6 continued



acid substitutions can abolish NAC DNA-binding or structural integrity (Olsen et al. 2005). CcNAC1 and CcNAC2, like most other NACs, contain conserved NAC N-terminal and variable C-terminal domains. Protoplast transformation experiments indicated that both CcNAC1 and CcNAC2 are localized in the nucleus, which is where most TFs function. Research has indicated that C-terminal regions of many NACs possess trans-activation activity (He et al. 2005; Peng et al. 2009). Yeast assay experiments showed that CcNAC1 has trans-activation activity, while CcNAC2 does not have that ability. Some NACs have been reported to function as transcriptional repressors. Hao et al. (2010) reported that NARD (NAC Repression domain) contributed to the transcriptional repression function of GmNAC20, with the LVFY motif essentially required for suppression.

Examination of *CcNAC1* and *CcNAC2* expression in different plant tissues pointed to similar expression patterns, although both genes were expressed mainly in male flowers and the hypocotyl. Tissue-specific expression of members of the NAC gene family has also been studied in other species. For example, ATAF2, which is a pathogenesis-related gene in *Arabidopsis,* showed expression mainly in roots, leaves and mature flowers (Delessert et al. 2005). ANAC036 which caused a dwarf phenotype in *Arabidopsis thaliana,* was expressed mainly in rosette leaves (Kato et al. 2010). AtNAC2 was expressed mainly in root tissues and involved in salt stress responses and lateral root development (He et al. 2005). ATAF1 in *Arabidopsis* showed expression in every tissue, but mainly in stems, flowers and seedlings. Its overexpression resulted in severe developmental defects in *Arabidopsis* (Kleinow et al. 2009).

Phytohormone and stress treatments induced CcNAC1 and CcNAC2 expression to different levels. Since the promoters of both genes contain the anaerobic related motif ARE, this indicates that both genes might be correlated with oxidative stress. Salt and drought stress are worldwide problems, effecting global crop production and quality. Both genes were regulated by salt and drought stress, and a drought related motif was also detected in the *CcNAC2* promoter. Elevated levels of ABA, JA and anthocyanin are metabolic signatures of oxidative stress (Steppuhn et al. 2010). While the impact of the different treatments was similar on both genes, differences in gene expression were detected. NAC TFs are candidate

molecules that potentially regulate aspects of both biotic and abiotic signaling (Fujita et al. 2006). In signaling pathways, different hormones play different parts and crosstalk with each other. Earlier experiments had indicated that many NACs play a role in phytohormone pathways (Kim et al. 2008). NTL8, a membrane-bound NAC TF, plays a role in GA-mediated salt signaling in *Arabidopsis* (Kim et al. 2008). Studies of gene expression in wild-type and mutant *Arabidopsis* genotypes in response to pathogens revealed interactions among SA, JA, and ethylene (Maleck et al. 2000; Tao et al. 2003; Salzman et al. 2005). It has been suggested that ethylene produced during wounding can activate JA biosynthesis, and ethylene can also interact with the JA pathway to induce a number of pathogenesis-related and defense genes (Laudert and Weiler 1998; Kunkel and Brooks 2002). JA operates in a distinct defense pathway, which interacts with the SA pathway. JA is known to effectively mediate the defense of necrotrophic pathogens, while SA is effective against biotrophic fungi, bacteria and viruses (Murphy and Carr 2002). SA is thought to be antagonistic to JA, indicating that SA can block the JA induction pathway (Doares et al. 1995). Ethylene and JA are also associated with pathogen-induced wounding (Kunkel and Brooks 2002). JA is one of the main components of the wound repair signal in plant tissues, and the formation of JA is activated by ABA, ethylene, hydrogen peroxide, UV, whereas SA and nitric oxide inhibit the synthesis of JA (Vasyukova et al. 2011). Both JA and wounding did induce CcNAC1 and CcNAC2 expression, which indicates that both genes might play a role in the signaling of pathogen resistance and wounding response. Similar to AtNAC2, CcNAC1, and CcNAC2 were also up-regulated by ethylene. SA is a major component of the systemic acquired resistance (SAR) response, which refers to induced resistance to pathogens (Bostock 2005). *ANAC055, ANAC092,* and *GmNAC6* genes were identified as SA signaling components (Delessert et al. 2005; Faria et al. 2011). SA similarly induced changes in *CcNAC1* and *CcNAC2* expression, especially to a large degree in *CcNAC2* expression. The fungal elicitor motif Box-W1 was detected in the *CcNAC2* promoter, which is further evidence that *CcNAC2* has a function in biotic stress responses. JAZ (JASMONATE-ZIM DOMAIN) family proteins are JA co-receptors and transcriptional repressors in JA signaling in *Arabidopsis*. Research has indicated that JAZ orchestrates the crosstalk between JA and other hormone signaling pathways such as ethylene, gibberellic acid, SA and auxin (Kazan and Manners 2012). Both CcNAC1 and CcNAC2 were regulated by GA as well. It is known that some NACs act as regulators in several phytohormone pathways. AtNAC2 is a TF downstream of the ethylene and auxin signaling pathway (He et al. 2005).

In conclusion, two NAC TFs CcNAC1 and CcNAC2 were identified in *C. colocynthis*. Different stresses and phytohormones did induce *CcNAC1* and *CcNAC2* gene expression, which may provide clues for a better understanding of NAC gene family in this drought-tolerant cucurbit species. The identification of novel TFs regulating abiotic stress tolerance will enable further enhancement of stress tolerance in cultivated cucurbit species.

Author contribution Z. Wang designed, conducted the experiments and wrote the manuscript. A.G. Moss and A. Rashotte helped with the fluorescence microscopy experiments, and F. Dane supervised and edited the manuscript.

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