Tree Genetics & Genomes (2011) 7:1123–1134
DOI 10.1007/s11295-011-0400-8

ORIGINAL PAPER

Analysis of the *NAC* transcription factor gene family in citrus reveals a novel member involved in multiple abiotic stress responses

Tahise M. de Oliveira · Luciana C. Cidade · Abelmon S. Gesteira · Maurício A. Coelho Filho · Walter S. Soares Filho · Marcio G. C. Costa

Received: 15 October 2010 / Revised: 22 February 2011 / Accepted: 11 May 2011 / Published online: 8 June 2011
© Springer-Verlag 2011

Abstract The *NAC* (NAM, ATAF1, -2, and CUC2) gene family encodes a large family of plant-specific transcription factors that play diverse roles in plant development and stress regulation. In this study, we performed a survey of citrus NAC transcription factors in the HarvEST: Citrus database, in which 45 NAC domain-containing proteins were identified and phylogenetically classified into 13 different subfamilies. The results suggest the existence of a structurally diversified family of NAC transcription factors in citrus, which has not been previously characterized. One of these *NAC* genes, *CsNAC1* was found to be a member of the stress-NAC subfamily, whose homologs from other plant species function in pathways of environmental stress response and tolerance, and was further characterized. The CsNAC1 deduced protein was shown to contain the five N-terminal A through E NAC subdomains, a C-terminal region containing three transcriptional activation motifs, and a predicted NAC nuclear localization signal, consistent with its putative role as a NAC transcription factor. *In silico* analysis indicated that *CsNAC1* was primarily expressed in leaves and shoot meristems, and was involved in general stress responses. Quantitative real-time reverse transcription PCR analysis revealed that *CsNAC1* was strongly induced by drought stress in leaves of *Citrus reshni* and *Citrus limonia*, and also by salt stress, cold, and ABA in leaves and roots of *C. reshni*. Collectively, these results suggest that *CsNAC1* encodes a novel stress-responsive NAC transcription factor that is potentially useful for engineering tolerance to multiple abiotic stresses in citrus.

Keywords Plant-specific transfactors · Drought · Salt · Cold · Abscisic acid · Genome

Communicated by F. Gmitter

Electronic supplementary material The online version of this article (doi:10.1007/s11295-011-0400-8) contains supplementary material, which is available to authorized users.

T. M. de Oliveira · L. C. Cidade · M. G. C. Costa (✉)
Centro de Biotecnologia e Genética,
Departamento de Ciências Biológicas,
Universidade Estadual de Santa Cruz,
Rod. Ilhéus-Itabuna km 16,
Ilhéus, BA 45662-900, Brazil
e-mail: marciogc.costa@gmail.com

A. S. Gesteira · M. A. Coelho Filho · W. S. Soares Filho
Embrapa Mandioca e Fruticultura,
Cruz das Almas, BA 44380-000, Brazil

Introduction

Environmental stress conditions are major factors limiting growth, productivity, and the distribution of plants in the world (Boyer 1982). One third of the earth surface is classified as arid or semiarid, and in most of the humid regions where much of the world food is produced, the crops are subjected to periods of severe drought and extreme temperatures. Moreover, nearly 40% of the world land surface can be categorized as having potential high salinity problems (Boyer 1982). As a major horticultural crop worldwide, citrus production is not safe against these environmental threats. Citrus cultivation is mainly located in semiarid zones, where water supply is restricted, and primarily limited to the region between 40°N and 40°S latitudes, where low temperatures occasionally affect production. In addition, the use of low-quality water for irrigation has increased the salt concentration in citrus-producing areas (Storey and Walker 1999).

Responses to abiotic stresses require the production of important functional proteins, such as those involved in the synthesis of osmoprotectants, and regulatory proteins operating in the signal transduction pathways, such as kinases and transcription factors (TFs) (Saibo et al. 2009). Because most of these responses imply the control of gene expression, TFs play a critical role in the abiotic stress responses (Chaves and Oliveira 2004). At least four different TF families, designed according to their DNA-binding domain (Riechmann et al. 2000), were identified to be involved in the plant response to abiotic stresses: (1) CBF/DREB (C-repeat binding factor/DRE binding protein), (2) NAC (NAM, ATAF, and CUC) and ZF-HD (zinc-finger homeodomain), (3) AREB/ABF (ABA-responsive element-binding protein/ABA-binding factor), and (4) MYC (myelocytomatosis oncogene)/MYB (myeloblastosis oncogene). Some of these TFs are controlled by ABA, while others are not, indicating the involvement of both ABA-dependent and ABA-independent regulatory systems controlling stress-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki 2006).

NAC domain-containing proteins comprise one of the largest plant-specific TF families, with more than 100 members identified in both *Arabidopsis* and rice (Ooka et al. 2003; Fang et al. 2008), which are expressed in several tissues and developmental stages. Proteins of this family are characterized by a highly conserved N-terminal domain involved in DNA binding, known as NAC domain, and a C-terminal region highly divergent in sequence and length containing the transcriptional activation domain (Ooka et al. 2003). The NAC domain comprises about 160 amino acid residues, divided into five subdomains (A–E) of conserved blocks intercalated with heterogeneous blocks or gaps.

NACs play important roles in plant development, including the formation and maintenance of shoot apical meristems (Souer et al. 1996), floral development and morphogenesis (Sablowski and Meyerowitz 1998), embryo development (Duval et al. 2002), hormone signaling (Fujita et al. 2004), and formation of secondary walls (Zhong et al. 2006). NACs have also been implicated in the response to abiotic and biotic stresses, and some of them have been used as targets for engineering stress tolerance in plants. Several *NAC* genes from *Brassica napus* (Hegedus et al. 2003) and the *SsNAC23* gene from *Saccharum* sp. (Nogueira et al. 2005) were induced by cold, dehydration, and herbivorous insect attack. *OsNAC6* from *Oryza sativa* was induced by abscisic acid (ABA), cold, dehydration, and salt stress (Ohnishi et al. 2005). Transgenic rice plants overexpressing *OsNAC6* constitutively exhibited tolerance to dehydration, high-salt stress, and blast disease (Nakashima et al. 2007). *anac019*, *anac055*, and *anac072* from *Arabidopsis* were also induced by ABA, drought, and salt stress, and the transgenic lines overexpressing these genes displayed an increased tolerance to drought (Tran et al. 2004).

While a portion of the *NAC* genes has been primarily characterized in model plants such as *Arabidopsis* and rice, *NAC* genes remain poorly characterized in woody fruit species like citrus. Recently, Fan et al. (2007) reported for the first time the molecular cloning of a citrus *NAC*-like gene involved in the peel pitting stress response. This gene was induced under several postharvest abiotic stresses, including wounding, anoxia, low temperature, and ethylene. More recently, Liu et al. (2009) isolated another citrus *NAC*-like gene that was found to be involved in fruit development and senescence. In the present study, the identification of 45 citrus *NAC* genes belonging to 13 different subfamilies is reported as part of an effort to identify novel genes involved in abiotic stress responses, especially drought stress, in citrus. One of them, *CsNAC1*, was showed to be a member of the stress-NAC subfamily. Given its potential as target for engineering tolerance to environmental stresses in citrus, this gene was further characterized. It is suggested that citrus *CsNAC1* is involved in the response to multiple abiotic stresses and in ABA signaling.

Materials and methods

Plant materials and stress treatments

Seeds of 'Cleopatra' mandarin (*Citrus reshni* hort. ex Tanaka) and 'Rangpur' lime (*Citrus limonia* Osbeck) were collected at the Citrus Germplasm Collection of Embrapa Cassava & Fruits (Cruz das Almas, Bahia, Brazil). For the dry-down experiments (drought stress treatment), citrus seeds were germinated in plastic dibble tubes (115 cm^3 and 180 cm^3) containing a mixture of Plantmax (Eucatex Agro., Brazil) substrate and coconut fiber, at 1:1 proportion, under greenhouse conditions. Four months after germination, the plants were transplanted to 3-l pots containing a mixture of soil, sand, and Plantmax substrate, at 2:1:1 proportion. Eight months after germination, plants of nucellar origin were selected based on their uniformity and divided into two subsets: (1) a set of 10 plants of each genotype that were daily irrigated (well-watered plants—WW) and (2) a set of 10 plants of each genotype that were drought stressed (DS). Prior to the stress initiation, the pots of both treatments were saturated with water and left overnight to drain the excess water. On the next morning, all the pots were closed with aluminum foil in order to allow the water loss only by transpiration. The water content of the soil in each pot was thereafter measured with a time-domain reflectometry (TDR) probe. The normalized transpiration rate (NTR) was calculated for each DS plant by dividing their transpiration rate determined from the differences in

daily soil moisture by the mean transpiration rate of the WW plants (Wahbi and Sinclair 2007). The explored soil volume (Vs) and the total leaf area (m^2) of plants were taken into account in order to allow comparisons among plants of different sizes. The fraction of transpirable soil water (FTSW) was calculated for each pot on every day. This was performed by subtracting for each pot the lower limit soil moisture from the soil moisture measured each day, and dividing it by the total transpirable soil water for the pot (Sinclair and Ludlow 1986). Data collection for each plant was stopped when the NTR value decreased to less than 0.1. Leaf tissue samples were collected at different FTSWs, following the initiation of the experiments.

Values of NTR and FTSW obtained during the dry-down experiments for each plant on each day were all combined in order to obtain the NTR response curve as a function of FTSW (Wahbi and Sinclair 2007). A linear-plateau analysis was used to calculate the FTSW threshold value where NTR begins to decline. A plateau region, where NTR was approximately 1.0, was defined for the region where the soil medium was wet. The region of the linear decline in NTR as the soil dried further was fitted by linear regression. The intersection of the linear region with NTR=1.0 was calculated as the plateau intercept where drying induced a response in plant gas exchange. This model has been one that has been shown to fit well the response of plants grown on mineral soil (Wahbi and Sinclair 2007). All regressions were carried out using GraphPad Prism ver. 4.0 (GraphPad Software, USA).

For salt, cold, and ABA treatments, citrus seeds were germinated in vitro (Oliveira et al. 2009) and the seedlings were maintained at 27±2°C on a 16:8 h light/dark regime with a photon flux density of 250 μmol m^{-2} s^{-1}. Eight weeks after germination, seedlings of nucellar origin were selected based on their uniformity, divided into three subsets (six replicated plants), and transferred from the basal MS (Murashige and Skoog 1962) solution to the MS solution containing 200 mM NaCl or 100 μM ABA. For the cold treatment, seedlings were transferred to and kept at 4°C. Leaves and roots were harvested after 3, 6, and 12 h of treatment. Control plantlets were transferred to basal MS solution and kept at 27±2°C. All tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Identification and sequence analysis of NAC domain-containing proteins of citrus

The NAC domain-containing protein sequences of citrus were identified in the HarvEST: Citrus database, which contains more than 200,000 citrus expressed sequence tags (ESTs), using the NAC domain of proteins from *Arabidopsis thaliana* and *O. sativa* (Ooka et al. 2003) as driver sequences for searching. The set of non-redundant citrus assembled sequences with an *E* value $\leq 10^{-20}$ were considered to represent putative citrus NAC domain-containing genes. Only the sequences that contained at least four out of five conserved N-terminal NAC subdomains (A through E) were considered and used for the multiple alignments and phylogenetic analysis.

Multiple sequence alignments were performed using the default parameters of ClustalW (Thompson et al. 1994) and the dendrogram was constructed by the MEGA4 program (Tamura et al. 2007) that used the neighbor-joining method (Saitou and Nei 1987) and bootstrap re-sampling analysis (1,000 replications). The identification of the sequences used is provided by the accession number in the corresponding figure.

Digital gene expression profiling

Digital gene expression profile of the *CsNAC1* gene was calculated as previously reported (Chen et al. 2010). Briefly, tissue specificity and treatment were summarized, respectively, from the tissue sources and treatments of all the 89 EST libraries contained in the HarvEST: Citrus database. Approximate expression intensity in each tissue/treatment was inferred from the ratio of the count of CsNAC1 ESTs to the total number of clones in non-normalized EST libraries of the same tissue/treatment. For comparison, the ratio was converted into transcripts per million (TPM) in each tissue/treatment. The ESTs from those normalized, subtracted, or otherwise biased libraries were not counted or used for TPM calculation.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from leaf and root tissues with the RNAqueous® kit (Applied Biosystems, USA), treated with 100 U of RNase-free DNase (Amersham Pharmacia Biotech, USA) and further purified with RNeasy Mini kit (Qiagen, USA). The purity and concentration of the isolated RNA samples were checked on 1% (w/v) agarose gels and in a spectrophotometer. Reverse transcription was carried out using the RETROscript kit (Ambion, USA) and 2 μg of total RNA, according to the manufacturer's instructions. The cDNA concentration was determined spectrophotometrically.

Quantitative real-time reverse transcription PCR (qPCR)

All the procedures for qPCR, including tests, validations, and experiments, were carried out according to the recommendations of Applied Biosystems. The qPCR reactions were performed on an ABI Prism 7500 instrument and Sequence Detection System software, version 1.6.3 (Applied Biosystems). Based on the expression stability

observed under the experimental conditions analyzed, actin was amplified along with the target gene as an endogenous control to normalize expression among different samples. The control primers were: forward 5′-CATCCCTCAG-CACCTTCC-3′ and reverse 5′-CCAACCTTAG-CACTTCTCC-3′. The primers for the *CsNAC1* gene were: forward 5′-GGCACCGACAAGATTATCACAA-3′ and reverse 5′-GCTGCCATTTTTGCGAGAAG-3′. Reactions were performed in triplicate, containing 100 ng of cDNA, 0.4 μl of each primer (5 pmol), 10 μl Power SYBR Green Master Mix (Applied Biosystems), and sterile Milli-Q water for a final volume of 25 μl. The amplification reactions were performed under the following conditions: (1) activation of Taq DNA polymerase at 50°C for 2 min, (2) initial denaturation at 95°C for 10 min, (3) denaturation at 95°C for 15 s, (4) annealing at 60°C for 30 s, and (5) extension at 60°C for 1 min. Steps 3–5 were repeated for 40 cycles. To verify that only a single PCR product was generated for the amplified transcript, the multicomponent data for each sample was subsequently analyzed using the Dissociation Curve 1.0 program (Applied Biosystems). Gene expression was quantified using the comparative methods Ct: $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$, with data obtained from a pool of at least three biological replicates that were individually validated.

Results

Identification of citrus *NAC* genes and sequence analysis

NAC domain protein sequences from *A. thaliana* and *O. sativa* were used as queries to identify citrus NAC domain-containing sequences encoded by the assembly of 229,570 ESTs of *Citrus* and *Poncirus* available at the HarvEST: Citrus database. Forty-five open reading frames (ORFs) encoding proteins with at least four out of five conserved NAC subdomains (A through E) were identified in this study and sequentially named as *Citrus* sp. NACs (CsNACs). These included sequences from both *Citrus* and *Poncirus*. Each gene was further confirmed to contain a NAC domain through the BLASTP program against the non-redundant database. The CsNAC proteins identified in the present study are listed in Table 1. To our knowledge, this is the first report describing the NAC gene family in citrus.

The deduced amino acid sequences of the identified CsNACs [see electronic supplementary material (ESM) S1], including the two previously characterized CsNACs together with their respective orthologs from other plant species, were aligned for sequence comparison and phylogenetic analysis (Fig. 1). The phylogenetic analysis classified the CsNACs into 13 different subfamilies with an internal stability supported by bootstrapping testing. These results indicate the existence of a diversified family of NAC domain-containing protein in citrus, with distinct roles in stress response and/or development. One of these proteins, CsNAC1, showed to be a member of the stress-NAC subfamily. This group also encompasses GhNAC4 from cotton, GmNAC3 and GmNAC4 from soybean, and anac019, anac055, and anac072 from *Arabidopsis*, which have been shown to be induced by ABA, drought, cold, and salt stresses and to enhance tolerance to drought stress in transgenic plants (Tran et al. 2004, 2009; Meng et al. 2009; Pinheiro et al. 2009).

Since members of the stress-NAC subfamily are involved in general responses to abiotic stresses and, thereby, they may constitute potential targets for engineering plant tolerance to abiotic stresses, we have selected the *CsNAC1* gene for further characterization. The *CsNAC1* gene contains a 1,044-bp ORF encoding a 347 amino acid protein (38.8 kDa) that shared 76% identity and 84% similarity at the amino acid level to GhNAC4 from cotton (*Gossypium hirsutum*). Sequence analysis of the deduced CsNAC1 protein showed that it contains features typical of known NAC domain proteins, including the five N-terminal A through E NAC subdomains (amino acid 14–164) and a C-terminal region containing three transcriptional activation motifs according to Ooka et al. (2003) (Fig. 2a). Sequence alignment of NAC domains from different plant species showed that CsNAC1 contains all the conserved residues from A through E subdomains present in other plants (Fig. 2b). A degenerate bipartite nuclear localization signal (NLS) conserved in several NAC domain proteins among different plant species (Greve et al. 2003) was identified between the amino acids 113–131 (Fig. 2b).

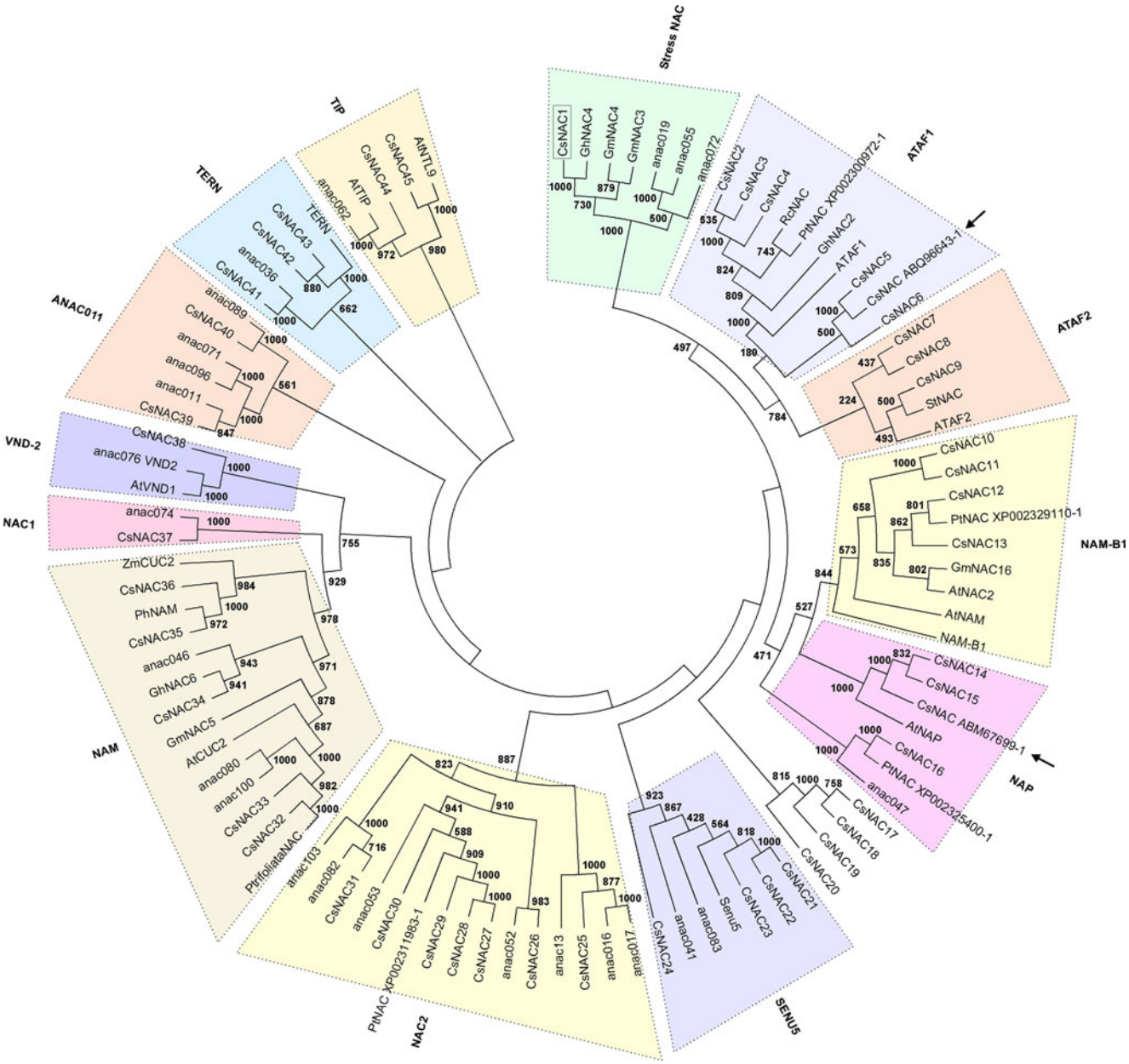
Digital gene expression profile of *CsNAC1*

The inferred transcripts per million (TPM) in each tissue or stress treatment provided a comparable basis to reflect the tendency of the *CsNAC1* expression, though not representing the true-to-type expression level. CsNAC1 ESTs were distributed among 1,897 non-normalized EST clones of shoot meristem (one library), 1,950 (three libraries) of leaves, 10,922 (four libraries) of floral organs, and 24,463 (12 libraries) of fruits, obtained from different *Citrus* species (Table 2). Leaves (2,051.3 TPM) and shoot meristems (1,581.4 TPM) were the tissues where *CsNAC1* was more highly expressed (Table 2). However, it is noteworthy that the absence of CsNAC1 ESTs in any other tissue does not necessarily imply that they are not expressed in those tissues, but due to the lack of non-normalized libraries from other tissue sources that could be considered for TPM calculation.

CsNAC1 ESTs were distributed in libraries of different *Citrus* species subjected to seven distinct stress treatments, including cold, salt, and iron deficiency (Table 3). TPM calculation indicated that *CsNAC1* was highly expressed

Table 1 CsNAC proteins identified in the HarvEST: Citrus database

Name	HarvEST: Citrus ID	GenBank accession no.	Subfamily	Best BLAST and GenBank accession no.	*E* value
CsNAC1	C18015B04Rv	CX306190	Stress NAC	NAC4 [*Gossypium hirsutum*] ACI15345.1	9e−145
CsNAC2	CGF1004750_D03	CK939458	ATAF1	NAC domain protein [*Populus trichocarpa*] XP_002300972.1	6e−134
CsNAC3	USDA-FP/ARO_12035	DN797167	ATAF1	NAC domain protein [*Populus trichocarpa*] XP_002300972.1	9e−134
CsNAC4	UCRPT01_0010L22_f	CV708062	ATAF1	NAC domain protein [*Populus trichocarpa*] XP_002300972.1	3e−131
CsNAC5	IC0AAA24CE04RM1	DY268591	ATAF1	NAC domain protein [*Citrus sinensis*] ABQ96643.1	0.0
CsNAC6	IC0AAA79BE01RM1	DY291872	ATAF1	NAC domain protein [*Citrus sinensis*] ABQ96643.1	1e−65
CsNAC7	IC0AAA84AA06RM1	DY293874	ATAF2	ATAF2 [*Arabidopsis thaliana*] NP_680161	8e−84
CsNAC8	UFPtC01_101	CX065999	ATAF2	ATAF2 [*Arabidopsis thaliana*] NP_680161	3e−48
CsNAC9	IC0AAA36CG09RM1	DY274441	ATAF2	NAC domain protein [*Solanum tuberosum*] CAC42087.1	2e−65
CsNAC10	IC0AAA29CF03RM1	DY271536	NAM-B1	ATNAC2 [*Arabidopsis thaliana*] NP_188170.1	4e−98
CsNAC11	IC0AAA17AE01RM1	DY265372	NAM-B1	ATNAC2 [*Arabidopsis thaliana*] NP_188170.1	5e−81
CsNAC12	UCRCS06_0005G18_r	CN191564	NAM-B1	NAC domain protein [*Populus trichocarpa*] XP_002329110.1	3e−89
CsNAC13	IC0AAA80DD04RM1	DY292748	NAM-B1	NAC domain protein [*Populus trichocarpa*] XP_002329110.1	1e−86
CsNAC14	CGF1004443_H04	CK940145	NAP	NAC domain protein [*Citrus sinensis*] ABM67699.1	2e−151
CsNAC15	CGF1004510_D11	CK937389	NAP	NAC domain protein [*Citrus sinensis*] ABM67699.1	5e−147
CsNAC16	IC0AAA17CD04RM1	DY265530	NAP	NAC domain protein [*Populus trichocarpa*] XP_002325400.1	3e−96
CsNAC17	C05075E10SK	CX295755	–	NAC4 [*Gossypium hirsutum*] ACI15345.1	5e−30
CsNAC18	IC0AAA36BC05RM2	DY274299	–	NAC domain protein [*Ricinus communis*] XP_002532683.1	3e−26
CsNAC19	CGF1004387_E05	CK936050	–	NAC domain protein [*Citrus sinensis*] ABM67699.1	1e−49
CsNAC20	USDA-FP/ARO_14380	DN958550	–	NAC domain protein [*Populus trichocarpa*] XP_002300866.1	8e−71
CsNAC21	UCRPT01_03cc08_g3	CD576134	SENU5	SENU5 [*Solanum lycopersicum*] CAA99760.1	7e−107
CsNAC22	UCRCS11_01M19_r	DN617870	SENU5	SENU5 [*Solanum lycopersicum*] CAA99760.1	3e−81
CsNAC23	C16016D11SK	CX304855	SENU5	SENU5 [*Solanum lycopersicum*] CAA99760.1	4e−80
CsNAC24	Volcani_7_03_C2	EX446934	SENU5	anac041 [*Arabidopsis thaliana*] NP_001118435.1	4e−59
CsNAC25	IC0AAA21AH06RM1	DY267350	NAC2	anac017 [*Arabidopsis thaliana*] NP_564440.1	1e−74
CsNAC26	Volcani_7_16_H1	EX446238	NAC2	anac052 [*Arabidopsis thaliana*] NP_566375.1	5e−88
CsNAC27	KN0AAP13YB13FM1	DY260614	NAC2	NAC domain protein [*Populus trichocarpa*] XP_002311983.1	5e−134
CsNAC28	UCRCS11_05L16_r	DN620727	NAC2	NAC domain protein [*Populus trichocarpa*] XP_002311983.1	3e−86
CsNAC29	UCRPT01_5_020_E09_T3	CX545227	NAC2	NAC domain protein [*Populus trichocarpa*] XP_002311983.1	1e−66
CsNAC30	UCRCS08_0008H13_f	CV717146	NAC2	NAC domain protein [*Populus trichocarpa*] XP_002311983.1	2e−61
CsNAC31	UCRCS01_01de02_g1	CB290772	NAC2	anac082 [*Arabidopsis thaliana*] NP_196495.1	6e−77
CsNAC32	UCRCS04_2_029D05_T3	CV887139	NAM	NAC domain protein [*Poncirus trifoliata*] FJ619349.1	4e−168
CsNAC33	KN0AAH3AC06RM1	DY305241	NAM	NAC domain protein [*Poncirus trifoliata*] FJ619349.1	3e−53
CsNAC34	C05068G11SK	CX295122	NAM	NAC6 [*Gossypium hirsutum*] ACI15343.1	3e−77
CsNAC35	IC0AAA91DC06RM1	DY297096	NAM	NAM [*Petunia × hybrida*] CAA63101.1	6e−84
CsNAC36	IC0AAA98BA03RM1	DY299386	NAM	CUC2 [*Zea mays*] ACG42560.1	2e−70
CsNAC37	wca24e12	–	NAC1	anac074 [*Arabidopsis thaliana*] NP_567811.1	5e−67
CsNAC38	IC0AAA72AA09RM1	DY289288	VND-2	VND2 [*Arabidopsis thaliana*] NP_195339.1	4e−82
CsNAC39	UCRCP01_038_H05_T7	CX668643	ANAC011	anac071 [*Arabidopsis thaliana*] NP_193532.1	2e−66
CsNAC40	wcb12a05	–	ANAC011	anac089 [*Arabidopsis thaliana*] NP_568414.1	2e−71
CsNAC41	USDA-FP_121000-242	CF505747	TERN	anac036 [*Arabidopsis thaliana*] NP_565404.2	2e−85
CsNAC42	CGF1004388_H10	DY270729	TERN	TERN [*Nicotiana tabacum*] BAA78417.1	3e−81
CsNAC43	IC0AAA81AD10RM1	DY292843	TERN	TERN [*Nicotiana tabacum*] BAA78417.1	5e−80
CsNAC44	IC0AAA1CE09RM1	DY266727	TIP	TIP [*Arabidopsis thaliana*] NP_197847.3	1e−71
CsNAC45	KN0AAQ7YL07RM1	DY304096	TIP	NTL9 [*Arabidopsis thaliana*] NP_001119122.1	2e−96



under salt (2,656 TPM), iron deficiency (1,763.7 TPM), exocortis (1,587.3 TPM), and cold (1,307.8 TPM) treatments. Gibberellic acid (956.9 TPM), hot water (351.2 TPM), and thrips (541.7 TPM) were also treatments in which *CsNAC1* was expressed. Thus, the digital expression data suggest an involvement of *CsNAC1* in general stress responses, providing useful information for further experimental validation.

The *CsNAC1* gene is induced by drought stress

To investigate whether the *CsNAC1* gene is induced by drought stress, plants of two important citrus rootstocks (*C. reshni* and *C. limonia*) were subjected to dry-down experiments, which mimic the different phases that a plant experiences under drought in natural conditions, and gene expression was monitored by qPCR. The dry-down experiments showed that the onset of transpiration decline occurred at FTSW of 0.31 for *C. reshni* and 0.36 for *C. limonia*, as indicated by the linear-plateau model (Fig. 3a). These FTSW values represent the transition from stage I to stage II dehydration according to Sinclair and Ludlow (1986). The results of the qPCR analysis revealed that *CsNAC1* expression was significantly induced by drought stress in both citrus rootstocks (Fig. 3b). *CsNAC1* expression was induced in leaves at an early stage of soil drying (FTSW of 0.6), and enhanced and maintained following further soil drying (FTSWs of 0.5–0.2). It is interesting to note that the induction of *CsNAC1* expression in both citrus

Fig. 1 Phylogenetic tree of NAC domain-containing proteins from citrus. Amino acid sequences were aligned using ClustalW and a neighbor-joining tree was constructed with a 1,000-bootstrap replication support. The subfamilies within the NAC family, as designated by Ooka et al. (2003), are grouped as indicated. The NAC domain-containing proteins of citrus are identified as listed in Table 1. CsNAC1 protein is highlighted by *box*. *Arrows* indicate the previously described CsNAC proteins of citrus. Abbreviations for the name of the subfamilies are as follows—*stress NAC* stress NAC transcription factor family, *ATAF1 Arabidopsis* transcription factor 1-like family, *ATAF2 Arabidopsis* transcription factor 2-like family, *NAM-B1* no apical meristem B1 transcription factor-like family, *NAP* NAC-like activated by APETALA 3/PISTILLATA family, *SENU5* tomato senescence up-regulated 5-like family, *NAC2 Arabidopsis thaliana* NAC protein 2-like family, *NAM* no apical meristem transcription factor-like family, *NAC1 Arabidopsis thaliana* NAC protein 1-like family, *VND-2* vascular-related NAC domain 2 family, *ANAC011 Arabidopsis* NAC protein 011 transcription factor-like family, *TERN* tobacco elicitor-responsive NAC protein-like family, *TIP* turnip crinkle virus interacting protein-like family. Accession numbers of NAC sequences from other plant species used in the analysis are in *parentheses*. anac011 (NP_174529.2); anac013 (NP_001117401.1); anac016 (NP_564439.1); anac017 (NP_564440.1); anac019 (NP_175697.1); anac036 (NP_565404.2); anac041 (NP_001118435.1); anac046 (NP_187056.1); anac052 (NP_566375.1); anac053 (NP_566376.1); anac055 (NP_188169.1); anac062 (NP_190522.1); anac071 (NP_193532.1); anac072 (NP_001078452.1); anac074 (NP_567811.1); anac076 (NP_195339.1); anac080 (NP_568182.2); anac082 (NP_196495.1); anac083 (NP_196822.1); anac089 (NP_568414.1); anac096 (NP_199471.1); anac100 (NP_200951.1); anac103 (NP_201211.1); ATAF1 (NP_171677); ATAF2 (NP_680161); AtCUC2 (BAA19529.1); AtNAC2 (NP_188170.1); AtNAM (AAM65392.1); AtNAP (NP_564966.1); AtNTL9 (NP_001119122.1); AtTIP (NP_197847.3); AtVND1 (NP_179397.1); CsNAC (ABM67699.1; ABQ96643.1); GhNAC2 (ACI15342.1); GhNAC4 (ACI15345.1); GhNAC6 (ACI15343.1); GmNAC3 (AAX85980.1); GmNAC4 (AAX85981.1); GmNAC5 (AAX85982.1); GmNAC16 (ACD39374.1); NAM-B1 (ABI15896 .1); PhNAM (CAA63101.1); PtNAC (XP002300972.1; XP002311983.1; XP002325400.1; XP002329110.1); PtrifoliataNAC (ACM90162.1); RcNAC (XP_002520341.1); Senu5 (CAA99760.1); StNAC (CAC42087.1); TERN (BAA78417.1); ZmCUC2 (ACG42560.1)

rootstocks has occurred prior to the transition between the dehydration stages I and II. It suggests a modulation of *CsNAC1* expression by early signal(s) triggered by the initial decline in the soil water potential, before the drought stress took place.

The *CsNAC1* gene is induced by salt, cold, and ABA

We further analyzed the effects of salt stress, cold, and ABA, a hormone involved in abiotic stress signaling (Mahajan and Tuteja 2005), on the expression of the *CsNAC1* gene by qPCR analysis. *C. reshni* was selected for these assays based on the high level of *CsNAC1* expression (Fig. 3b). Figure 4 shows that the *CsNAC1* gene was strongly induced within 3, 6, and/or 12 h after salt, cold, and ABA treatments in both leaves and roots of *C. reshni*. Thus, these results also suggest the involvement of *CsNAC1* in salt and cold stress response pathways, in a regulation that may be dependent on ABA. Taken together, the results indicate that *CsNAC1* is involved in general responses to abiotic stresses, as it is induced by drought stress, salt, cold, and ABA, and therefore may constitute a potential target for engineering tolerance to multiple abiotic stresses in citrus.

Discussion

The diversified NAC domain-containing protein family of citrus

Although several members of the *NAC* domain gene family have been characterized in *Arabidopsis* and rice (*O. sativa*), the characterization of *NAC* genes in woody fruit species remains scarce. Aiming to identify novel genes involved in abiotic stress responses in citrus, we carried out a comprehensive analysis that has identified 45 members of the NAC citrus family using *Arabidopsis* and rice sequences as drivers to screen the HarvEST: Citrus database. This number, however, can be considered a conservative estimate for the number of *NAC* domain genes in citrus because we did not include in the analysis assembled contig sets or singlets containing less than four out of five conserved NAC subdomains (A through E). Only 26 members of the NAC citrus family contained the five conserved NAC subdomains, which is in accordance with the number of full-length *NAC* domain genes reported for *Citrus sinensis* in the Plant Transcription Factor Database (PlantTFDB 2.0; http://planttfdb.cbi.pku.edu.cn/family.php?sp=Csi&fam=NAC).

The phylogenetic analysis placed the 45 identified citrus NAC-deduced proteins into 13 different subfamilies according to the Ooka et al. (2003) classification. Members of the different subfamilies have been implicated in functionally diverse roles, including response to abiotic stresses, defense against biotic stresses, and regulation of developmental programs (Olsen et al. 2005). Members of the stress-NAC subfamily have been demonstrated to be induced by ABA, drought, and salt stress (Tran et al. 2004, 2009; Meng et al. 2009; Pinheiro et al. 2009), to function as positive regulators in the drought stress response (Tran et al. 2004), and to increase drought tolerance in transgenic plants (Tran et al. 2004). ATAF1 and ATAF2 clades encompass proteins that are strongly induced by wounding (Collinge and Boller 2001; Delessert et al. 2004) and associated with response to several pathogens (Hauck et al. 2003). TERN and TIP members have been also associated with defense responses against pathogens (Ren et al. 2000). NAM-B1 has been shown to stimulate leaf senescence and increase the remobilization of nutrients from the leaves to the developing grains in wheat (Uauy et al. 2006). NAP and SENU5 members have been demonstrated to be also involved in leaf senescence (Guo and

Gan 2006). Members of the NAM subfamily are involved in embryogenesis and shoot apical meristem formation and development (Souer et al. 1996; Olsen et al. 2005), while NAC1 and NAC2 of *Arabidopsis* are involved in the regulation of lateral root development and auxin signaling (Xie et al. 2000; He et al. 2005).

The results suggest that the 45 citrus NAC proteins described may constitute functionally non-redundant transcription factors, which could be involved in the response to distinct types of stresses and/or events of development and morphogenesis. This functional diversity is supported by the recent observations that sequence similarity-based clustering of the members of the NAC superfamily correlates with their function (Olsen et al. 2005; Pinheiro et al. 2009).

The *CsNAC1* gene is involved in abiotic stress response pathways and in ABA signaling

CsNAC1 shares an extensive similarity with GhNAC4, GmNAC3, GmNAC4, anac019, anac055, and anac072, all of which have been shown to function in pathways of abiotic stress response and tolerance (Tran et al. 2004, 2009; Meng et al. 2009; Pinheiro et al. 2009). Consistent with its putative role as a stress-NAC transcription factor containing all the five NAC subdomains (A through E), the CsNAC1-deduced protein also possesses a predicted NAC nuclear localization signal (Xie et al. 2000; Greve et al. 2003) and three transcriptional activation motifs (Ooka et al. 2003) on its primary structure. Several NAC proteins containing these conserved sequences in the primary structure have been demonstrated to be addressed to the nucleus and to show transactivation activity (Xie et al. 2000; Greve et al. 2003; Fujita et al. 2004; Nogueira et al. 2005; Nakashima et al. 2007; Hu et al. 2008; Pinheiro et al. 2009; Tran et al. 2009; Zheng et al. 2009).

A number of reports have suggested that the tissue specificity of NACs provides clues about their biological function (Xie et al. 2000; Sharp et al. 2004; He et al. 2005; Mitsuda et al. 2007; Yoo et al. 2007; Tran et al. 2009). We have therefore explored the HarvEST: Citrus database to

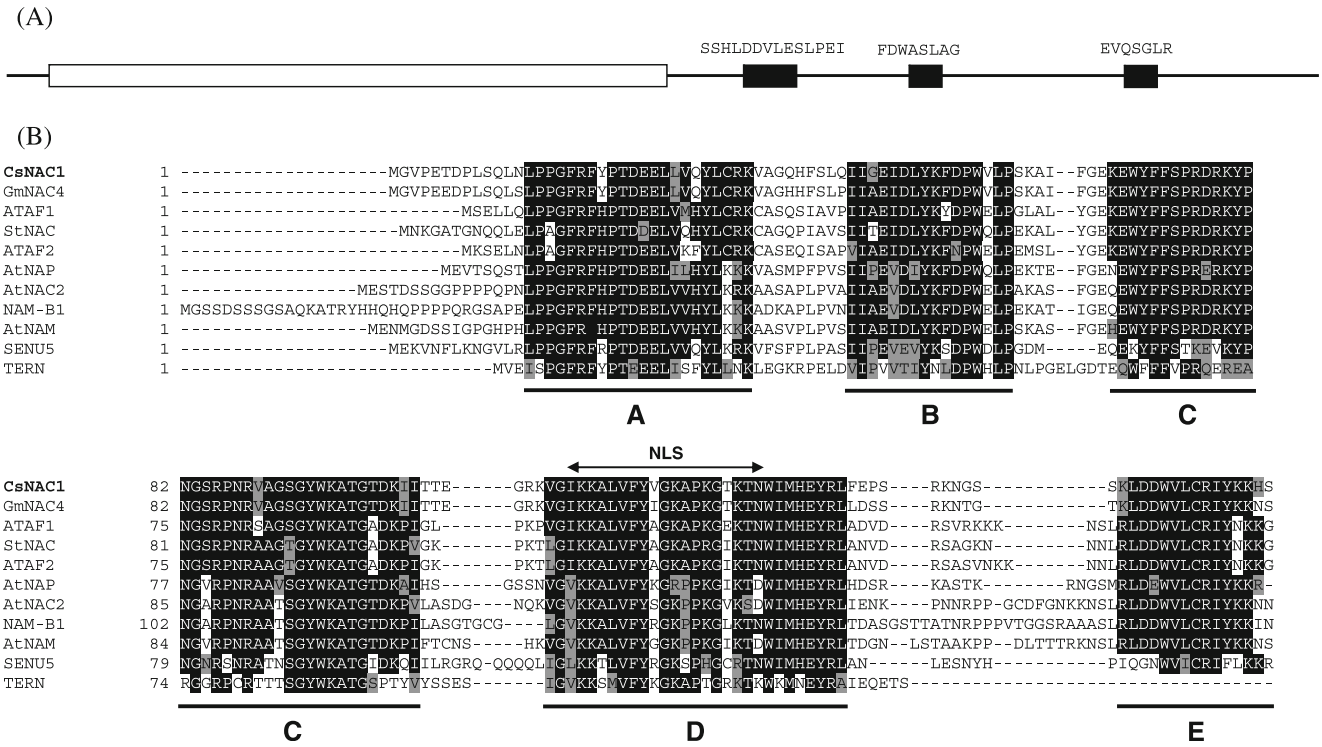


Fig. 2 Structural analysis of CsNAC1 protein. **a** Diagrammatic representation of CsNAC1. NAC domain is identified by *white box* while transcriptional activation motifs, as reported by Ooka et al. (2003), are identified by *black boxes*. Amino acid sequences of motifs are indicated above each *black box*. **b** Sequence alignment of NAC domains from different plant species. Only amino acids belonging to the NAC domain sequence were used in the alignment. Subdomains A through E are shown by *lines* below the sequences. Identical amino acid residues in a given position are in *white text on a black background*, while those conserved residues are in *white text in a gray background*. *Dashes* denote a gap in the amino acid sequence. *Arrow* above the sequences denotes the degenerate bipartite nuclear localization signal (NLS) that is conserved in several NAC domain proteins (Greve et al. 2003). Accession numbers for protein sequences of other plant species are in parentheses. GmNAC4 (AAX85981.1); ATAF1 (NP_171677); StNAC (CAC42087.1); ATAF2 (NP_680161); AtNAP (NP_564966.1); AtNAC2 (NP_188170.1); NAM-B1 (ABI15896.1); AtNAM (AAM65392.1); SENU5 (CAA99760.1); TERN (BAA78417.1). CsNAC1 protein is in *bold*

Table 2 CsNAC1 EST distribution and digital gene expression profile (TPM) in different tissues/organs

Tissue/organ[a]	EST library ID[b]	*Citrus* species	Total clones	CsNAC1 members	TPM[c]
Shoot meristem	UCRCS03	*C. sinensis*	1,897	3	1,581.4
Leaves	ExocortL, FerrChlL, SLH	*C. clementina*, *C. sinensis*, *C. medica*	1,950	4	2,051.3
Floral organs	OF1, VOLCANI7, VOLCANI8, USDA03	*C. clementina*, *C. reticulata* × *C. temple*, *C. sinensis*	10,922	4	366.2
Fruits	AbsCFrt, FLAVEDO5, FLAVEDO6, FlavFr1, Fruit-TF, UCDCS01, UCDCS02, UCDCS05, UCRCS01, UCRCS05, UCRCS06, UCRCS07	*C. clementina*, *C. paradisi*, *C. sinensis*	24,463	24	981.1

[a] EST libraries were grouped according to the source of tissues/organs from which they were derived

[b] Identification of each EST library within the HarvEST: Citrus database

[c] TPM inferred from the ratio of CsNAC1 ESTs to the total number of clones in non-normalized EST libraries, converted into transcripts per million

provide the first approximation of *CsNAC1* transcript abundance in different tissues and under different treatments. The digital gene expression profile analysis demonstrated that *CsNAC1* was primarily expressed in leaves and shoot meristems and was involved in general stress responses. The tissue specificity of some stress-NAC members has been previously determined. *GhNAC4* of cotton was shown to be abundantly expressed in leaves, but it has little or no expression in stems and roots (Meng et al. 2009). On the other hand, *GmNAC3* and *GmNAC4* of soybean were shown to be primarily expressed in roots and flowers (Tran et al. 2009). In our *in silico* analysis, we were not able to observe *CsNAC1* expression in roots due to the absence of non-normalized libraries from this tissue source in the HarvEST: Citrus database. Yet, it was then confirmed through qPCR analysis (Fig. 4).

We have further demonstrated by qPCR analysis that *CsNAC1* is not only structurally similar to the stress-NAC subgroup but it also shares a conserved stress-inducible expression profile as it was strongly induced by drought stress, salt, cold, and ABA. *GhNAC4* was shown to be induced by drought, salt, cold, and ABA (Meng et al. 2009). *GmNAC3* and *GmNAC4* were induced by osmotic stress, salt, cold, ABA, and jasmonic acid (JA) (Pinheiro et al. 2009; Tran et al. 2009). *anac019*, *anac055*, and *anac072* of *Arabidopsis* were mainly induced by drought stress, salt, and ABA, and some of them also by JA (Tran et al. 2004). These findings suggest that *CsNAC1* likely plays a role in stress responses similar to that of its counterparts in other plant species.

It has been shown that NACs can regulate abiotic stress responses through both ABA-dependent and ABA-

Table 3 CsNAC1 EST distribution and digital gene expression profile (TPM) under different treatments

Treatment[a]	EST library ID[b]	*Citrus* species	Total clones	CsNAC1 members	TPM[c]
Normal[d]	AbsCFrt, Fruit-TF, UCRCS03, USDA03 VOLCANI7	*C. clementina, C. reticulata* × *C. temple*, *C. sinensis*	15,284	9	588.8
Gibberellic acid	OF1, VOLCANI8	*C. reticulata* × *C. temple*, *C. sinensis*	2,090	2	956.9
Cold	FLAVEDO5, FlavFr1, UCDCS01, UCDCS02, UCRCS01, UCRCS05, UCRCS06	*C. clementina*, *C. paradisi*, *C. sinensis*	12,999	17	1,307.8
Salt	SLH	*C. sinensis*	753	2	2,656.0
Iron deficiency	FerrChlL	*C. clementina*	567	1	1,763.7
Hot water	FLAVEDO6	*C. paradisi*	2,847	1	351.2
Exocortis	ExocortL	*C. medica*	630	1	1,587.3
Thrips	UCRCS07	*C. sinensis*	1,846	1	541.7

[a] EST libraries were grouped according to the treatment from which the tissues/organs were submitted

[b] Identification of each EST library within the HarvEST: Citrus database

[c] TPM inferred from the ratio of CsNAC1 ESTs to the total number of clones in non-normalized EST libraries, converted into transcripts per million

[d] Non-treated tissues

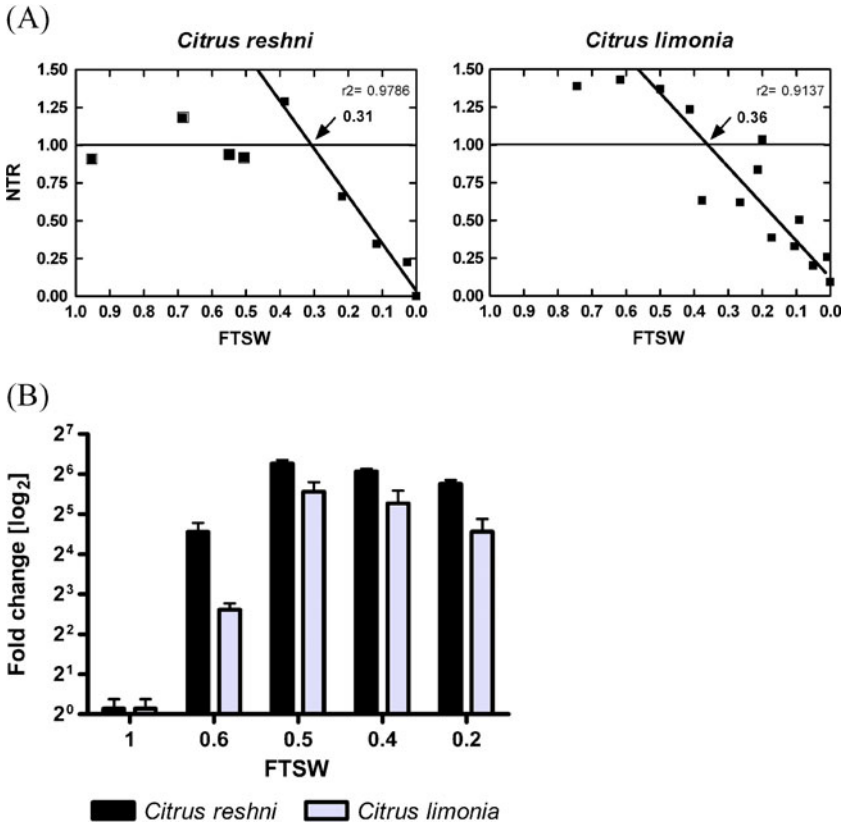


Fig. 3 Expression of *CsNAC1* in response to drought. **a** Soil dry-down experiments. Eight-month-old *Citrus reshni* and *C. limonia* plants were subjected to progressive soil drying and a normalized transpiration rate (NTR) response curve was obtained as function of the fraction of transpirable soil water (FTSW). *Arrow* indicates the fraction of extractable water whereby the transpiration rates began to decline in an approximately linear manner in each genotype. **b** quantitative RT–PCR analysis of *CsNAC1* expression during the soil dry-down experiments. The transcript levels were measured in leaves and standard errors were calculated from three biological replicates in which β-actin transcripts were used as internal controls. The fold induction by each treatment as related to the control (FTSW=1) is indicated

independent pathways (Fujita et al. 2004; Tran et al. 2004). Since the expression of *CsNAC1* was induced under exogenous ABA treatment, its participation in abiotic stress responses through an ABA-dependent signaling pathway is suggested, as demonstrated for its closest orthologs (Tran et al. 2004, 2009; Meng et al. 2009; Pinheiro et al. 2009). This hypothesis is additionally supported by the fact that the *cis*-acting element ABRE (ABA-responsive element) has been identified in the promoter region of several *NAC* genes (Fujita et al. 2004; Tran et al. 2004; Nakashima et al. 2007; Fang et al. 2008). Nevertheless, the induction of *CsNAC1* as well through an ABA-independent signaling pathway cannot be ruled out since the *cis*-element DRE (dehydration-responsive element)/CRT (C-RepeaT) that functions in

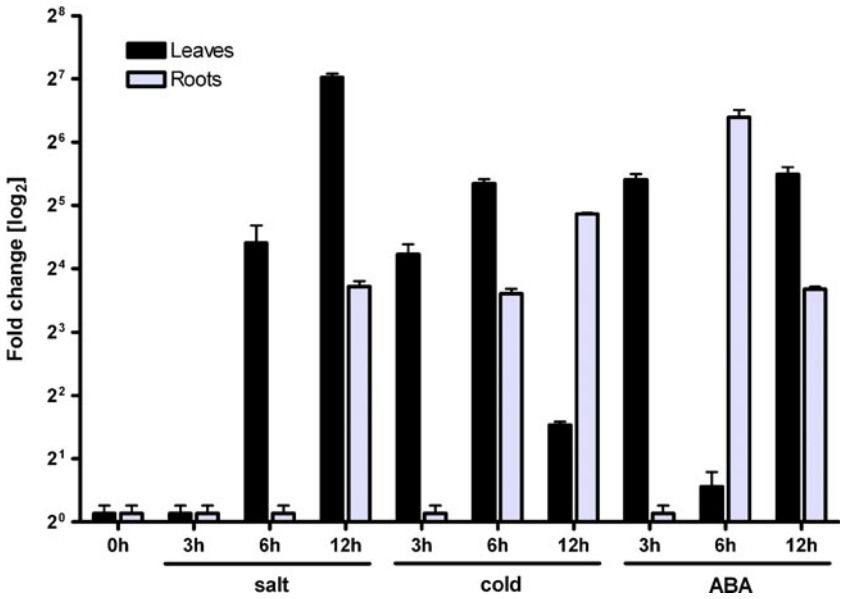


Fig. 4 Expression of *CsNAC1* in response to salt, cold, and ABA treatments. Eight-week-old *Citrus reshni* plants were treated with 200 mM NaCl (salt), 100 μM ABA, or exposed to 4°C (cold) for 3, 6, and 12 h. The transcript levels were measured in leaves and roots through quantitative RT–PCR and standard errors were calculated from three biological replicates in which β-actin transcripts were used as internal controls. The fold induction by each treatment as related to the untreated control is indicated

ABA-independent gene expression (Yamaguchi-Shinozaki and Shinozaki 2006) has also been identified in the promoter of some *NAC*s (Fujita et al. 2004; Fang et al. 2008). DRE/CRT has been shown to function in the early process of stress-responsive gene expression whereas ABRE functions after the accumulation of ABA (Yamaguchi-Shinozaki and Shinozaki 2006). The presence of both *cis*-elements in the promoter region of *CsNAC1* could explain its induction observed in leaves since an early stage of soil drying (FTSW of 0.6), before the threshold soil moisture level for decreasing transpiration rate and establishing drought stress are to be reached (Fig. 3). However, further analysis of the promoter elements of *CsNAC1* and their relation with the induction of its expression is required to determine the regulatory systems controlling the stress-inducible expression of *CsNAC1*.

In conclusion, our data provide the first analysis of the previously uncharacterized CsNAC family of citrus and reveal a novel member involved in the response to multiple abiotic stresses. Our results also suggest that the *CsNAC1* gene may represent a potential target for engineering stress tolerance in citrus. We are currently testing this hypothesis through the ectopic expression of *CsNAC1* in tobacco and citrus.

Acknowledgments This research was supported by research grants from Embrapa (Macroprograma 2), CNPq (Brasília, Brazil), and FAPESP (São Paulo, Brazil). We gratefully acknowledge the Masters Degree scholarships to T.M. de Oliveira and L.C. Cidade by FAPESB (Salvador, Brazil) and CAPES (Brasília, Brazil), respectively. We thank Dr. Claudia Fortes Ferreira (Embrapa, Brazil) for helpful advices and the critical reading of the manuscript.

References

Boyer JS (1982) Plant productivity and environment. Science 218:443–448

Chaves MM, Oliveira MM (2004) Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J Exp Bot 55:2365–2384

Chen C, Costa MGC, Yu Q, Moore GA, Gmitter FG Jr (2010) Identification of novel members in sweet orange carotenoid biosynthesis gene families. Tree Genet Genome. 6:905–914

Collinge M, Boller T (2001) Differential induction of two potato genes, *Stprx2* and *StNAC*, in response to infection by *Phytophthora infestans* and to wounding. Plant Mol Biol 46:521–529

Delessert C, Wilson IW, Van Der Straeten D, Dennis ES, Dolferus R (2004) Spatial and temporal analysis of the local response to wounding in *Arabidopsis* leaves. Plant Mol Biol 55:165–181

Duval M, Hsieh T, Kim S, Thomas T (2002) Molecular characterization of AtNAM: a member of the *Arabidopsis* NAC domain superfamily. Plant Mol Biol 50:237–248

Fan J, Gao X, Yang Y-W, Deng W, Li Z-G (2007) Molecular cloning and characterization of a NAC-like gene in "navel" orange fruit response to postharvest stresses. Plant Mol Biol Report 25:145–153

Fang Y, You J, Xie K, Xie W, Xiong L (2008) Systematic sequence analysis and identification of tissue-specific or stress-responsive genes of NAC transcription factor family in rice. Mol Genet Genomics 280:535–546

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran L-SP, Yamaguchi-Shinozaki K, Shinozaki K (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J 39:863–876

Greve K, La Cour T, Jensen MK, Poulsen FM, Skriver K (2003) Interactions between plant RING-H2 and plant-specific NAC (NAM/ATAF1/2/CUC2) proteins: RING-H2 molecular specificity and cellular localization. Biochem J 371:97–108

Guo Y, Gan S (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J 46:601–612

Hauck P, Thilmony R, He SY (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. Proc Natl Acad Sci USA 100:8577–8582

He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, Chen SY (2005) AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. Plant J 44:903–916

Hegedus D, Yu M, Baldwin D, Gruber M, Sharpe A, Parkin I, Whitwill S, Lydiate D (2003) Molecular characterization of *Brassica napus* NAC domain transcriptional activators induced in response to biotic and abiotic stress. Plant Mol Biol 53:383–397

Hu H, You J, Fang Y, Zhu X, Qi Z, Xiong L (2008) Characterization of transcription factor gene *SNAC2* conferring cold and salt tolerance in rice. Plant Mol Biol 67:169–181

Liu Y-Z, Baig MNR, Fan R, Ye J-L, Cao Y-C, Deng X-X (2009) Identification and expression pattern of a novel NAM, ATAF, and CUC-like gene from *Citrus sinensis* Osbeck. Plant Mol Biol Report 27:292–297

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 444:139–158

Meng C, Cai C, Zhang T, Guo W (2009) Characterization of six novel NAC genes and their responses to abiotic stresses in *Gossypium hirsutum* L. Plant Sci 176:352–359

Mitsuda N, Iwase A, Yamamoto H, Yoshida M, Seki M, Shinozaki K, Ohme-Takagi M (2007) NAC transcription factors, NST1 and NST3 are key regulators of the formation of secondary walls in woody tissues of *Arabidopsis*. Plant Cell 19:270–280

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Nakashima K, Tran LS, Van Nguyen D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J 51:617–630

Nogueira FTS, Schlogl PS, Camargo SR, Fernandez JH, De Rosa VE, Jr PP, Arruda P (2005) *SsNAC23*, a member of the NAC domain protein family, is associated with cold, herbivory and water stress in sugarcane. Plant Sci 169:93–106

Ohnishi T, Sugahara S, Yamada T, Kikuchi K, Yoshiba Y, Hirano HY, Tsutsumi N (2005) *OsNAC6*, a member of the NAC gene family, is induced by various stresses in rice. Genes Genet Syst 80:135–139

Oliveira MLP, Febres VJ, Costa MGC, Moore GA, Otoni WC (2009) High-efficiency *Agrobacterium*-mediated transformation of citrus via sonication and vacuum infiltration. Plant Cell Rep 28:387–395

Olsen AN, Ernst HA, Leggio LL, Skriver K (2005) NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci 10:79–87

Ooka H, Satoh K, Doi K et al (2003) Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*. DNA Res 10:239–247

Pinheiro GL, Marques CS, Costa MDBL, Reis PAB, Alves MS, Carvalho CM, Fietto LG, Fontes EPB (2009) Complete inventory

of soybean NAC transcription factors: sequence conservation and expression analysis uncover their distinct roles in stress response. Gene 444:10–23

Ren T, Qu F, Morris TJ (2000) HRT gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to turnip crinkle virus. Plant Cell 12:1917–1926

Riechmann JL, Heard J, Martin G et al (2000) *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. Science 290:2105–2110

Sablowski RW, Meyerowitz EM (1998) A homolog of NO APICAL MERISTEM is an immediate target of the floral homeotic genes APETALA3/PISTILLATA. Cell 92:93–103

Saibo NJM, Lourenço T, Oliveira MM (2009) Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Ann Bot 103:609–623

Saitou N, Nei M (1987) The Neighbor-Joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sharp RE, Poroyko V, Hejlek LG, Spollen WG, Springer GK, Bohnert HJ, Nguyen HT (2004) Root growth maintenance during water deficits: physiology to functional genomics. J Exp Bot 55:2343–2351

Sinclair TR, Ludlow MM (1986) Influence of soil water supply on the plant water balance of four tropical grain legumes. Aust J Plant Physiol 13:329–341

Souer E, van Houwelingen A, Kloos D, Mol J, Koes R (1996) The no apical meristem gene of *Petunia* is required for pattern formation in embryos and flowers and is expressed at meristem and primordial boundaries. Cell 85:159–170

Storey R, Walker RR (1999) Citrus and salinity. Sci Hortic 78:39–81

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Thompson JD, Higgins DG, Gibson TJ (1994) Clustal-W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the *early responsive to dehydration stress 1* promoter. Plant Cell 16:2481–2498

Tran L-SP, Quach TN, Guttikonda SK, Aldrich DL, Kumar R, Neelakandan A, Valliyodan B, Nguyen HT (2009) Molecular characterization of stress-inducible *GmNAC* genes in soybean. Mol Genet Genomics 28:647–664

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J (2006) A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314:1298–1301

Wahbi A, Sinclair TR (2007) Transpiration response of *Arabidopsis*, maize, and soybean to drying of artificial and mineral soil. Environ Exp Bot 59:188–192

Xie Q, Frugis G, Colgan D, Chua NH (2000) Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Genes Dev 14:3024–3036

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803

Yoo YS, Kim Y, Kim YS, Lee JS, Ahn HJ (2007) Control of flowering time and cold response by a NAC-domain protein in *Arabidopsis*. PLoS ONE 2:e642

Zheng X, Chen B, Lu G, Han B (2009) Overexpression of a NAC transcription factor enhances rice drought and salt tolerance. Biochem Biophys Res Commun 379:985–989

Zhong R, Demura T, Ye ZH (2006) SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of *Arabidopsis*. Plant Cell 18:3158–3170