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Molecular Cloning and Characterization of a NAC-like Gene in "Navel" Orange Fruit Response to Postharvest Stresses

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Abstract A cDNA subtraction library had been constructed to identify differentially expressed genes in peel pitting of citrus fruit. Based on the sequence of a cDNA fragment homologous to *NAC* gene family, the full-length cDNA of 1,203 nucleotides was cloned from "navel" orange by rapid amplification of cDNA ends. It was designated as *CsNAC*, encoding a protein of 305 amino acids. The calculated molecular weight of the CsNAC protein was 35.2 kDa, and theoretical isoelectric point was 6.72. Sequence comparison showed that the CsNAC protein had a strikingly conserved region at the N terminus, which is considered as the characteristic of the NAC protein family. CsNAC protein was orthologous to *Arabidopsis thaliana* ATAF1. Phylogenetic analysis confirmed CsNAC belonged to the ATAF subfamily, which plays an important role in response to stress stimuli. RNA gel blot analysis showed that the expression of *CsNAC* gene was rapidly and strongly induced by stresses such as wounding and no oxygen. Low temperature (4°C) and exposure to ethylene also increased the expression level of *CsNAC* gene. However, its expression was suppressed by high temperature (40°C) but not affected by low oxygen (3%). Our results may provide the basis for future research of NAC-like gene's role in stress-induced citrus peel pitting.

Keywords *Citrus sinensis* osbeck · Cloning · Expression · NAC · Peel pitting

Sequence data of *CsNAC* from this article have been deposited at GenBank under accession number EF596736.

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Abbreviations

CUC	Cup-shaped cotyledon
NAM	No apical meristem
NLS	Nuclear location site
ORF	Open reading frame
RACE	Rapid amplification of cDNA ends

Introduction

Plant-specific transcription factors play diverse and vital roles in the process of plant development (Kusano et al. 1995; Nagano et al. 2001; Singh et al. 2002). NAC domain proteins have been identified as a novel class of transcriptional regulators recently. They are characterized with a highly conserved region at their N-terminal ends and a highly diverged C terminus. Conserved N terminus may function as DNA-binding region of NAC domain proteins, including NO APICAL MERISTEM (NAM), ATAF1 and 2, and CUP-SHAPED COTYLEDON (Aida et al. 1997), whereas C terminus may serve as a transcriptional activation region (Duval et al. 2002; Ren et al. 2000; Xie et al. 2000).

So far, plant NAC genes have been shown to be involved in many processes of plant development, such as lateral root formation in auxin signaling (Xie et al. 2000), leaf senescence (John et al. 1997), formation of flower organ promordia (Sablowski and Meyerowitz 1998), and responses to biotic or abiotic stresses (Fujita et al. 2004; Hegedus et al. 2003).

Citrus fruit is prone to develop peel pitting, which has been described as a severe disorder with characteristics of extensive collapsed areas of the flavedo (outer colored part of the peel) and part of the albedo (inner part of the peel) that become brown with time. It could affect the quality and decrease the market value of citrus fruits. Efforts have been made to elucidate the triggering factors of peel pitting, but little has been reported about the molecular mechanism. In previous work, we established a suppression subtractive library of citrus peel pitting, and a cDNA fragment of NAC-like gene was confirmed as a pitting-related gene in “navel” orange (Gao et al. 2006).

In this study, we report cloning of a full-length NAC-like gene in navel orange and its expression patterns under stress stimuli. We found that the deduced amino acid sequence of *CsNAC* (GenBank accession no. EF596736) showed high homology with NAC domain proteins in other plants, especially with the ATAF subfamily. Furthermore, the expression of *CsNAC* was induced under several postharvest abiotic stresses. Based on the data, we propose that the *CsNAC* gene is involved in stress responses related to citrus peel pitting.

Materials and Methods

Plant Material and Treatments

Navel orange (*Citrus sinensis* Osbeck) were obtained from local orchards. For stress treatment, three replications of five fruits as a set were wounded using a sharp knife

and held at 20°C. Two sets of fruits were placed in airtight 30-L barrels and exposed to a constant gas flow of 600 mL min^{-1} containing 100% N_2 and 0% O_2 or 97% N_2 and 3% O_2 at 20°C, respectively. Another two sets of fruits were treated with 4°C (low temperature) and 40°C (high temperature), respectively. The last set of fruits was dipped in ethylene (600 μg L^{-1}) and held at 20°C. For time course experiments, the fruit peels were stripped at 6, 12, 24 h after treatments, frozen immediately in liquid nitrogen, and stored at −80°C for further use. The peels stripped from untreated fruits at 0 h were sampled as controls.

Amplification of Full-length CsNAC cDNA

A NAC-like cDNA fragment of 308 bp was isolated from a peel pitting subtraction library of navel orange fruit (Gao et al. 2006). Sequence comparison of this cDNA fragment with other plant NAC gene sequences indicated that this fragment already included the 5′ end of the NAC-like gene. Rapid amplification of cDNA ends (RACE) was performed to amplify its unknown 3′ end.

Total RNA was isolated from peel tissue of navel orange using Trizol reagent (Dingguo, Beijing, China). mRNA was purified by Quick Prep™ Micro mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ, USA) and used to synthesize the first-strand cDNA according to the manufacturer's recommendation of 3′-Full Race Core Set (TaKaRa, Shiga, Japan). Based on the cDNA sequence already obtained, a gene-specific primer P1 (5′-AACATAGAAAGACCAAGCG-3′) was designed according to the NAC-like cDNA fragment sequence. The primer P1, together with the three sites adaptor primer provided by 3′-Full Race Core Set, was used to amplify the 3′ cDNA end of this gene under the following conditions: predenaturation at 94°C for 5 min, followed by 32 cycles of 94°C 40 s, 52°C 40 s, and 72°C 1 min, then further prolonged at 72°C for 10 min. PCR product was separated by electrophoresis on a 1% agarose gel stained with ethidium bromide, purified using the Quiquick gel extraction kit (Tiangen Biotech, Beijing, China), then cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* strain JM109. Positive clone was sequenced by Shanghai Invitrogen Biotech (Shanghai, China).

Sequence Analysis

Identification of nucleotide sequence was established using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST, Altschul et al. 1997). The bioinformatics tools at the web site http://www.expasy.org were used to analyze the deduced protein. Sequence alignments were made by the DNAMAN program, version 5.2.2. Theoretical isoelectric point and mass values for the protein were also predicted and calculated using DNAMAN program. Structural domains were annotated according to PredictProtein (http://www.predictprotein.org/newwebsite/, Rost et al. 2004) with default parameters.

For phylogenetic analysis, other plant NAC-like gene sequences were retrieved from GenBank database: *Arabidopsis thaliana* ANAC (AY117224), ATAF1 (X74755), ATAF2 (X74756), AtNAC3 (AB049070), NAC2 (AF201456), NAC1 (AF198054), and ANAC055 (At3g15500); *Oryza sativa* OsNAC1 (AB028180),

OsNAC2 (AB028181), OsNAC5 (AB028184), OsNAC6 (AB028185), and OsNAC8 (AB028187); *Triticum sativa* GRAB2 (AJ010830); *Petunia × hybrida* NAM (X92205); *Lycopersicon esculentum* SENU5 (Z75524); and *Nicotiana tabacum* TERN (AB021178). Using DNAMAN program, overall amino acid sequences were aligned, and then the unrooted phylogenetic tree was generated. This tree was setup with the distance matrix using the neighbor-joining method. Poisson correction with the complete deletion of gaps was used to calculate protein distances. Bootstrap values are based on 100 iterations.

RNA Gel Blot Analysis

For RNA gel blot analysis, total RNA was extracted from flavedo tissues and then denatured at 65°C and separated on a 1% (w/v) formaldehyde denatured agarose gel (10 μg per lane). The RNA was transferred to a Hybond-N^+ membrane (Amersham Biosciences) for at least 15 h and fixed on the membrane using a UV Crosslinker. Blots were prehybridized in Church buffer (Church and Gilbert 1984) [7% sodium dodecyl sulfate (SDS), 300 mM sodium phosphate pH 7.4, 1 mM EDTA] at 65°C for at least 1 h. The DNA probe consisted of a PCR-amplified fragment corresponding to a conserved amino-terminal region of CsNAC and labeled to high specific activity by random priming at 37°C with [^{32}P]-dCTP according to Random Primer Labeling Kit (Invitrogen, Carlsbad, CA, USA) in Church buffer. After a 20-h hybridization period, the membrane was washed three times with 0.5×SSC and 0.1% SDS at 65°C and exposed to autoradiography film at −80°C.

Results

Cloning of CsNAC cDNA

Based on the sequence of 308-bp NAC-like cDNA fragment isolated from a peel-pitting subtraction library of navel orange fruit, we obtained the 1,203-bp full-length cDNA of putative NAC-like gene in navel orange by RACE, tentatively designated as *CsNAC* (GenBank accession no. EF596736). The cDNA of *CsNAC* contains a 915-bp open reading frame encoding a protein of 305 amino acids with a calculated molecular mass of 35.2 kDa and an isoelectric point of 6.72.

Sequence Analysis of CsNAC Protein

An alignment of the predicted amino acid sequence of CsNAC with the cloned NAC-like genes from many organisms was conducted using the DNAMAN program. As shown in Fig. 1, the deduced CsNAC protein contained a highly conserved region in its N-terminal sequence that may function as a DNA-binding domain. The N-terminal 158 residues contained five subdomains (A–E) (Fig. 1) according to Ooka et al. (2003). The C terminus of CsNAC protein, serving as transcription activation domain, showed low sequence similarity to other plant NAC proteins. It was indicated that NAC proteins may perform different functions accompanied by diverse C-terminal transcriptional activation domains.

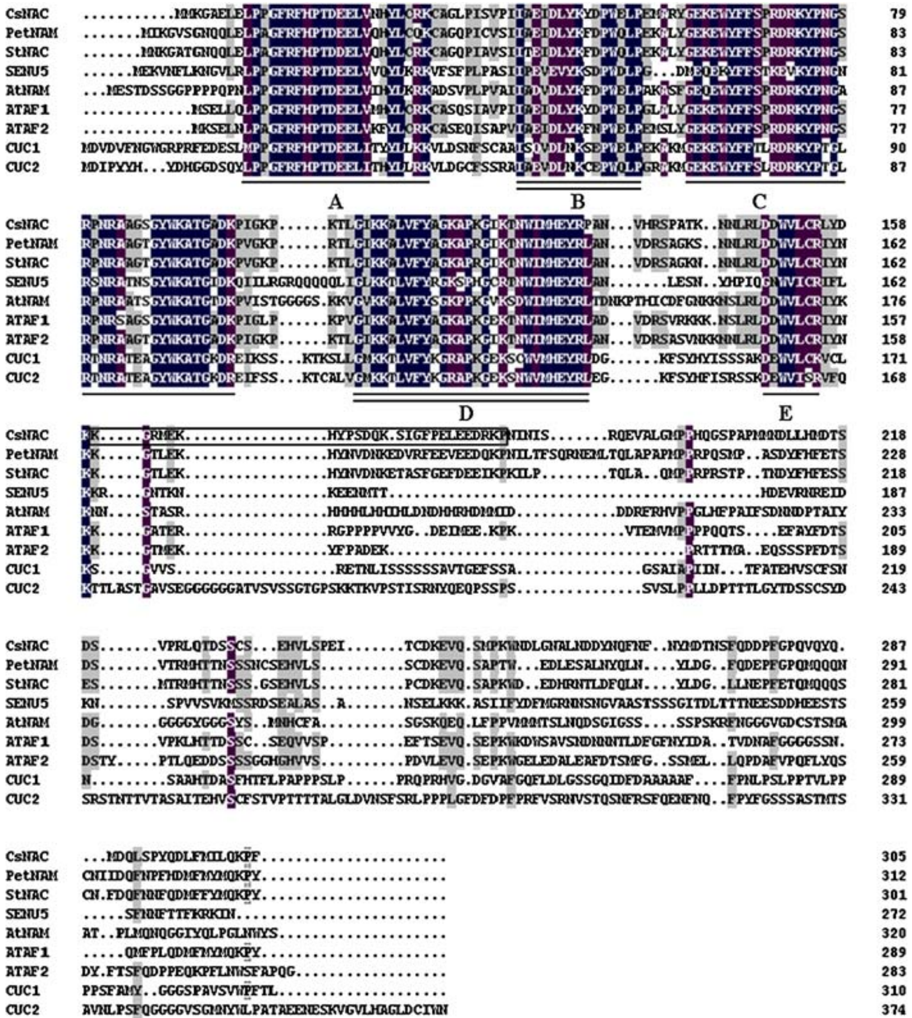

Fig. 1 Sequence alignment of CsNAC protein with other NAC-domain proteins, including *Petunia* × *hybrida* PetNAM (AAM34766); *Solanum tuberosum* StNAC (CAC42087); *L. esculentum* SENU5 (Z75524); and *A. thaliana* AtNAM (AF123311), ATAF1 (NP_171677), ATAF2 (NP_680161), CUC1 (AB049069), and CUC2 (AB002560). The amino acids with identity over 75% are *shaded in black*, whereas those with identity between 50 and 75% are *shaded in gray*. The five subdomains of NAC domain are shown with *A*, *B*, *C*, *D*, and *E*. The unstructured region is *boxed*

In addition, the putative CsNAC protein had some common features, including N-glycosylation sites, protein kinase C phosphorylation sites, casein kinase II phosphorylation sites, tyrosine kinase phosphorylation sites, and N-myristoylation sites (data not shown). It also contained an unstructured region (KGRMEKHYPS DQKSIGFPELEEDRKP) (Fig. 1). Nuclear location site was found in many NAC proteins (Taoka et al. 2004; Tran et al. 2004), whereas it was not found in the CsNAC protein.

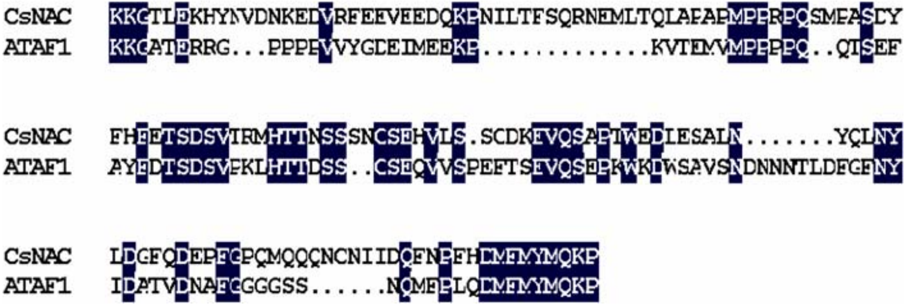

Fig. 2 Alignment of translated CsNAC variable carboxy-terminal domain with corresponding region of the most closely related plant homologue ATAF1. The identical residues are *highlighted in black*

The variable carboxy-terminal domain (residues 159–305) of the CsNAC protein was compared to the corresponding regions of NAC domain proteins in *A. thaliana* to identify homologues. Analysis showed that CsNAC was closely related to ATAF1 with 44.4% identity in their carboxy-terminal domains (Fig. 2). To confirm this relationship, phylogenetic analysis was performed using overall amino acid sequences of CsNAC and other plant NAC-domain proteins, and the result was slightly different from that of Ooka et al. (2003). To compare simplicity and facility, only major subfamilies were shown in Fig. 3. It was indicated that the putative CsNAC protein belonged to the ATAF subfamily, which was composed of ATAF1, ATAF2, OsNAC5, and OsNAC6 proteins.

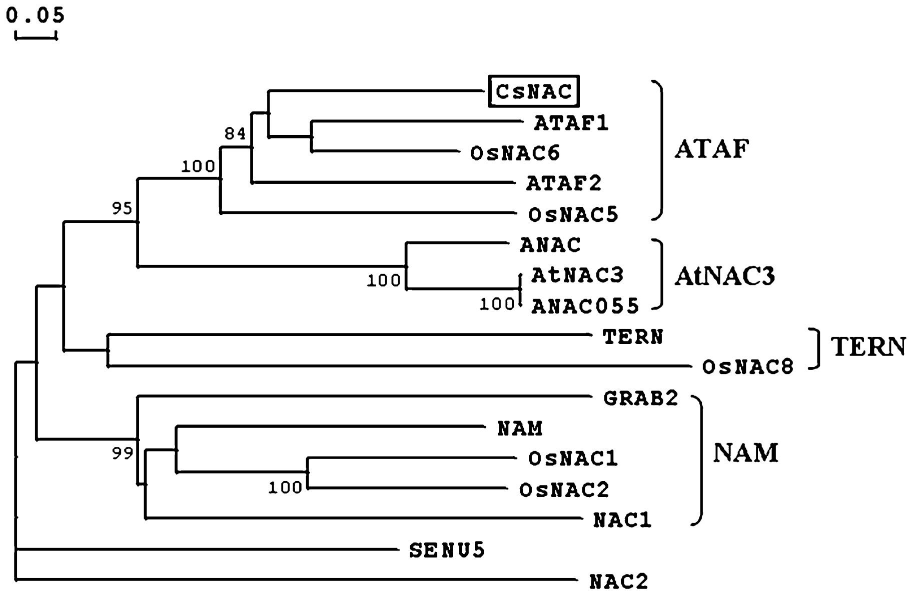

Fig. 3 Phylogenetic relationship between CsNAC protein and other plant NAC-domain proteins. Bootstrap values are based on 100 iterations. *Numbers* at the *nodes* indicate the bootstrap values over 75. The CsNAC protein is *boxed*

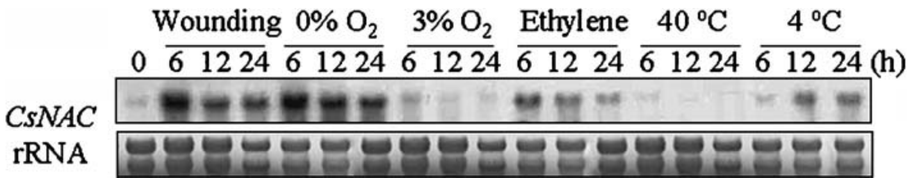

Fig. 4 RNA gel blot analysis of *CsNAC* gene expression in response to wounding, 0% O_2, 3% O_2, exposure to ethylene, and different temperatures (40, 4°C) at 6, 12, and 24 h after treatments. The sample derived from untreated fruits at 0 h was used as control. A 10-μg portion of total RNA was loaded in each lane. Equal loading, integrity, and transfer were observed by methylene blue staining of ribosomal RNA

Expression of CsNAC Gene Following Treatments with Abiotic Stresses and Ethylene

To elucidate the expression patterns of *CsNAC* gene under stresses such as wounding, no/low oxygen (0, 3%), low and high temperatures (4, 40°C), and exposure to plant hormone ethylene, RNA gel blot analysis was performed. The results show that *CsNAC* was up-regulated under stresses of wounding, exposure to ethylene, anoxia (0% O_2), and low temperature (4°C) (Fig. 4). It was found that the expression of *CsNAC* was suppressed by high temperature (40°C). Additionally, it was not obviously affected by hypoxia (3% O_2) (Fig. 4).

The expression levels of *CsNAC* were rapidly and strongly induced under wounding and anoxia (0% O_2). As shown in Fig. 4, the *CsNAC* transcripts started to accumulate at 6 h after wounding and anoxia (0% O_2) and remained at a high level over the 24-h posttreatment period. Additionally, exposure to ethylene also induced *CsNAC* expression, and the level decreased a little with time. The expression level of *CsNAC* was not increased until 12 h under low temperature (4°C). These results collectively indicated that *CsNAC* gene expression was preferentially related to the stresses of wounding and anoxia (0% O_2).

Discussion

In this study, we isolated the full-length cDNA of *CsNAC* from navel orange on the basis of 308-bp NAC-like cDNA fragment from the suppression subtractive library (Gao et al. 2006). Sequence analysis of CsNAC protein indicated that it contained a conserved NAC domain, which had five subdomains: A, B, C, D, and E (Fig. 1). It has been reported that subdomain C may be involved in DNA binding (Kikuchi et al. 2000). Subdomain E might be involved in functional and/or developmental stages and/or tissue-specific diversity. It might also be involved in DNA binding in cooperation with subdomain D (Duval et al. 2002). Together, these findings suggest the CsNAC might be a transcription factor.

Phylogenetic analysis showed that the putative CsNAC protein belonged to the ATAF subfamily. It has been reported that members of subgroup ATAF, such as ATAF1, ATAF2, and StNAC, could be rapidly and transiently induced by wounding. OsNAC6, in subgroup ATAF, is involved in the stress response (Collinge and Boller 2001). SsNAC23 in sugarcane, which belongs to the ATAF subfamily, is also

involved in the response to extreme low-temperature stress and could also be induced by water stress and herbivory (Nogueira et al. 2005). These reports and results provide strong support for the idea that the NAC family members in subfamily ATAF share a conserved role in the response to stress stimuli (Collinge and Boller 2001).

Wounding, especially mechanical damage, which happens during postharvest handling, storage, and transportation, is an important stress that induces citrus peel pitting. It was also reported that pitting of grapefruit increased with decreasing internal O_2 level and subjecting grapefruit to low O_2 (4%) induced pitting (Petracek et al. 1998). In this research, the expression of *CsNAC* gene was induced rapidly and strongly by wounding and anoxia (0% O_2) but not affected by low oxygen (3% O_2). It is indicated that the *CsNAC* is not only a wound-inducible gene but also an anoxia-related gene, which may play an important role in citrus postharvest peel pitting caused by abiotic stresses.

The small gaseous hormone ethylene is a regulator both of endogenous developmental programs and externally stimulated stress adaptations and plant defenses (Dreher and Callis 2007). It has been reported that *AtNAC2*, a NAC-type transcription factor gene induced by the ethylene precursor ACC, may be a common downstream component of ethylene signaling pathway (He et al. 2005). In our research, *CsNAC* was also induced by exogenous ethylene. It suggests that *CsNAC* may be also involved in ethylene signaling.

As an important plant-specific transcription factor gene, *CsNAC* is involved in stress responses. In future study, it will be necessary to elucidate its role in the process of peel pitting induced by stress stimuli.

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