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Research article

Genome-wide characterization and expression profiling of the *NAC* genes under abiotic stresses in *Cucumis sativus*

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ABSTRACT

The NAC (standing for no apical meristem [NAM], Arabidopsis transcription activation factor [ATAF] and cup-shaped cotyledon [CUC]) proteins pertain to one of the plant-specific transcription factor families that play important roles in plant development, abiotic stress resistance and signalling transduction. In the present study, the genomic features of the *NAC* genes in cucumber were analysed in depth using in silico tools. To reveal a tissue-specific, abiotic stress and hormone-responsive expression profile of *CsNAC* genes, RT-qPCR was performed under different treatments. Phylogenetic analyses and genome-wide annotation indicated that 82 high-confidence *CsNAC* genes were clustered into 13 sub-groups with uneven distribution in the cucumber genome. Furthermore, the *CsNAC* genes exhibited different tissue-specific expression patterns in 10 tissues under normal growth conditions, while 13 (16%) and 28 (34%) genes displayed preferential expression in roots and flowers, respectively. Moreover, *CsNAC* genes were more sensitive to salinity than other stresses; however, their responses were relatively rapid and transient to nutrition deprivation. Several *CsNAC* genes, including *CsNAC35*, which is an orthologue of the known stress-responsive *Arabidopsis RD26*, were identified as highly responsive to abiotic stresses and hormones. Overall, our findings revealed the genomic landscape and expression profiling of the *CsNAC* genes in response to multiple stresses and hormones, offering clues for further function analyses and molecular breeding.

1. Introduction

Cucumber (*Cucumis sativus* L.) is an economically and nutritionally important vegetable crop that is cultivated worldwide. Since the root of cucumber largely spreads in shallow soil, it is easily affected by environmental constraints and is sensitive to surrounding abiotic stresses, such as high salinity, drought, low temperature and deficiency of nutrient elements, which seriously limit the quality and quantity of the cucumber yield (Boyer, 1982). Thus, increased tolerance of cucumber to these abiotic stresses may significantly improve cucumber production. Tolerance or susceptibility to these stresses is often regulated by specific transcription factors (TFs).

The NAC (standing for no apical meristem [NAM], *Arabidopsis* transcription activation factor [ATAF] and cup-shaped cotyledon [CUC]) protein family is one of the largest transcription factor families that specifically exist in plants (Souer et al., 1996). The number of *NAC* genes is variable among different plant species, such as 163 genes in poplar (Hu et al., 2010), 110 in potato (Singh et al., 2013) and 204 in Chinese cabbage (Liu et al., 2014b). Moreover, NAC proteins regulate multiple biological processes, such as root development (Bennett et al., 2010), secondary cell wall formation (Yoshida et al., 2013), cell division (Kim et al., 2006), embryonic and floral development (Souer et al., 1996; Sablowski and Meyerowitz, 1998) and hormone signalling (Fujita et al., 2004; Kim et al., 2006). In cucumber, an *NAC* gene is differentially expressed between gynoecious and hermaphroditic plants (Guo

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et al., 2010). However, studies on the functions of *NAC* genes in cucumber growth and tissue development are relatively rare.

Furthermore, many NAC proteins regulate the plant's responses to abiotic stresses (Tran et al., 2004; Lee et al., 2014; Jeong et al., 2010; Hong et al., 2016). In *Arabidopsis*, the overexpression of any of the genes *ANAC019*, *ANAC055*, *ANAC072*/*RD26* or *NTL4* (*ANAC053*) significantly improved drought, salinity or heat stress tolerance in transgenic plants (Tran et al., 2004; Fujita et al., 2004; Lee et al., 2014). Similarly, the overexpression of various *NAC* genes confers improved tolerance to abiotic stresses in transgenic rice (Jeong et al., 2010; Hong et al., 2016). In vegetable crops, *SlNAC4* and *SlSRN1* (*Solanum lycopersicum Stress-related NAC1*) in tomato (Zhu et al., 2014; Liu et al., 2014a) improved the susceptibility of transgenic plants to salt and drought, respectively, whereas *CaNAC2* improved the susceptibility of pepper to cold stress (Guo et al., 2015). Identifying and characterising the NAC TF families in economically and nutritionally vegetable crops, such as cucumber, can help select potential *NAC* genes, which can generate crops with improved stress tolerance. In cucumber, five *NAC* genes were responsive to nitrogen deficiency treatments (Zhao et al., 2015). However, the potential functions of *NAC* genes in cucumber under other stresses (e.g. salinity, drought, cold and other nutrient deficiency) remain unclear so far.

Genome information has grown dramatically in the past few years, and the completed genome sequencing of cucumber provides an opportunity to identify protein families at the genome-wide level. To study the roles of NAC TFs in cucumber, we identified an NAC TF family in the cucumber genome and phylogenetically analysed the members of the *NAC* gene family. To explore the function of NAC genes, RT-qPCR was performed at different developmental stages and under different abiotic stress and hormone treatments. Non-redundant *CsNAC* genes responding to various stresses at multiple treatment time points were selected through comparison of the induced *CsNAC* genes, which provided clues for the functional characterization of cucumber NAC TFs and their use in cucumber molecular breeding.

2. Methods

2.1. Identification and basic feature analyses of members of the NAC gene family in cucumber

The sequences and annotation of the cucumber genome were extracted from the latest version (V2) of the cucumber database (http://www.icugi.org/cgi-bin/ICuGI/index.cgi). To perform genome wide identification of *CsNAC* genes, Hidden Markov Model (HMM) searching of the NAM domain (PF02365, from Pfam 29.0, http://Pfam.sanger.ac.uk/) (Finn et al., 2016) was performed for all the cucumber proteins. In this step, the cut-off of the expected value was set at 1e-3 to ensure the confidence. Later, our annotated results were further compared with the annotated *CsNAC* genes in the current cucumber genome. After overlap comparison, the information was integrated into one *CsNAC* dataset. *Arabidopsis thaliana* orthologues for cucumber NAC proteins were identified using BLASTp search against the *Arabidopsis* proteins TAIR10 release (http://www.arabidopsis.org).

The names of the *CsNAC* genes were based on their physical positions in the cucumber genome, which were adapted from the rules for potato (Singh et al., 2013). For the basic characteristic analyses, domain information was obtained by HMM searching and multiple sequence alignment of the ClustalX program. Additionally, we investigated the chromosome distribution of the *CsNAC* genes. The total number and distribution density of the *CsNAC* genes in their respective chromosomes were calculated to generate genome information on the *CsNAC* genes.

2.2. Intraspecific comparisons of CsNAC members in phylogeny, conserved motifs and membrane-bound structures

Intraspecific comparisons of Cs*NAC* members were performed to analyse the features of *CsNAC* genes from three aspects. First, the CsNAC protein sequences were aligned by the ClustalX program with default parameters (Jeanmougin et al., 1998). Subsequently, the phylogenetic tree of these NAC proteins was plotted using MEGA6.06 software by the neighbour-joining method with 1000 bootstrap replicates (Tamura et al., 2013). The phylogenetic tree extensively separated the *CsNAC* genes into different sub-groups. Second, the conserved motifs for all the CsNAC proteins were identified by using the Multiple Expectation Maximization for Motif Elicitation (MEME) program (version 4.11.1). In this analysis, the maximum number of motifs for CsNAC proteins was set to 10, whereas the other parameters were set to the default values (Bailey et al., 2009). Finally, membrane-bound CsNAC proteins were predicted by using the TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001).

2.3. Plant, tissues and stress treatments

To understand the regulatory functions of the *CsNAC* genes in cucumber growth and development and in stress tolerance and hormone signal transduction, we surveyed the expression patterns of *CsNAC* genes under both normal growth conditions and stress and hormone treatments. First, the expression patterns of *CsNAC* genes were investigated in 10 tissues under normal conditions. These 10 tissues were as follows: root, stem, leaf, cotyledon, tendril, male flower, female flower, ovary, 1-day fruit and 5-day fruit. Then, expression profiling of *CsNAC* genes was performed for six abiotic stresses (salt, drought, cold, nitrogen deprivation, phosphorus deprivation and potassium deprivation) and two hormones treatments (ABA and MeJA) in roots.

The *Cucumis sativus* L. 'Chinese long' inbred line 9930 was used as the experimental material. Seedlings were cultured as previously described (Zhao et al., 2015). For the normal conditions, the sampling time for different tissues was determined by the growth stages of the cucumber plants. Four tissues (roots, stems, leaves and tendrils) were sampled when the fourth leaf was fully expanded; five tissues (male flowers, female flowers, ovaries, 1-day fruits and 5-day fruits) were sampled at 0 days, 0 days, 0 days, 1 day and 5 days after anthesis; and the cotyledons were sampled when they had fully expanded. These samples were collected separately, frozen quickly in liquid nitrogen and stored at −80 °C until used for RNA isolation.

Stress and hormone treatments were performed when the fourth leaf was fully expanded, whereas the control was kept in a stable condition at the seedling time. The treatments for five stresses were completed by modifying the Hoagland nutrient solution: salinity (NaCl, 200 mM), drought (PEG6000, 10%), nitrogen deprivation (0 mM NO_3^-, 0 mM NH_4^+), phosphorus deprivation (0 mM $H_2PO_4^-$) and potassium deprivation (0 mM K^+). One stress (cold) was applied via temperature change (4 °C). Additionally, cucumber leaves were subjected to hormone treatment (ABA and MeJA) by using spray solutions (100 μM ABA and 100 μM MeJA). Sampling for each treatment was performed in roots at six time points: five treatment points (1 h, 3 h, 6 h, 12 h and 24 h) and one control point (0 h). Sampling in this section was the same as described earlier.

2.4. RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.