Ectopic Expression of Pumpkin NAC Transcription Factor CmNAC1 Improves Multiple Abiotic Stress Tolerance in Arabidopsis

Haishun Cao, Li Wang, Muhammad A. Nawaz, Mengliang Niu, Jingyu Sun, Junjun Xie, Qiusheng Kong, Yuan Huang, Fei Cheng and Zhilong Bie*

Key Laboratory of Horticultural Plant Biology, Ministry of Education, College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan, China

Drought, cold and salinity are the major environmental stresses that limit agricultural productivity. NAC transcription factors regulate the stress response in plants. Pumpkin (Cucurbita moschata) is an important cucurbit vegetable crop and it has strong resistance to abiotic stress; however, the biological functions of stress-related NAC genes in this crop are largely unknown. This study reports the function of CmNAC1, a stress-responsive pumpkin NAC domain protein. The CmNAC1-GFP fusion protein was transiently expressed in tobacco leaves for subcellular localization analysis, and we found that CmNAC1 is localized in the nucleus. Transactivation assay in yeast cells revealed that CmNAC1 functions as a transcription activator, and its transactivation domain is located in the C-terminus. CmNAC1 was ubiquitously expressed in different organs, and its transcript was induced by salinity, cold, dehydration, H₂O₂, and abscisic acid (ABA) treatment. Furthermore, the ectopic expression (EE) of CmNAC1 in Arabidopsis led to ABA hypersensitivity and enhanced tolerance to salinity, drought and cold stress. In addition, five ABA-responsive elements were enriched in CmNAC1 promoter. The CmNAC1-EE plants exhibited different root architecture, leaf morphology, and significantly high concentration of ABA compared with WT Arabidopsis under normal conditions. Our results indicated that CmNAC1 is a critical factor in ABA signaling pathways and it can be utilized in transgenic breeding to improve the abiotic stress tolerance of crops.

Keywords: pumpkin, ABA, NAC domain protein, ABRE, abiotic stress tolerance

INTRODUCTION

Crops are affected by all kinds of abiotic stresses such as drought, cold, heat, and salinity stress that could lead toward extensive production losses worldwide. Therefore, understanding the stress response and improving the stress tolerance of crops is critical to boost agricultural productivity, ensure food security, and environmental sustainability (Mittler, 2006; Zhu, 2016). As a largest

Abbreviations: ABA, abscisic acid; ABRE, ABA response elements; DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast images; GA, gibberellin; NARD, NAC repression domain; SNACs, stress-responsive NAC transcription factors.
plant-specific transcription factor family, NAC domain proteins play an important role in plant development and regulation of abiotic stress tolerance. These proteins have recently received the attention as a major regulator in various stress signaling pathways and have been found to improve the abiotic stress tolerance of different crops through genetic engineering (Hu et al., 2006; Nakashima et al., 2007; Hong et al., 2016; Huang L. et al., 2016; Wang et al., 2017). As transcriptional factors, NAC domain proteins contain highly conserved DNA-binding domain in the N-terminal and diverse transcription activation or repression domain in the C-terminal (Olsen et al., 2005; Kim et al., 2007; Hao et al., 2010).

The NAC domain transcription factors in Arabidopsis function in plant development, senescence, and stress regulation. NAC1 and AtNAC2 regulate root development (Xie et al., 2000; He et al., 2005; CUC1, CUC2, and CUC3 control leaf serration and axillary bud development (Nikovics et al., 2006; Raman et al., 2008); SND1 and VND7 trigger the de novo xylem formation and regulate secondary wall synthesis in fibers (Zhong et al., 2006; Reusche et al., 2012). According to some recent reports, NAC domain proteins are also associated with senescence, such as ORE1, NAP, ATAF1, and JUNGBRUNNEN1 play an important role in promoting or delaying senescence (Balazadeh et al., 2010; Wu et al., 2012; Yang et al., 2014; Garapati et al., 2015; Qiu et al., 2015; Takasaki et al., 2015). NAC domain proteins also act as major regulators in abiotic stress response, for instance ATAF1, RD26, ANAC096, and ANAC013 are involved in the regulation of salinity, oxidative, and drought stress tolerance (Fujita et al., 2004; Wu et al., 2012; Yang et al., 2014; Garapati et al., 2015; Qiu et al., 2015; Takasaki et al., 2015). NAC domain proteins also act as major regulators in abiotic stress tolerance, we expressed CmNAC1 abiotic stress tolerance, we expressed CmNAC1 ectopically. Morphological assays revealed that the EE of CmNAC1 altered the plant phenotypes, such as relative dwarfism and large roots under normal growth conditions. Moreover, CmNAC1-EE plants showed good tolerance under abiotic stress treatment. In summary, our results suggest that CmNAC1 plants positive roles in plant stress response and can be a candidate gene for the improvement of crops stress tolerance in the future.

MATERIALS AND METHODS

Plant Materials and Abiotic Stress Treatment

N15 an inbred line of pumpkin (Cucurbita moschata Duch.) developed at our laboratory was utilized as experimental material in this study. The seeds were germinated in distilled water and cultivated under a 12h light/12h dark cycle at 28°C/18°C in a growth chamber and photosynthetic photon flux density of 200 µmol m−2 s−1. The seedlings preparation method is described in our previous study (Xie et al., 2015). Fourteen-day-old pumpkin seedlings were treated with dehydration, 100 mM NaCl, 100 µM exogenous GA, 100 µM exogenous ABA, and 10 mM H2O2 at 4°C for 0, 0.5, 1, 4, 8, and 12 h. Different organs, including roots, young leaves, cotyledons, and hypocotyls (at the three-leaf stage); mature leaves and stems (at the six-leaf stage); and fruits (3 days after pollination) were used in measuring the organ-specific expression patterns of CmNAC1. All collected samples were immediately frozen in liquid nitrogen and stored at −80°C. Arabidopsis thaliana Columbia-0 plants cultured in substrate were used for the transgenic study of CmNAC1.

RNA Isolation and RT-qPCR

Total RNA was extracted using Tranzol (TransGen Biotech, Inc., Beijing, China), and reverse transcription was performed using 2 µg of RNA using HiScript II One Step RT-PCR Kit (Vazyme, Piscataway, NJ, United States) according to the manufacturer’s instructions. RT-qPCR was performed on Applied Biosystems QuantStudio system using an ABI 7500 real-time PCR machine (Applied Biosystems, Foster City, CA, United States) on it default PCR program with a reaction mixture volume of 10 µl. The melting curve was recorded after 40 cycles to verify the primer specificity by being conventional at 65°C to 95°C. One microliter of RNase-free H2O was also included in each plate as a control template. CmEF-1α, CmCAC (Obrero et al., 2011), and AtActin2 were used as the internal references. The 2−ΔΔCt method was used to calculating relative gene expression values (Livak and Schmittgen, 2001). The Ct value of two reference genes was the square root of CtCmEF−1α × CtCmCAC.
The primers are listed in Supplementary Table S2. All RT-qPCR reaction results were obtained from three independent replicates.

**Cloning and Analysis of CmNAC1 and CmNAC1 Promoter**

The CmNAC1 cDNA sequence was obtained from the transcriptome data of salt-treated pumpkin root in our laboratory. The NCBI accession number of the transcriptome is SRP066227. Primer pairs (Supplementary Table S2) were designed to amplify the coding sequence (CDS). The PCR products were cloned into pEASYT1-Blunt vector (TransGen Biotech, Inc., Beijing, China) and transfected into *Escherichia coli* Tran5αT1 competent cells (TransGen Biotech, Inc., Beijing, China). Finally, the target gene in positive clone strains was sequenced (Tsingke, Inc., Beijing, China). Multiple alignments of amino acid sequences of NAC domain proteins were performed using MEGA software (version 5.1) and DNAMAN (version 7). CmNAC1 promoter sequence was cloned from pumpkin genomic DNA using GenomeWalker Universal Kits (Takara, Shiga, Japan). The 1069 bp upstream from the start codon (ATG) of CmNAC1 was selected for further analysis. The online search tool PlantCARE was used to detect putative cis-acting regulatory elements.

**Subcellular Localization and Transactivation Assay Analysis**

The full-length CDS of CmNAC1 was amplified by PCR using 2x High-Fidelity Master Mix (Tsingke, Inc., Beijing, China), and the fragments were inserted into the *Stu*I site of the pH7LIC5.0-N-eGFP vector by using ClonExpress II One Step Cloning Kits. The construct and negative control (pH7LIC5.0-N-eGFP) were transformed into *Agrobacterium* strain GV3101 and were infiltrated into tobacco (*Nicotiana benthamiana*) leaves via *Agrobacterium*-mediated transformation method (Sheludko et al., 2007). Laser scanning confocal microscope was used to detect the GFP fluorescence signal. The nucleus was stained with DNA dye 4,6-diamidino-2-phenylindole (DAPI).

The CDS of CmNAC1 and the sequence encoding the N-terminus (1–450 bp) and C-terminus (450–879) were cloned. The PCR products were amplified with primers listed in Supplementary Table S2 and inserted into the *BamHI* site of pGBDKT7 vector and fused with the DNA-binding domain by using ClonExpress II One Step Cloning Kits to obtain pGBDKT7-CmNAC1-FL (1–292 aa), pGBDKT7-CmNAC1-N (1–150 aa) and pGBDKT7-CmNAC1-C (150–292 aa) constructs. These vectors and the pGBDKT7 (negative control) were separately transformed into the yeast strain Y2HGold. The transformed yeast cells were incubated on SD/-Trp, SD/-Trp/-His-Ade and SD/-Trp-His-x-gal plates. The transactivation activity was detected according to their growth status and α-galactosidase activity.

1. http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
et al., 2003). The relative chlorophyll content of 6-week-old seedling leaves from the control and salt-treated plants were measured as SPAD value using chlorophyll meter SPAD-502. For ABA content measurement, 0.10 g of 5-week-old seedling leaves were sampled and grinded on ice with cold extraction buffer (methanol:water:acetic acid, 80:19:1, v:v:v), shaken for 16 h at 4°C, centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was supplemented with internal standards, filtered using a syringe-facilitated 13-mm diameter nylon filter, dried by evaporation, and dissolved in 200 µl methanol. Simultaneous quantification of ABA by liquid chromatography was conducted using a UFLC with an autosampler according the method (Pan et al., 2008; Liu et al., 2012).

The experiment was repeated three times at least. All data were compared using Student's t-test for statistical analysis.

RESULTS

CmNAC1 Is a Stress-Responsive Gene

RT-qPCR was performed to elucidate the gene expression pattern in different tissues and the response of CmNAC1 to various abiotic stresses. Temporal and spatial expression results revealed that CmNAC1 has relative high expression levels in mature leaves, stem, roots, and the highest in hypocotyl. However, its expression was low in the new leaves and fruits (Figure 1A). Under H2O2 and NaCl stress conditions, CmNAC1 transcript was upregulated specifically at 0.5 and 8 h (Figures 1B,C). The expression of CmNAC1 was induced during 0.5–8 h under dehydration and ABA treatment, but only the transcript levels in ABA treatment reverted at 12 h (Figures 1E,F). However, GA consistently inhibited CmNAC1 expression during the treatment (Figure 1D). The cold stress repressed the CmNAC1 expression within 4 h, and then the transcript levels were induced until 12 h (Figure 1E). The qRT-PCR results suggest that similar to other stress-associated NAC domain proteins, CmNAC1 participates in plant stress response, and its expression is sensitive to ABA, H2O2, and GA signaling molecules.

The 1 kb upstream of ATG start codon CmNAC1 promoter sequence was cloned to detect the putative, stress-responsive cis-acting regulatory elements (Supplementary Figure S5). Stress response-related cis-acting elements, that include five ABA-responsive elements (ABREs), one low-temperature responsive motif, two GC motifs involved in anoxic specific inducibility, and two TGACG motifs involved in methyl jasmonate responsiveness (Table 1), were found in the promoter. These stress-related cis-acting elements could be necessary for the stress-regulated expression of CmNAC1. These findings indicate that CmNAC1 plays a critical role in early stress responsiveness.

CmNAC1 Belongs to ATAF Subfamily with Transcriptional Activity and Is Localized in the Nucleus

CmNAC1 cDNA had a length of 1200 bp and was predicted to contain an 879 bp CDS encoding 292 amino acid protein. The CmNAC1 gene accession number in NCBI GenBank database was MG199592. Amino acid sequence alignment shows that CmNAC1 shared an identity of 65.35% with AtATAF1 protein from Arabidopsis. CmNAC1 protein also contained a diverse activation domain in C-terminal and a conserved NAC domain in the N-terminal region including subdomains A to E (Supplementary Figure S1). To further reveal the divergence of CmNAC1 protein during evolution, we analyzed the phylogenetic relationship of CmNAC1 with SNACs orthologues, which were functionally characterized in Arabidopsis and crops. Phylogenetic analysis showed that CmNAC1 and AtATAF1 gene are in the same clade (Supplementary Figures S2, S7).

The eGFP-CmNAC1 fusion construct and the eGFP control in pH7LIC5.0-N-eGFP vector driven by CaMV35S promoter were transiently expressed in tobacco epidermal cells and visualized under a laser scanning confocal microscope to determine the subcellular localization of CmNAC1. The eGFP fluorescence signal was widely observed from the cytoplasm to nucleus, whereas the eGFP-CmNAC1 fusion protein fluorescence signal was mainly detected in the nucleus as confirmed by DAPI staining (Figure 2). Thus, above results demonstrated that CmNAC1 is a nuclear protein.

The entire coding region, N-terminal and C-terminal domain coding sequence were inserted into the pGBDKT7 vector, which contains the GAL4 DNA-binding domain, to investigate the transcriptional activity of CmNAC1 protein (Figure 3A). These constructs and empty vector pGBDKT7 (negative control) were transformed into the yeast strain Y2HGold. The transactivation results show that all transformed yeast cells grew well on SD/-Trp medium. The yeast strain containing the full-length CmNAC1 (pGBDKT7-CmNAC1-A) and the C-terminus of CmNAC1 (pGBDKT7-CmNAC1-C) could grow well on the selected medium SD/-Trp/-His/-Ade, whereas the cells with the N-terminus of CmNAC1 (pGBDKT7-CmNAC1-N) and pGBDKT7 empty vector could not grow normally. Furthermore, the yeast cells that grew well on the SD/-Trp/-His-x-gal medium appeared blue in the presence of α-galactosidase, indicating the activation of the reporter gene LacZ (Figure 3B). These results indicate that CmNAC1 is a transcriptional activator, and its transactivation domain localizes in the C-terminus.

Ectopic Expression of CmNAC1 Changes the Phenotype of Arabidopsis

When grown in the substrate, the CmNAC1-EE plants showed two different phenotypes. One was similar to WT, and the other was dwarf and light green in color (Supplementary Figure S3B). Eight transgenic positive lines with sufficient seeds and stable phenotype were selected for further gene expression experiment. RT-PCR results show that three EE lines had the same phenotype that is different from that of WT, and PCR analysis using vector specific primer indicated that all transgenic lines are positive (Supplementary Figure S3A). Therefore, CmNAC1-EE lines EE19 and EE26 with high CmNAC1 expression levels were selected for further analysis.
FIGURE 1 | Expression patterns of CmNAC1 in pumpkin seedlings under different stress treatments or in different organs. (A) Expression patterns of CmNAC1 in various organs (root, RT; young leaves, YL; mature leaves, ML; cotyledon, CN; hypocotyl, HL; stem, ST; fruit, FT). Expression patterns of CmNAC1 in leaves after \( \text{H}_2\text{O}_2 \) (B), NaCl (C), GA (D), cold (E), ABA (F) and dehydration (G) treatments by qRT-PCR analysis. Zero represents leaf sample without any treatment; 0.5, 1, 4, 8, and 12 represent samples after 0.5, 1, 4, 8, and 12 h treatment, respectively. The \( 2^{-\Delta \Delta CT} \) method was used in qRT-PCR analysis. Transcript levels were normalized to CmEF1α and CmCAC. Values are means ± SD of three replicates. Three independent experiments were performed. Asterisks indicate a significant difference (*P < 0.05; **P < 0.01) compared with the corresponding controls.
EE19 and EE26 lines showed sterility and dwarfism phenotype with light green and round-shaped leaves (Figures 4B,C). These plants also showed accelerated chlorophyll degradation during senescence (shown as white color) as compared with wild type plants with red senescence leaves (Figure 4A). When grown in the 1/2 MS dish, both EE line seedlings showed downward cotyledon and light color (Figures 4D,E). Also, both EE lines had larger roots with larger total root surface area, longer total root length, and more root tips than the WT plants (Figure 5). These findings suggest that CmNAC1 is an important regulator of plant phenotype development.

FIGURE 2 | Nuclear localization of CmNAC1. e-GFP and e-GFP-CmNAC1 fusion proteins were transiently expressed in tobacco leaves under control of the CaMV 35S promoter and observed under a laser scanning confocal microscope. e-GFP images, DAPI stained images, differential interference contrast images (DIC), and merged images were taken.

FIGURE 3 | Transactivation assay of CmNAC1 in yeast cell Y2HGold. (A) Full-length protein (CmNAC1-A), N-terminal fragment (CmNAC1-N) and C-terminal fragment (CmNAC1-C) were fused with GAL4 DNA binding domain and expressed in yeast strain Y2HGold. The pGBKTK7 vector was used as a negative control. (B) Transformed yeasts were dripped on the SD-Trp, SD-Trp-His-Ade, and SD-Trp-His-x-gal after being cultured for 3 days in the growth chamber.
Ectopic Expression of *CmNAC1* Improves Drought Tolerance and Increases ABA Content in *Arabidopsis*

The 4-week-old transgenic lines EE19, EE26 and WT *Arabidopsis* seedlings were transferred to water-saturated substrate to determine the function of *CmNAC1* to drought stress tolerance in plants. Water was suspended to gradually reduce water availability in the substrate. After 2 weeks of treatment, most WT plants wilted because of the extreme water deficit. By contrast, the *CmNAC1*-EE *Arabidopsis* plants showed little wilting and a slight leaf senescence phenotype. After re-watering, most of the *CmNAC1*-EE plants remained vigorous and survived, whereas only few of the WT plants recovered (Figure 6A). Furthermore, the survival ratio of the two *CmNAC1* transgenic lines (100%) was higher than those of the WT plants (19.5%) under drought stress (Figure 6B). The *CmNAC1*- EE plants showed lower water loss rate and better water retaining phenotype than WT during the 3 h dehydration stress (Figures 6C,D). The EE plants also had significantly high expression levels of key ABA biosynthetic gene *AtNCED3* in accordance to *CmNAC1* expression levels and ABA content (Figures 6E,F,G). These results suggest that *CmNAC1*-EE leads to high ABA content and efficient water use in *Arabidopsis*.

**CmNAC1** Ectopic Expressing Plants Are Tolerant to Salt and Cold stress

Salt tolerance of *CmNAC1*-EE plants were examined. Four-week-old seedlings cultivated in substrate were irrigated with 250 mM NaCl solution. After 30 days of salt treatment, *CmNAC1*-EE lines showed better growth status than the WT plants, thereby indicating serious chlorosis (Figure 7A). As shown in Figure 7C, the Fv/Fm value decreased significantly starting at the 10th day of treatment, this indicated that the leaves were damaged starting at the 10th day of salt treatment, and the EE lines consistently performed better than WT *Arabidopsis*. Moreover, the Fv/Fm images also revealed that salt damage at the 7th day of treatment was not visible. However, at the 14th day of treatment, the WT plants were significantly damaged compared with the EE plants (Figure 7B). Consistent with the Fv/Fm value changes, the transgenic plants had lighter green leaves (lower SPAD value) without salinity. However, after 2 weeks of salt stress treatment, their SPAD value was higher than that of the...
FIGURE 5 | CmNAC1-EE lines have enhanced root growth. (A) Root scanning picture of WT, EE19 and EE26 lines. (B) Quantification of total root surface area. (C) Quantification of total root length. (D) Quantification of total root tips. Three independent experiments were performed. Asterisks indicate statistically significant differences compared with WT (*P < 0.05; **P < 0.01).

FIGURE 6 | CmNAC1-EE lines have enhanced drought stress tolerance. Four-week-old seedlings of WT and EE plants were grown in substrate for treatment. (A) Phenotypes of WT and EE plants during drought stress. (B) Quantitative analysis of survival rate after drought stress. (C) Fully expanded leaves of Arabidopsis plants (5 weeks old) were excised and exposed to dehydration stress for 3 h on dry filter paper at room temperature. Pictures were taken at 0 and 3 h after treatment. (D) Water loss assay from detached leaves of 5-week-old seedings. (E) CmNAC1 expression levels in transgenic Arabidopsis EE19, EE26 lines and WT plants. (F) Key ABA biosynthesis gene AtNCED3 expression levels in transgenic Arabidopsis EE19, EE26 lines and WT plants. (G) Concentration of ABA in transgenic Arabidopsis EE19, EE26 lines and WT plants. Three independent experiments were performed. Asterisks indicate statistically significant differences compared with WT plants (**P < 0.01).
WT plants (Figure 7E). All EE lines also had high survival rate (Figure 7D). In conclusion, EE lines are more tolerant to salinity stress.

Four-week-old potted Arabidopsis seedlings were subjected to freezing at −10°C for 1 h to characterize the function of CmNAC1 in cold tolerance. After that, incubate the plants in a cold growth chamber (4°C) for 3 h, followed by transferring the plants to normal growth conditions (23 ± 1°C) for recovery. EE lines had less cold injury and better recovery than the WT plants after cold treatment (Supplementary Figure S4A). Moreover, the survival ratio of the two transgenic lines were significantly higher than WT plants (Supplementary Figure S4B). During cold treatment, the two CmNAC1 transgenic lines also had lower electrolyte leakage than WT plants (Supplementary Figure S4C). These results indicate that EE of CmNAC1 also improves the tolerance of plants to low-temperature stress.

**DISCUSSION**

**CmNAC1 Ectopic Expressing Arabidopsis Lines Are ABA Hypersensitive**

Abscisic acid is a critical stress regulator in plants, and EE lines had high ABA content. Therefore, the ABA sensitivity of EE plants was assessed. ABA significantly inhibited Arabidopsis germination when the seeds were cultivated on 1/2 MS medium supplemented with 0 and 2 µM ABA (Figure 8A). However, the emergence ratio of WT seeds was higher than that of EE lines seeds in 1/2 MS medium containing 2 µM ABA (Figure 8C). The seedlings of 7-day-old WT and EE lines were transplanted to vertical 1/2 MS medium containing 0 and 10 µM ABA for ABA sensitivity analysis. Both EE lines showed yellow and senescent shoot phenotypes compared with the control (Figure 8B). Additionally, when the seedlings were cultivated in 1/2 MS medium supplemented with 2 µM ABA for 2 weeks, both transgenic lines showed yellow seedling cotyledon leaves compared with the light green seedling cotyledon leaves of WT plants (Figure 8D). Therefore, we suggested that CmNAC1 transgenic line shoots are more hypersensitive to ABA than WT plants.
FIGURE 8 | Hypersensitivity of CmNAC1-EE lines to ABA. Phenotypes of WT and EE plants grown in 1/2 MS medium supplemented with ABA for sensitivity quantitation. (A) Pictures of germination phenotype with 0 and 2 𝜇M ABA were taken after 8 days of treatment. (B) Phenotype of WT and EE lines in vertical 1/2 MS medium with 0 and 10 𝜇M ABA. (C) Germination rate were scored at five, six, seven, and 8 days after planting in the 1/2 MS plates supplemented with 0 and 2 𝜇M ABA. (D) Phenotype of WT and EE lines grown in 1/2 MS medium with 0 and 2 𝜇M ABA for 2 weeks and EE lines showing yellow phenotype compared with WT green phenotype.

TABLE 1 | Stress-related cis-acting regulatory elements identified in the promoters region of CmNAC1.

| Site name | Position | Strand | Sequence | Function |
|-----------|----------|--------|----------|----------|
| ARE       | 120,1391 | -      | TGGTTT   | cis-acting regulatory element essential for anaerobic induction |
| TC-rich repeats | 927 | +      | ATTTTCTCCA | cis-acting element involved in defense and stress responsiveness |
| HSE       | 447      | +      | AAAAAATTTTC | cis-acting element involved in heat stress responsiveness |
| MBS       | 591, 1592| +      | CAACTG   | MYB binding site involved in drought-inducibility |
| MBS       | 158      | -      | CCGTCA   | MYB binding site |
| LTR       | 860,1052 | +      | CCGAAA   | cis-acting element involved in low-temperature responsiveness |
| Crepeat/DRE | 329 | +      | TGGCCGAC | Regulatory element involved in cold- and dehydration-responsiveness |
| ABRE      | 118,152 379,520 | - | CACGTG | cis-acting element involved in the abscisic acid responsiveness |
| ABRE      | 496      | +      | ACGTGC   | cis-acting element involved in the abscisic acid responsiveness |
| TCA-element | 1095 | +      | TCAAGAAGGG | cis-acting element involved in salicylic acid responsiveness |
| TCA-element | 750 | -      | CCATCTTT | cis-acting element involved in salicylic acid responsiveness |
| TGACG-motif | 362,1428 | + | TGACG | cis-acting regulatory element involved in the MeJA-responsiveness |
| CCGTCA-motif | 1775 | -      | CCGTCA   | cis-acting regulatory element involved in the MeJA-responsiveness |
| TATACT/C-motif | 1894 | - | TATCAT | cis-acting regulatory element; associated with G-box like motif; involved in sugar repression responsiveness |
| W box 1514, 1344 | - | TTGACC | Elicitation; wounding and pathogen responsiveness. Binds WRKY type transcription factors |
| W box 631, 783 | + | TTGACC | Elicitation; wounding and pathogen responsiveness. Binds WRKY type transcription factors |

transcriptome data (Supplementary Figure S6), CmNAC1 is the greatest induced NAC genes by salinity. CmNAC1 is also the first identified stress-related NAC transcription factor in pumpkin containing diverse C-terminal activation domain and conserved N-terminal NAC domain. Amino acid sequence alignment shows that CmNAC1 has high sequence identity with AtATAF1 of stress-responsive NAC domain proteins from Arabidopsis (Supplementary Figure S1). Recently plenty of NAC domain proteins were identified to be stress-responsive and those proteins have important function in different stress signaling pathways (Hao et al., 2011; Xu et al., 2013; Yang et al., 2015; Hong et al., 2016; Zhang et al., 2016). The expression of CmNAC1 is induced by drought, salt, ABA and H₂O₂ (Figures 1B,C,F,G), and several stress-related cis-elements are present in promoter
of CmNAC1 (Table 1), suggesting it is a stress-responsive NAC domain protein. Most of transcription factors are localized in nucleus and have transactivation activity, CmNAC1 protein is also localized in nucleus (Figure 2) and both the C-terminal and full-length CmNAC1 have high transactivation ability in yeast (Figure 3B). However previous studies reported that the conserved hydrophobic LVFY motif in N-terminal of NARD would lead to abolished transactivation (Hao et al., 2010; Wang et al., 2017). CmNAC1 also contains NARD, but we have not observed significant difference of transactivation ability between the C-terminal and full-length CmNAC1. This might be due to its high transcriptional activation motif in C-terminal, but the mechanisms need to be further investigated. These results suggest that CmNAC1 is a nucleus localized stress-responsive transcriptional activator.

CmNAC1 Is Involved in Developmental Processes

Growth retardation with a dwarf phenotype has been observed in Arabidopsis EE of NTM1, AtATAF1, OsNAC2, MINAC5, and ANAC036 (Kim et al., 2006; Wu et al., 2009; Kato et al., 2010; Chen et al., 2015; Yang et al., 2015). CmNAC1-EE also leads to a dwarf phenotype (Figure 4C), indicating that CmNAC1 regulates shoot development. Plants can change their phenotypes to alleviate the harmful impacts of environmental stresses (Pierik and Testerink, 2014). AtATAF1 are reported to play an important role in promoting senescence and chlorophyll degradation (Garapati et al., 2015). CmNAC1-EE also showed light color and accelerated chlorophyll degradation during senescence with white leaves (Figure 4A). The results suggested the function of CmNAC1 are conserved in some aspects. Interestingly, we also found different root phenotype in EE lines. Several studies have proved that rice, Arabidopsis, and tobacco plants with optimized root systems have good tolerance to drought and limited nutrient supply (Yu et al., 2008; Werner et al., 2010; Uga et al., 2013). These findings are in agreement with our results that EE plants have large roots and good stress tolerance (Figure 5). NAC1 and AtNAC2 from Arabidopsis can promote later root development (Xie et al., 2000; He et al., 2005), and NAC domain proteins function as homo- or heterodimers (Olsen et al., 2005). However, the mechanisms of CmNAC1 in regulating root development and its cooperation with other NAC transcriptional factors related with auxin and root architecture development are still unknown.

CmNAC1 Improves Tolerance to Multiple Abiotic Stress

Recently, numerous NAC domain proteins from different crops were reported to play a positive role in stress responsiveness and regulation of abiotic stress tolerance (Hong et al., 2016; Huang L. et al., 2016; Zhang et al., 2016; Wang et al., 2017). The observations that CmNAC1 significantly improves the tolerance of Arabidopsis to salt, drought and cold stress and EE lines shows better growth performance and higher survival ratio under stress conditions (Figures 6A,B, 7A,D and Supplementary Figures S4A,B) indicates that CmNAC1 also functions as a positive stress-responsive transcription factor of salt, drought and cold stress tolerance in pumpkin. In the represent study, several physiological changes in transgenic Arabidopsis seem involved in the mechanisms of abiotic stress tolerance. First, CmNAC1 significantly improves the tolerance of Arabidopsis to salt stress, and reducing the accumulation of Na⁺ in leaves is one of plant salinity tolerance mechanisms (Roy et al., 2014). The leaves of CmNAC1-EE plants got less damaged when grown under salt stress (Figures 7B,E), it is might be due to less Na⁺ content in EE leaves. Secondly, as one of the most important phytohormones, ABA regulates plant development, closing of stomata, and stress tolerance (Raghavendra et al., 2010; Yoshida et al., 2014). It is observed that CmNAC1-EE Arabidopsis lead to higher ABA content and ABA hypersensitive (Figures 6G, 8), this could lead to increased closing of stomata under drought, and then control water loss ratio and enhance drought tolerance (Figures 6A,C,D). Thus increased ABA content and sensitivity in EE plants may partially account for the enhanced drought tolerance, which is consistent with previous findings that overexpression of MINAC5, AtATAF1, ONAC022, TaNAC47 in plants led toward improved drought stress (Wu et al., 2009; Yang et al., 2015; Hong et al., 2016; Zhang et al., 2016). Finally, cold-responsive genes in plants were also partially regulated by ABA, ABA-mimicking ligand application could improve cold stress tolerance (Cheng Z.M. et al., 2016), and we also found that CmNAC1 could improve cold tolerance (Supplementary Figures S4A,B). Our result are in accordance with the previous studies that ectopic expression of MINAC5, GmNAC20 and TaNAC47 conferred ABA hypersensitivity and enhanced cold tolerance in Arabidopsis (Hao et al., 2011; Yang et al., 2015; Zhang et al., 2016), indicating that CmNAC1 may function as a positive regulator of abiotic stress tolerance in ABA-dependent pathway.

CmNAC1 Plays a Critical Role in ABA-Dependent Signaling

Stress induced ABA biosynthesis in leaves was mainly controlled by AtNCED3 (Tan et al., 2003). AtATAF1 is a rapid stress response transcription factor that controls ABA biosynthesis by binding to AtNCED3 promoters and enhancing its expression (Jensen et al., 2013; Garapati et al., 2015). CmNAC1 has a close relationship with the AtATAF1 in Arabidopsis (Supplementary Figure S1), CmNAC1-EE lines have high AtNCED3 expression levels and ABA content (Figures 6F,G). Therefore we speculate that CmNAC1 could bind to the promoter of AtNCED3 to regulate its expression, and this might be a conserved pathway in regulating ABA synthesis in plants. In addition, the expression of CmNAC1 is significantly induced by dehydration and ABA (Figures 1E,G). These results indicate that CmNAC1 play dual role in ABA signaling responsiveness and ABA synthesis regulation. ABREs are recognized by a group of transcription factors, ABRE-binding factors (ABRE/ABFs) (Yoshida et al., 2014). In Arabidopsis AREB1, AREB2, and ABF3 collectively regulate ABRE-dependent ABA signaling involved in drought stress tolerance (Yoshida et al., 2010). Several conserved ABREs were enriched in almost the exact position from promoter of Arabidopsis AtATAF1, cucumber CsATAF1 (Supplementary
Table S1), and rice OsNAC6 (Nakashima et al., 2007). Similarly five ABREs are found in the promoter of CmNAC1 (Table 1); suggesting that ATAF1 homologous genes from different plant species have pivotal functions in ABRE-dependent ABA signaling. Therefore, CmNAC1 might be a downstream gene of ABRE/ABFs transcription factors and cooperatively regulate the ABRE-dependent gene expression. In summary, we can speculate that once the plants are exposed to stress, ABA quickly promotes CmNAC1 expression through the ABRE/ABF pathway. CmNAC1 expression subsequently produces additional NCED3 transcripts to finally obtain increased ABA in a positive feedback loop.

CONCLUSION

The function of CmNAC1 as a new stress-response transcription factor in pumpkin was evaluated in Arabidopsis plants. CmNAC1 plays a positive role in promoting root growth and adapting to salt, cold, and drought stress. The EE plants also had significantly high ABA level, indicating that the ABA signaling pathway is involved in CmNAC1-mediated abiotic stress tolerance. CmNAC1 is a functional transcription factor in pumpkin and a candidate gene for stress tolerance regulation and genetic modification of crops.

AUTHOR CONTRIBUTIONS

ZB, QK, YH, and FC designed the research. HC, LW, MLN, JS, and JX did experiment and analyzed the data. HC and MAN wrote the manuscript. The manuscript is revised by HC and ZB. All authors read and approved the manuscript.

FUNDING

This research work was supported by National Natural Science Foundation of China (31172000, 31372110), China Agriculture Research System (CARS-25), and the International Science and Technology Cooperation Program of China (2015DFG32310).

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ACKNOWLEDGMENTS

The authors are thankful to Prof. Junhong Zhang and Prof. Botao Song for providing plant expression vector Phellsgate8.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.02052/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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