PCP
PLANT & CELL PHYSIOLOGY

CsATAF1 Positively Regulates Drought Stress Tolerance by an ABA-Dependent Pathway and by Promoting ROS Scavenging in Cucumber

Jinfang Wang[1,4], Lei Zhang[1,4], Yunyun Cao[1,4], Chuandong Qi[1], Shuangtao Li[1], Lun Liu[1], Gongle Wang[1], Aijun Mao[2], Shuxin Ren[3] and Yang-Dong Guo[1,*]

[1]College of Horticulture, Beijing Key Laboratory of Growth and Developmental Regulation for Protected Vegetable Crops, China Agricultural University, Beijing 100193, China
[2]Beijing Key Lab of Vegetable Germplasm Improvement, Beijing Vegetable Research Center, Beijing Academy of Agriculture and Forestry Science, Beijing 100097, China
[3]School of Agriculture, Virginia State University, PO Box 9061, Petersburg, VA 23806, USA
[4]These authors contributed equally to this work.
*Corresponding author: E-mail, yaguo@cau.edu.cn; Fax, 86-10-62733404.
(Received November 2, 2017; Accepted January 31, 2018)

Regular Paper

The NAC transcription factors play vital roles in responding to drought stress in plants; however, the molecular mechanisms remain largely unknown in cucumber. Suppression of *CsATAF1* via RNA interference (RNAi) weakened drought stress tolerance in cucumber due to a higher water loss rate in leaves, a higher level of hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2\cdot^-$), increased malondialdehyde (MDA) content, lower F_v/F_m ratios and lower antioxidant enzyme activity. The analysis of root length and stomatal apertures showed that *CsATAF1*-RNAi cucumber plants were less responsive to ABA. In contrast, *CsATAF1*-overexpression (OE) plants showed increased drought stress tolerance and sensitivity to ABA. Quantitative PCR (qPCR) analysis showed that expression of several stress-responsive genes was significantly up-regulated in *CsATAF1*-OE transformants and down-regulated in *CsATAF1*-RNAi transformants. *CsABI5*, *CsCu-ZnSOD* and *CsDREB2C* were verified as direct target genes of CsATAF1. Yeast one-hybrid analysis and electrophoretic mobility shift assay (EMSA) further substantiated that CsATAF1 bound to the promoters of *CsABI5*, *CsCu-ZnSOD* and *CsDREB2C*. Transient expression in tobacco leaves and cucumber protoplasts showed that CsATAF1 directly up-regulated the expression of *CsABI5*, *CsCu-ZnSOD* and *CsDREB2C*. Our results demonstrated that CsATAF1 functioned as a positive regulator in response to drought stress by an ABA-dependent pathway and decreasing reactive oxygen species (ROS) accumulation in cucumber.

Keywords: ABA-dependent pathway • *Cucumis sativus* L • Drought stress • NAC transcription factor • ROS scavenging.

Abbreviations: ABI, abscisic acid insensitive; 3-AT, 3-amino-triazole; 6-BA, 6-benzylaminopurine; CAT, catalase; DAB, diaminobenzidine; DREB, dehydration-responsive element binding; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; GUS, β-glucuronidase; H_2O_2, hydrogen peroxide; LUC, luciferase; MDA, malondialdehyde; MeJA, methyl jasmonate; NAC, NAM, ATAF and CUC; NACRS, NAC recogntion sequences, ATAF and CUC; NBT, nitro blue tetrazolium; $O_2\cdot^-$, superoxide radicle; OE, overexpression; ORF, open reading frame; qPCR, quantitative PCR; PEG, polyethylene glycol; POD, peroxidase; RNAi, RNA interference; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase; WT, wild type.

Introduction

Plants often suffer from different harmful conditions, which have an adverse effect on plant growth and productivity of crops, such as water deficit, high salinity, flooding and extreme temperature (Nakashima et al. 2007). Harmful environmental conditions cause changes in plant morphology and damage to plant cells. Excessive accumulation of reactive oxygen species (ROS) affects cell membrane stability, reduces photosynthetic efficiency, accelerates protein deformation and leads to leaf wilting (Hanin et al. 2011, Choudhury et al. 2013, Choudhury et al. 2017). Plants have evolved various physiological and biochemical abilities to adapt to such harmful conditions. Many stress-induced proteins have been reported in previous studies, including key enzymes in the ABA signaling pathway, numerous protein phosphatases, protein kinases, osmotic adaptive proteins, cellular protective enzymes and transcription factors (Zhu 2002, Zhu 2016).

The NAC (NAM, ATAF and CUC) transcription factor superfamily is only found in plants, and is one of the largest transcription factor families (Olsen et al. 2005). NAC proteins undertake different functions and participate in the regulation of plant development, including lateral root formation, leaf senescence and fruit ripening (He et al. 2005, Guo and Gan 2006, Zhong et al. 2006, Fang et al. 2008, Zhu et al. 2014).

The NAC transcription factor is an important regulator in responding to abiotic stress. Overexpressing *ANAC019*, *ANAC055* and *RD26/ANAC072*, well-characterized NAC stress-responsive genes, in Arabidopsis increased drought stress

Plant Cell Physiol. 59(5): 930–945 (2018) doi:10.1093/pcp/pcy030, Advance Access publication on 5 February 2018, available online at www.pcp.oxfordjournals.org

tolerance (Fujita et al. 2004, Tran et al. 2004). The expression of the rice *SNAC1* gene was induced by salt, drought and ABA, and overexpressing *SNAC1* increased drought stress tolerance in transgenic rice (Hu et al. 2006). ABA is an important plant stress hormone and mediates stress-responsive networks by regulating several NAC genes. The NAC transcription factors regulate the genes which are involved in the ABA pathway. Overexpressing *OsNAP* and *OsNAC022* in rice produced higher drought tolerance via the ABA-dependent pathway and up-regulated stress- and ABA-responsive genes (Chen et al. 2014, Hong et al. 2016). Several studies showed that NAC transcription factors also play important roles in ROS metabolism. OsNAC3 functioned as a transcriptional activator in improving heat and drought tolerance in transgenic rice, and five ROS-associated genes were verified to be direct target genes of OsNAC3 (Fang et al. 2015). GmNAC2 negatively responded to abiotic stress and down-regulated the expression of genes in the ROS signaling pathway (Jin et al. 2013).

In Arabidopsis, there are seven NAC genes belonging to the ATAF1 subfamily (Ooka et al. 2003, Fujita et al. 2004). ATAF1 and ATAF2 function as negative regulators in response to both biotic and abiotic stress (Lu et al. 2007, Mauch-Mani and Flors 2009). *ATAF1* (AT1G01720) was first identified in Arabidopsis, and was induced by drought, salt, ABA and wounding treatments. However, the function of ATAF1 is still under debate. The recovery rate of wild-type (WT) plants was lower than that of the *ataf1* mutants under drought stress. The expression of stress-responsive genes increased much more in the *ataf1* mutants than in the WT plants under stress conditions. In contrast, Wu et al. (2009) reported that *ATAF1*-overexpressing plants showed a tolerance phenotype to drought stress, which is quite different from the result reported by Lu et al. (2007). Overexpression of *ATAF1* conferred salt tolerance to trangenic rice (Liu et al. 2016). Overexpressing *GhATAF1* increased the salt stress tolerance and biotic stress by regulating stress-responsive genes and participating in the phytohormone signaling networks in cotton (He et al. 2016).

The cucumber (*Cucumis sativus* L.) is one of the most important horticultural crops. It often suffers from water deficit, high salinity, flooding and extreme temperature. These stresses significantly reduce yield and even lead to complete failure of production (Wang et al. 2014). A draft of the *C. sativus* L. genome sequence has been reported (Huang et al. 2009). Seven pairs of chromosomes were found in cucumber, and the haploid genome was 367 Mbp. A total of 82 *CsNAC* genes encoding 84 CsNAC transcription factors have been identified in cucumber. *CsATAF1* was reported as *CsNAC41* in a previous study in which it was found that the expression of *CsNAC41* was induced by drought and high salt stress; however, the function and molecular mechanism of this gene in response to drought were still unknown (Zhang et al. 2017).

In this study, *CsATAF1* was isolated and characterized in cucumber. *CsATAF1* was induced by drought, salt, hydrogen peroxide (H_2O_2), ABA and methyl jasmonate (MeJA) treatments. *CsATAF1*-RNA interference (RNAi) plants had weakened drought resistance and were less responsive to ABA, while the *CsATAF1*-overexpressaion (OE) plants showed increased drought tolerance and were hypersensitive to ABA. Furthermore, we demonstrated that *CsATAF1* directly increased the expression level of some stress-responsive genes. Yeast one-hybrid analysis and electrophoretic mobility shift assay (EMSA) further substantiated that CsATAF1 bound to the promoters of *CsABI5*, *CsCu-ZnSOD* and *CsDREB2C*, and directly up-regulated the expression of these three genes. Our results further suggested that CsATAF1 functioned as a crucial positive regulator in drought stress response by an ABA-dependent pathway and promoted ROS scavenging in cucumber.

Results

Sequence analysis of the *CsATAF1* gene

CsATAF1 (Csa4M361820.1) with high homology to the *ATAF1* gene (AT1G01720) was cloned from cucumber. The sequence with an open reading frame (ORF) of 888 codons a peptide of 296 amino acids. The multiple sequence alignment revealed that the CsATAF1 protein contained a highly conservedly N-terminal domain, which was also found in other NAC proteins in rice, orange, Arabidopsis, soybean, tomato and cotton. However, in the C-terminal domain, the cucumber CsATAF1 had a low similarity with the NAC proteins in other species (Supplementary Fig. S1). A phylogenetic tree showed that CsATAF1 clustered in the same clade as ATAF1 and BnNAC2, belonging to the ATAF subfamily (**Fig. 1A**).

Nuclear localization and transcriptional activation of CsATAF1

Transient expression assays in onion epidermis and fluorescence microscopy were used to identify the subcellular location of CsATAF1. The results showed that CsATAF1 localized in the nuclei (**Fig. 1B**).

In order to investigate the transactivation activity of CsATAF1, the yeast one-hybrid system was used. Yeast cells transformed with pBD-*CsATAF1*-FL and pBD-*CsATAF1*-C survived well in SD/-Trp/-His medium and turned blue in the presence of X-α-gal, while yeast cells containing pBD-*CsATAF1*-N and pBD could not survive (**Fig. 1C, D**). Compared with the negative control, the relative β-galactosidase activity of the transformants with pBD-*CsATAF1*-FL and pBD *CsATAF1*-C increased >17-fold and 26-fold, respectively (**Fig. 1E**). These results indicated that the C-terminal region of CsATAF1 was the transcription activation domain, while the N-terminal region of CsATAF1 appeared to lack this activity.

Expression pattern of *CsATAF1*

Quantitative PCR (qPCR) analysis was used to test the expression of *CsATAF1* under abiotic stresses. As shown in Supplementary Fig. S2, after 9 h of dehydration and salt treatments, the relative expression level of *CsATAF1* increased >40- and 60-fold, respectively. Treatment with H_2O_2 increased the relative expression level of *CsATAF1* >140-fold at 6 h. Furthermore, the expression level of *CsATAF1* was examined under treatment with various phytohormones. ABA and MeJA significantly induced the *CsATAF1* transcript level; the relative

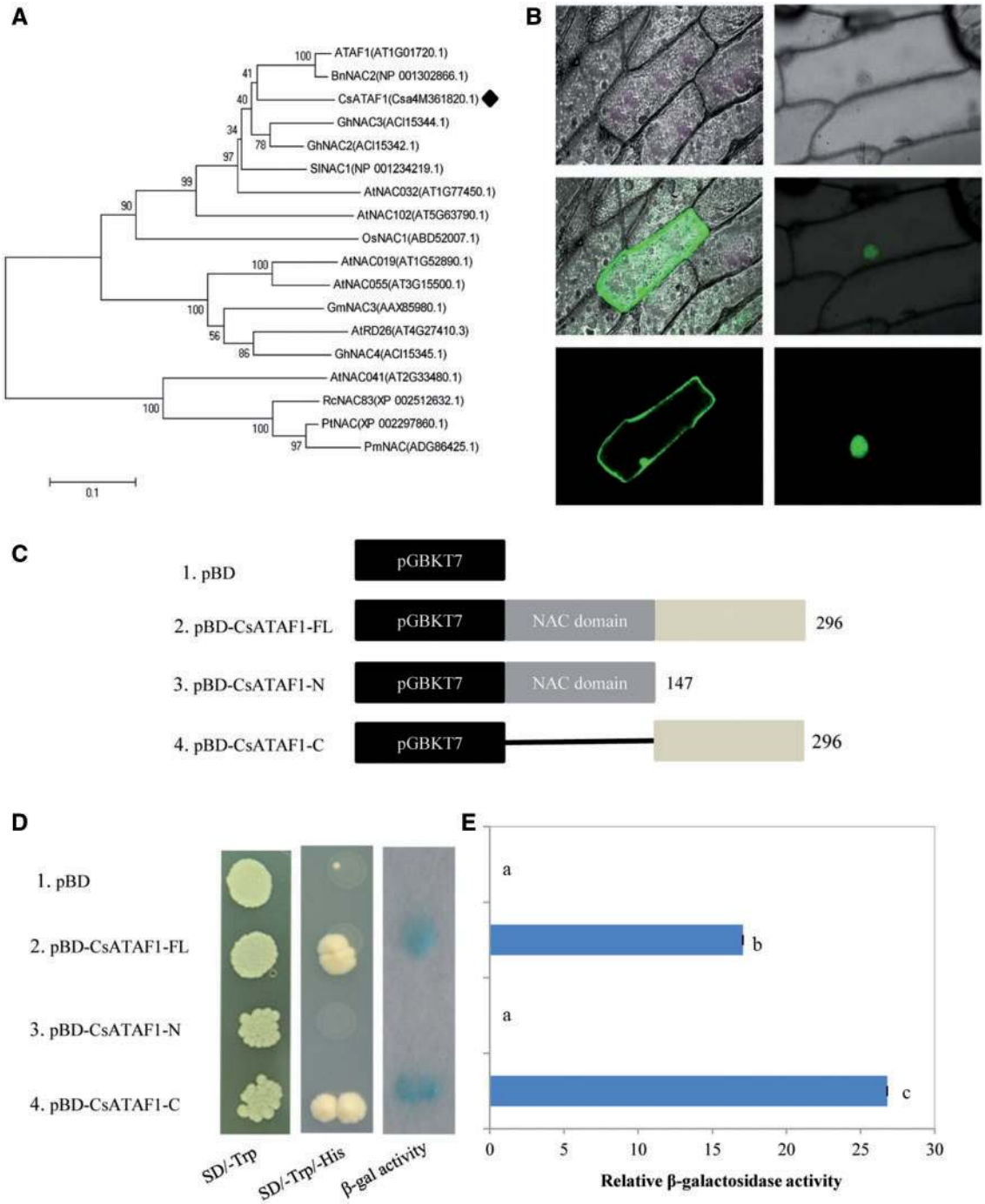

Fig. 1 Sequence analysis, nuclear localization and transcriptional activation of CsATAF1. (A) Phylogenetic tree of CsATAF1 and NAC transcription factors belonging to the NAC subfamily from other plant species. (B) Subcellular localization of the CsATAF1 protein in onion epidermal cells. (C) Transcription activation activity of transcription factor CsATAF1. The full-length protein (CsATAF1-FL), N-terminal fragment (CsATAF1-N) and C-terminal fragment (CsATAF1-C) were fused with the vector pGBKT7. (D) The plasmids containing the fusion genes and the empty control plasmid pGBKT7 were introduced into yeast cells. The pGBKT7 vector was used as a negative control. (E) The β-galactosidase activity of the yeast transformants that expressed the constructs as described above in the yeast strain AH109. Different letters above the columns indicate significant differences ($P < 0.05$).

expression level of *CsATAF1* increased >6- and 13-fold, respectively. Salicylic acid (SA) and 2,4-D slightly increased the relative expression level of *CsATAF1* by >2.4- and 3.5-fold at 3 h, respectively. No obvious changes were detected in response to gibberellin.

Suppressing *CsATAF1* via RNAi decreased tolerance to drought stress in cucumber

In order to illustrate the function of *CsATAF1* in responding to drought stress, RNAi plants were generated. More than 860

regenerated plants were assayed and a total of 19 independent RNAi transgenic cucumber lines were produced. Transgenic cucumber lines were confirmed via PCR and qPCR. In the RNAi plants, the *CsATAF1* transcript level in Ri-1 and Ri-6 was lower than that in other transgenic cucumber lines and WT plants (Supplementary Fig. S3C, D). Therefore, lines Ri-1 and Ri-6 were selected for further drought tolerance evaluation.

Under normal conditions, no obvious difference in growth performance was observed between WT plants and *CsATAF1*-RNAi plants. When water was withheld for 10 d, leaves displayed more serious and earlier wilting in Ri-1 and Ri-6 plants than in WT plants (**Fig. 2A**). The water loss rate of the *CsATAF1*-RNAi plants was much higher than that of the WT plants. After 5 h incubation, Ri-1 and Ri-6 transgenic plants showed a reduction of 55% and 53% in fresh weight, respectively, while WT plants showed a 45% reduction (**Fig. 2B**). After drought treatment, the stomatal apertures of *CsATAF1*-RNAi plants were larger than those of WT plants; these results were consistent with the water loss rates (**Fig. 2C, D**). The malondialdehyde (MDA) content was significantly increased in the *CsATAF1*-RNAi plants, indicating that the membrane damage of *CsATAF1*-RNAi plants was more severe than that of the WT plants under drought stress (**Fig. 2E**). The F_v/F_m ratios of the *CsATAF1-* RNAi lines were lower than those of the WT plants after drought treatment (**Fig. 2F**), indicating that the effect of drought stress on the photosynthetic efficiency of *CsATAF1*-RNAi plants was more serious. These results showed that suppressing *CsATAF1* decreased drought tolerance in transgenic cucumber.

Furthermore, under osmotic stress, the response of the *CsATAF1*-RNAi plants was tested after seed germination with 10% polyethylene glycol (PEG) for 3 d. The root length of the *CsATAF1*-RNAi plants was much shorter than that of the WT plants (**Fig. 2G, H**), implying that the *CsATAF1*-RNAi plants were much more sensitive to osmotic stress.

Overexpression of *CsATAF1* increased drought tolerance in cucumber

More than 750 regenerated plants were assayed, and a total of 27 independent overexpressing transgenic cucumber lines were produced. Transgenic cucumber lines were confirmed via PCR and qPCR. In the OE plants, the *CsATAF1* transcript level in OE-6 and OE-7 was significantly higher than that in the WT plants and other transgenic cucumber lines (Supplementary Fig. S3A, B). Consequently, we selected lines OE-6 and OE-7 for further drought tolerance evaluation.

Under normal conditions, no obvious different growth performance was observed between WT and *CsATAF1*-OE plants. For the drought tolerance analysis, water waas withheld from 4-week-old plants for 10 d. The overexpression plants exhibited wilted and leaf rolling later than the WT plants (**Fig. 3A**). The water loss rate of the *CsATAF1*-OE plants was much lower than that of the WT plants (**Fig. 3B**), and the stomatal apertures of *CsATAF1*-OE plants were smaller than those of WT plants (**Fig. 3C, D**). The MDA content was lower and the F_v/F_m ratios were significantly higher in the *CsATAF1*-OE plants than in the WT plants (**Fig. 3E, F**). After seed germination with 10% PEG for 3 d, the root length of the *CsATAF1*-OE plants was much greater than that of the WT plants (**Fig. 3G, H**). These results showed that overexpression of *CsATAF1* enhanced drought tolerance in transgenic cucumber.

Drought stress leads to the accumulatiuon of ROS. Histochemical staining by diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) was used to detect the accumulation of H_2O_2 and superoxide ($O_2\cdot^-$) radicals in leaves under normal and drought stress conditions. Under normal conditions, the leaves of the WT plants, *CsATAF1*-RNAi and *CsATAF1*-OE plants were not stained by DAB and NBT. The leaves of these three lines were stained brown by DAB and stained blue by NBT after drought stress, and the *CsATAF1*-RNAi plants were stained much more strongly than WT and *CsATAF1*-OE plants (**Fig. 4A, B**). The H_2O_2 and $O_2\cdot^-$ contents of the *CsATAF1*-RNAi plants were much higher than those in WT and *CsATAF1*-OE plants (**Fig. 4C, D**).

To verify the ability of transgenic cucumber to scavenge ROS, we detected the activity of three significant antioxidant enzymes in WT and *CsATAF1* transgenic plants under normal and drought stress conditions. Under both normal and drought conditions, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activity increased much more in the *CsATAF1*-OE plants than in the WT and *CsATAF1*-RNAi plants (**Fig. 4E–G**). These results showed that *CsATAF1*-RNAi plants were more severely damaged by ROS, while overexpression of *CsATAF1* protected transgenic lines from damage. *CsATAF1* positively participated in the ROS scavenging pathway by increasing the activity of SOD, POD and CAT in the antioxidant system under drought stress.

Exogenous ABA affects post-germination growth of *CsATAF1* transgenic plants

The expression level of *CsATAF1* was induced by ABA treatment (Supplementary Fig. S2). In order to test whether *CsATAF1* participated in the ABA signaling pathway, root elongation of transgenic plants was analyzed. Under normal conditions, no different growth performance was observed among the WT, *CsATAF1*-OE and *CsATAF1*-RNAi plants. However, when ABA was applied, the root length of the *CsATAF1*-RNAi plants was greater than that of the WT plants (**Fig. 5A, B**), while the root length of *CsATAF1*-OE plants is shorter than that of WT plants (Supplementary Fig. S4A, B). These results indicated that the *CsATAF1*-OE plants increased ABA sensitivity during the root elongation stage, while the *CsATAF1*-RNAi plants were less responsive to ABA.

ABA plays an important role in controlling stomatal closure (Chen et al. 2016). In order to elucidate the potential role of *CsATAF1* in stomatal regulation, cucumber cotyledon epidermis strips were treated with different concentrations of ABA and the stomatal apertures were measured using the WT and *CsATAF1* transgenic plants. There was no difference between the stomatal apertures of the *CsATAF1* transgenic plants and the WT plants under normal conditions. Under 1 μM ABA treatment for 2.5 h, the stomatal apertures of the

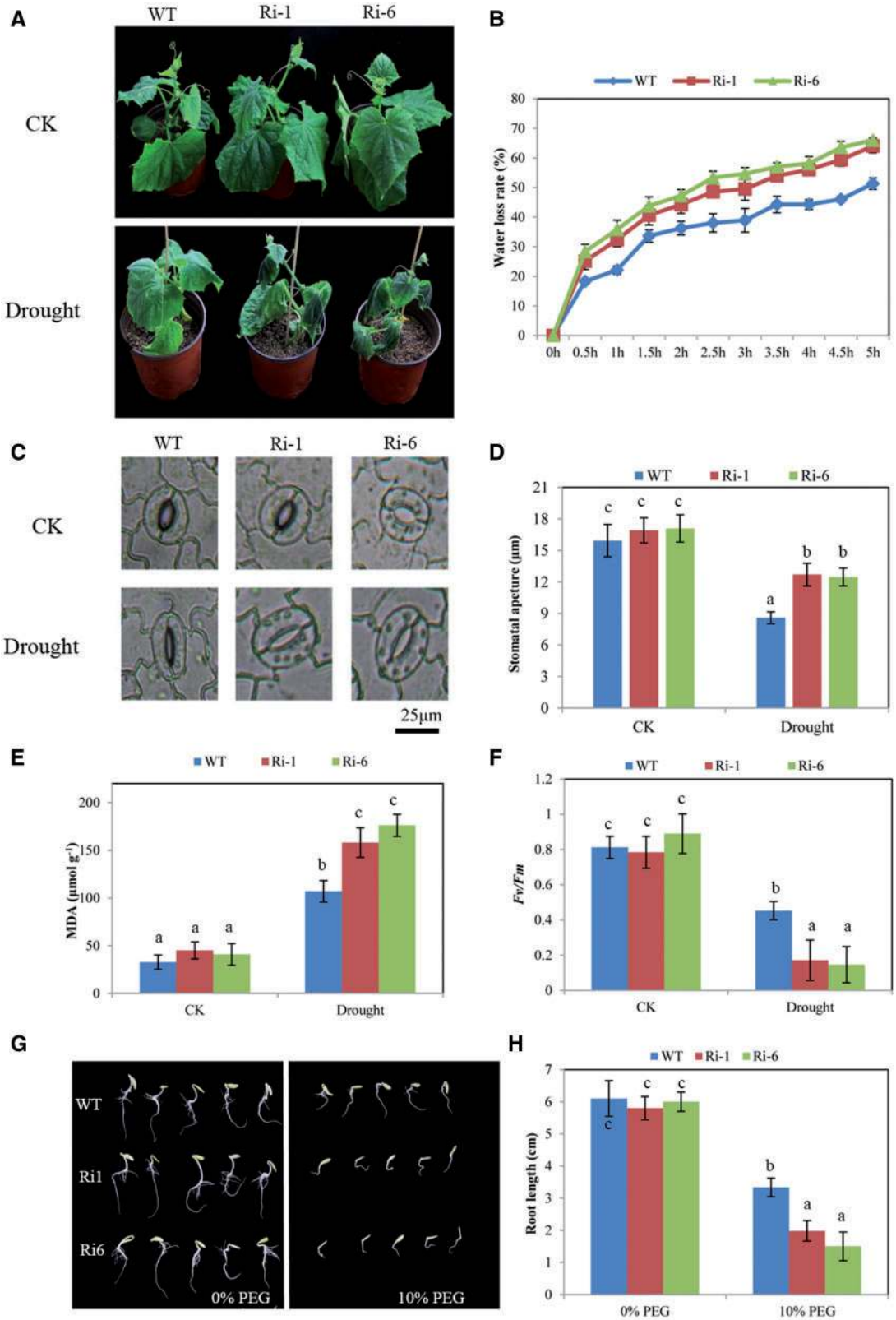

Fig. 2 Suppressing *CsATAF1* in cucumber decreased tolerance to drought stress. (A) Phenotypes of WT and Ri plants under normal and drought conditions. (B)Water loss rate of WT and Ri plants. (C and D) Representative images (C) and stomatal apertures (D) of WT and Ri plants under normal and drought conditions. (E) MDA content. (F) F_v/F_m ratios. Cucumber seedlings treated with water were used as a mock control (CK). (G) Phenotypes of WT and Ri plants grown for 3 d on filter paper supplemented with 0% PEG and 10% PEG. (H) Quantification of primary root length. Different letters above the columns indicate significant differences ($P < 0.05$).

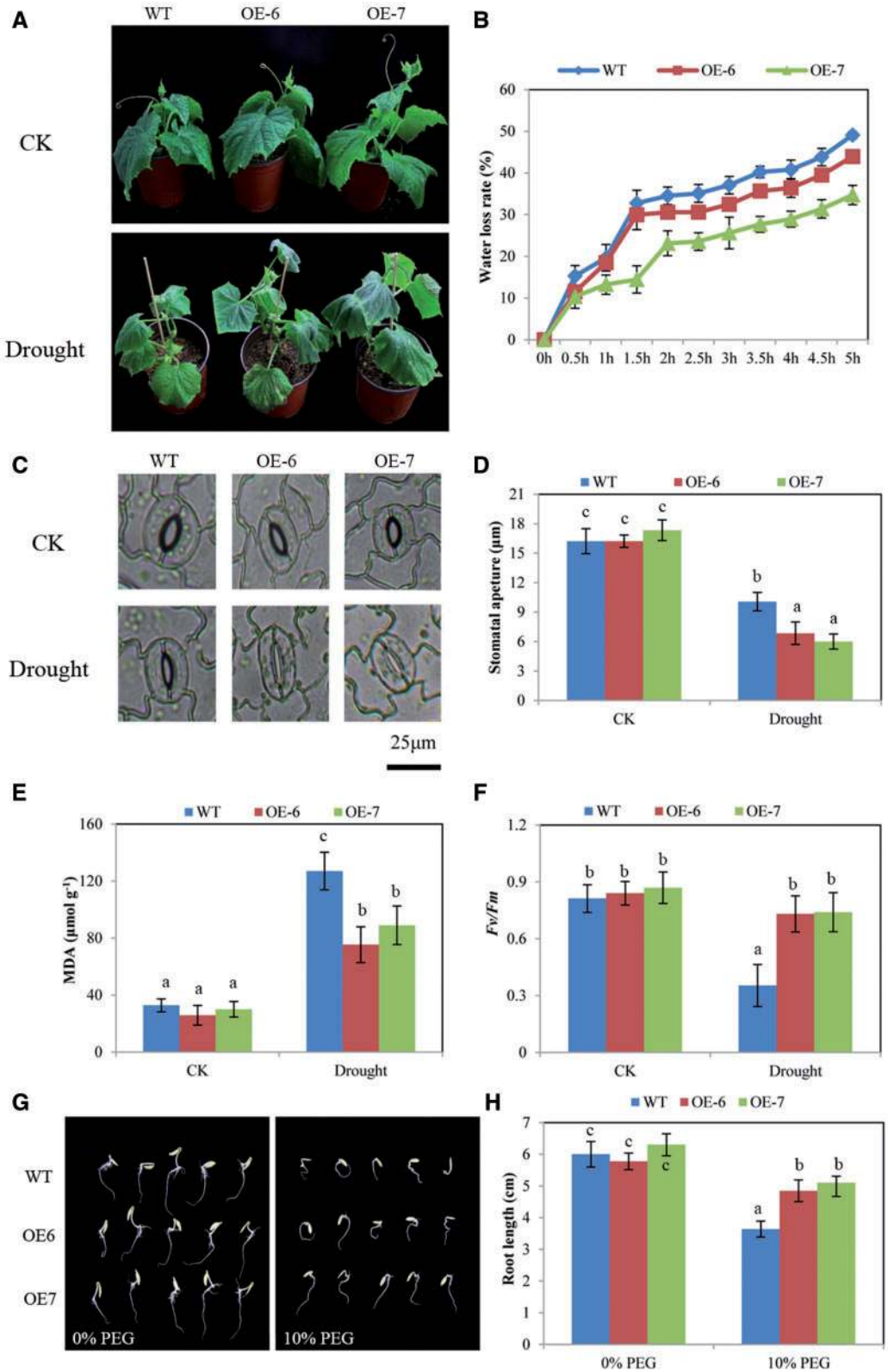

Fig. 3 Overexpression of *CsATAF1* in cucumber increased tolerance to drought stress. (A) Phenotypes of WT and OE plants under normal and drought conditions. (B) Water loss rate of OE and WT plants. (C and D) Representative images (C) and stomatal aperture (D) of WT and OE plants under normal and drought conditions. (E) MDA content. (F) F_v/F_m ratios. Cucumber seedlings treated with water were used as a mock control (CK). (G) Phenotypes of WT and OE plants grown for 3 d on filter paper supplemented with 0% PEG and 10% PEG. (H) Quantification of primary root length. Different letters above the columns indicate significant differences ($P < 0.05$).

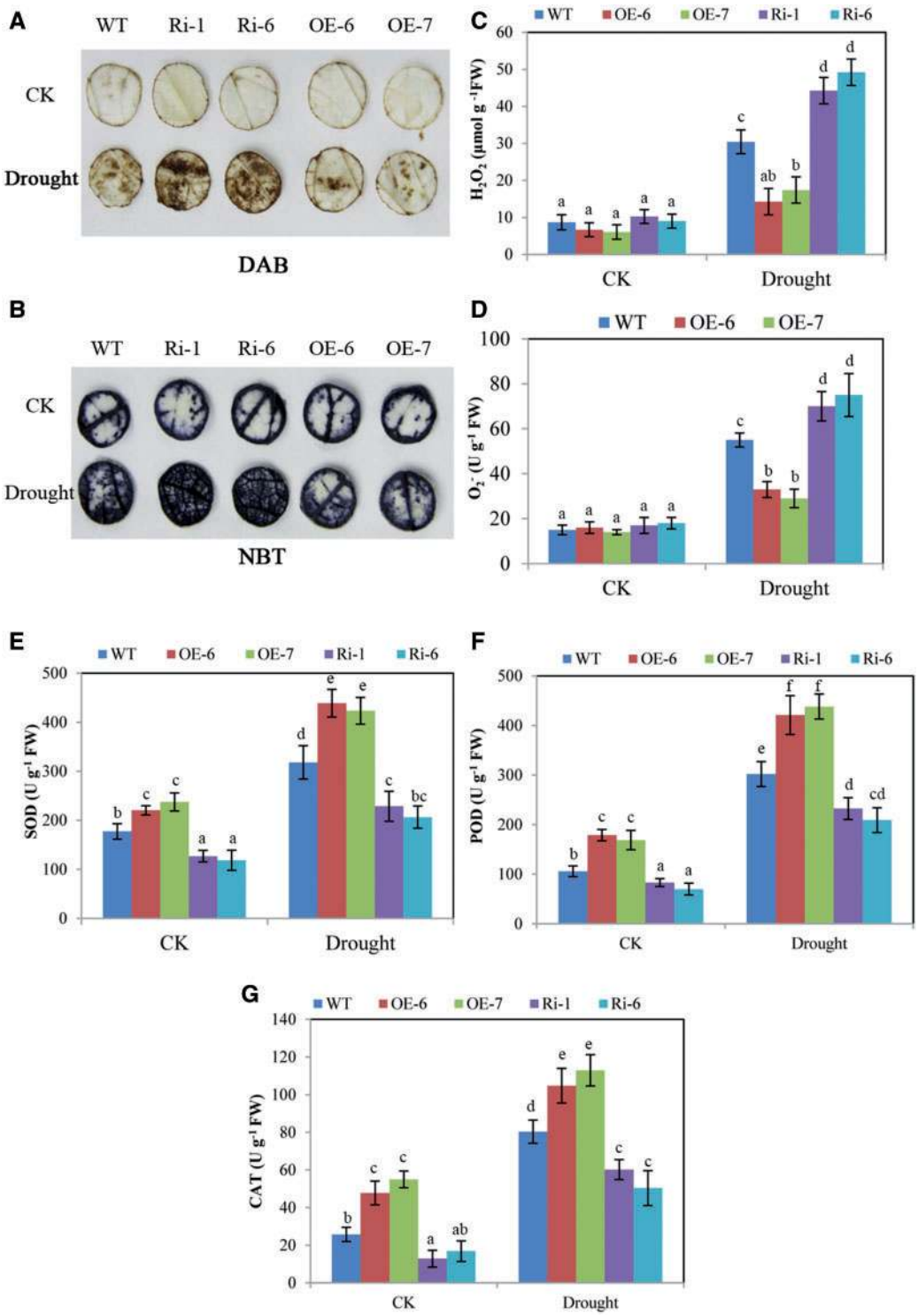

Fig. 4 Analysis of H_2O_2 and $O_2^{\cdot-}$ and three antioxidant enzyme activities in the WT and transgenic lines under both normal and drought conditions. (A and B) Histochemical staining with DAB (A) and NBT (B) for detection of H_2O_2 and $O_2^{\cdot-}$, respectively, in CsATAF1 transgenic plants and WT plants under normal and drought conditions. (C) H_2O_2 content. (D) $O_2^{\cdot-}$ content. (E and G) SOD, POD and CAT activity. Cucumber seedlings treated with water were used as a mock control (CK). Different letters above the columns indicate significant differences ($P < 0.05$).

CsATAF1-RNAi plants were not changed significantly, while the WT plants showed a 34% reduction of stomatal aperture and the stomatal apertures of the *CsATAF1*-OE plants were reduced >41% (**Fig. 5C, D;** Supplementary Fig. S4C, D). With 10 μM ABA, a 65% reduction of stomatal apertures was seen in *CsATAF1*-OE plants, while WT and *CsATAF1*-RNAi plants had reductions of 50% and 23% in stomatal aperture, respectively (**Fig. 5C, D;** Supplementary Fig. S4C, D). These results clearly

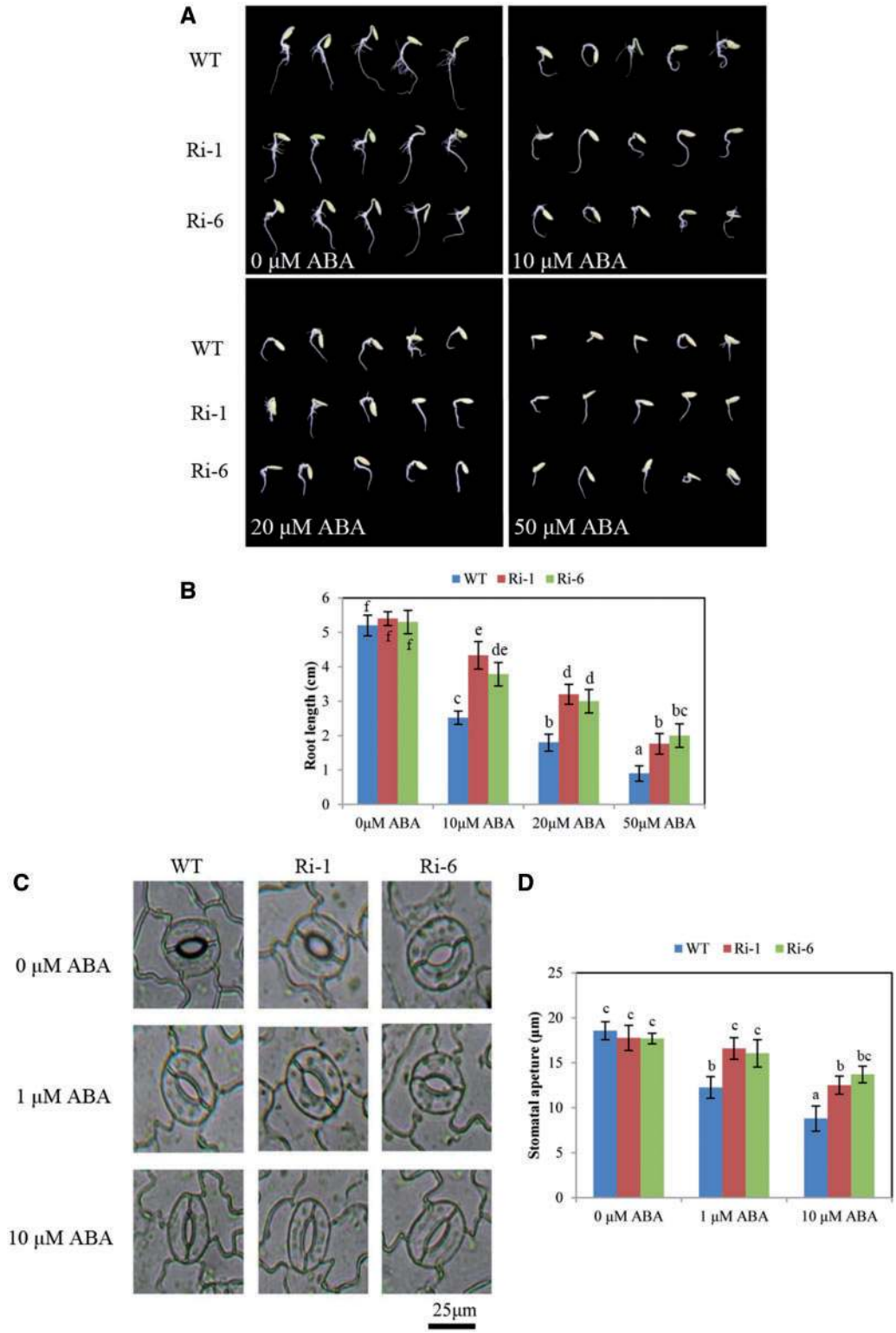

Fig. 5 Suppressing *CsATAF1* led to decreased ABA sensitivity. (A) Comparison of primary root length of transgenic and WT cucumber seedlings with ABA treatment. Seedlings grown on filter paper for 3 d supplemented with 0, 10, 20 and 50 μM ABA. (B) Quantification of primary root length. (C and D) Representative images (C) and stomatal aperture (D) of WT and Ri plants before and after ABA treatments. The epidermis strips of cucumber cotyledon were treated with 0, 1 and 10 μM ABA. Different letters above the columns indicate significant differences ($P < 0.05$).

demonstrated that *CsATAF1* promoted ABA-induced stomatal closure.

CsATAF1 regulates stress-responsive gene expression

Overexpression of *CsATAF1* increased the tolerance to drought stress, while the *CsATAF1*-RNAi plants had weakened tolerance. In order to elucidate the molecular mechanism of the response of *CsATAF1* to stress, 12 stress-responsive genes were selected for further study. The *CsATAF1*-OE plants contained lower H_2O_2 and higher activities of SOD, POD and CAT than the WT plants; these results hinted that *CsATAF1* might be involved in the regulation of ROS homeostasis. Therefore, the genes encoding SOD, POD and CAT were selected for further study. Overexpression of *CsATAF1* in cucumber increased hypersensitivity to ABA, and the root length of the *CsATAF1*-OE plants was inhibited under ABA treatment. Abscisic acid insensitive 3 (ABI3), ABI4 and ABI5 have been reported to have key roles in seed germination, root elongation and drought stress in Arabidopsis (Giraudat et al. 1992, Finkelstein et al. 1998, Finkelstein and Lynch 2000). Therefore, these CsABIs were selected for further research. Arabidopsis dehydration-responsive element binding 1 (*AtDREB1*) and *AtDERB2* are well-known genes responsive to drought stress (Pruthvi et al. 2014, Wei et al. 2016). We therefore selected six DREB genes (*CsDREB1B*, *CsDREB2A*, *CsDREB2C*, *CsDREB2D*, *CsDREB1E* and *CsDREB1A*) for further expression analysis. The proteins encoded by the above 12 genes shared a relatively high degree of amino acid sequence identity with those genes reported in Arabidopsis. The gene numbers of the closest homolog gene in Arabidopsis are shown in Supplementary Table S3. We then measured the expression levels of these 12 genes in 4-week-old *CsATAF1* transgenic plants and WT plants under normal conditions. The results revealed that in the *CsATAF1*-OE plants, the expression of *CsABI5*, *CsCu-ZnSOD*, *CsDREB2C*, *CsDREB1B*, *CsPOD* and *CsCAT* was higher than in the WT plants under normal conditions. In the *CsATAF1*-RNAi plants, the expression of these genes was lower than in the WT plants (**Fig. 6**). These results suggested *CsABI5*, *CsCu-ZnSOD*, *CsDREB2C*, *CsDREB1B*, *CsPOD* and *CsCAT* might be the target genes of *CsATAF1*.

Putative target genes of *CsATAF1*

Since so many stress-responsive genes were up-regulated in the *CsATAF1*-OE plants and down-regulated in the *CsATAF1*-RNAi plants, several of these genes might be the target genes of *CsATAF1*. To verify this hypothesis, the promoters (1.5 kb) of six stress-responsive genes were isolated via genomic PCR (see Supplementary Table S1). CGT (G/A) and CACG were reported to be the core NAC recognition sequences (NACRS) of NAC transcription factors (Simpson et al. 2003, Tran et al. 2004). TT(A,C,G)CGT and T(A,C,G)CGT(A,G) were reported to be the binding sites of *ATAF1* in Arabidopsis (Jensen et al. 2013). There was no ATAF1-binding site in the promoter of *CsPOD*; however, the promoters of the other five genes contained ATAF1-binding sites. The promoters of these six genes all contained supplementary Table S2 NACRS. We performed a yeast one-hybrid assay to test the interaction between CsATAF1 and the promoters of putative target genes. The result showed that the transformants of pGADT7-*CsATAF1*, along with pHIS2-*proCsCu-ZnSOD*, pHIS2-*proCsABI5* or pHIS2-*proCsDREB2C*, survived on the SD/-Trp/-Leu/-His medium containing 30 mM 3-aminotriazole (3-AT), whereas the co-transformants of pGADT7-*CsATAF1* along with pHIS2-*proCsDREB1B*, pHIS2-*proCsPOD* or pHIS2-*proCsCAT* could not survive (**Fig. 7A**). To examine whether *CsATAF1* directly bound to the promoters of *CsABI5*, *CsCu-ZnSOD* and *CsDREB2C* in vitro, an EMSA was performed. Probes were designed from the promoters of these genes containing CGT[G/A] or CACG, and CsATAF1 bound to all three promoters (**Fig. 7B**). The increasing concentrations of unlabeled core probes (competitor) reduced the binding of CsATAF1 to the labeled probes. These results suggested that CsATAF1 could directly bind to the promoters of *CsCu-ZnSOD*, *CsABI5* and *CsDREB2C*.

Furthermore, a transient expression assay of the promoter activity in tobacco leaves was used to test whether CsATAF1 regulated the target gene as a transcriptional activator. Compared with the control vector, the promoter activity, expressed as the β-glucuronidase (GUS)/luciferae (LUC) ratio, of those in the presence of CsATAF1 was significantly higher (**Fig. 7C**). To confirm this further, a dual-luciferase assay in cucumber protoplasts was performed. The promoter activity was shown as the LUC/REN ratio; the LUC/REN ratio of cucumber co-transformed with effector and reporter vectors was much higher than that of the control (**Fig. 7D**). These results showed that CsATAF1 might be a transcriptional activator in regulation of *CsCu-ZnSOD*, *CsABI5* and *CsDREB2C*.

Discussion

CsATAF1 functions as a positive regulator responding to drought stress in cucumber

The function of ATAF1 in response to drought stress in Arabidopsis is still under debate. ATAF1 was reported to regulate the expression of target genes negatively and to decrease the tolerance to drought (Lu et al. 2007, Jensen et al. 2008). However, Wu et al. (2009) reported that ATAF1 enhanced drought tolerance in overexpression lines. In this study, the CsATAF1 functioned as a positive regulator in response to drought stress and increased the ROS-scavenging ability in cucumber under drought stress (**Figs. 2, 3, 5**; Supplementary Fig. S4).

The way in which CsATAF1 participated in ROS scavenging was different from Arabidopsis ATAF1. After H_2O_2 treatment, *ATAF1*-overexpressng Arabidopsis plants significantly accumulated higher H_2O_2 and *ataf1* mutant plants accumulated less H_2O_2 (Lu et al. 2007, Wu et al. 2009, Garapati et al. 2015). Abiotic stresses led to oxidative damage and accumulation of $O_2\cdot^-$ and H_2O_2 (Alexieva et al. 2001, Hu et al. 2012). After drought treatment, the *CsATAF1*-OE plants accumulated less H_2O_2 and $O_2\cdot^-$ than the *CsATAF1*-RNAi plants and WT plants (**Fig. 4A–D**). The activity of antioxidant enzymes increased under drought stress to protect plants against oxidative damage (**Fig. 4E–G**).

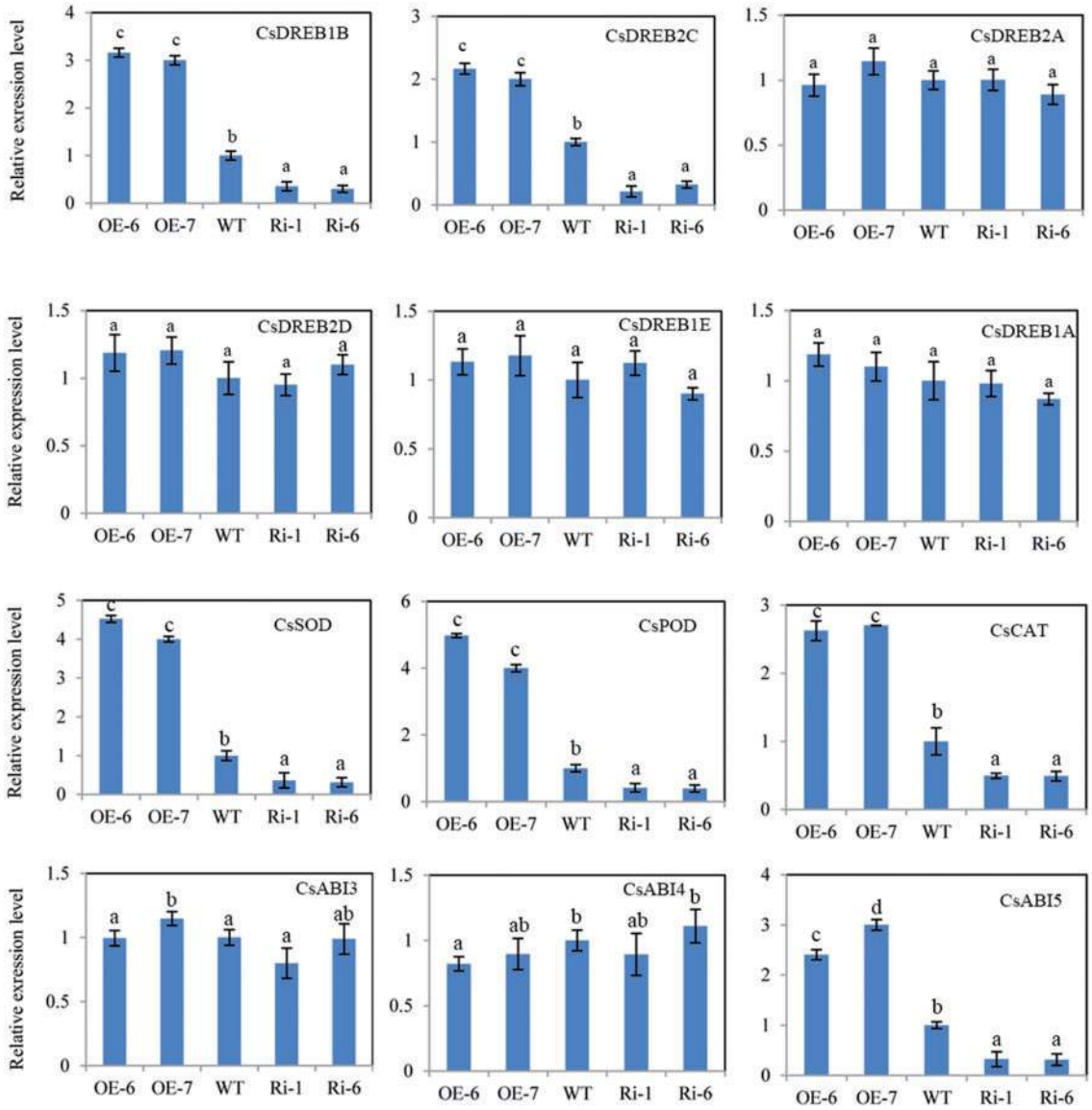

Fig. 6 Expression level of ROS-related and stress-responsive genes in the WT and *CsATAF1* transgenic lines under normal conditions analyzed by qPCR. The transcript level was normalized to *CsActin*. Different letters above the columns indicate significant differences ($P < 0.05$).

CsCu-ZnSOD, *CsPOD* and *CsCAT* encode three important antioxidant enzymes. These three genes were induced in *CsATAF1*-OE plants, and down-regulated in *CsATAF1*-RNAi plants. *CsCu-ZnSOD* was directly regulated by *CsATAF1*, while the yeast one-hybrid assay showed that *CsATAF1* could not bind to the promoter of *CsPOD* and *CsCAT* (**Fig. 7**). We suspected that there might exist an unknown factor which was regulated by CsATAF1 and in turn regulated the expression of *CsPOD* and *CsCAT*. To illustrate the detailed mechanisms of the interactions between CsATAF1 and ROS genes, further investigations are needed. These results indicated that CsATAF1 participated in drought stress resistance by regulating ROS scavenging-related genes. Furthermore, we speculated that the different transcriptional activation regions between AtATAF1 and CsATAF1 which determine the functions of NAC transcription factors might result in different mechanisms of regulation of downstream genes (see Supplementary Fig. S1).

CsATAF1 increased drought stress tolerance via an ABA-dependent signaling pathway

ABA plays an important role in many physiological processes, such as seed development and germination, root growth and stomatal movement (Fujita et al. 2011). In this study, *CsATAF1* was induced by ABA and increased hypersensitivity to ABA (**Fig. 5**; see Supplementary Figs. S2, S4).

ABI5 mediates seed germination, root elongation and abiotic stress by regulating ABA-responsive genes (Yao et al. 2011, Yang et al. 2016). In this study, we identified that CsATAF1 bound to the promoter of *CsABI5* by using yeast one-hybrid analysis and EMSA; tobacco leaf and cucumber protoplast transient expression experiments also revealed that CsATAF1 could directly activate *CsABI5* (**Fig. 7**). *EM1* and *EM6* encoding LEA (late embryogenesis abundant) proteins were the first target genes of ABI5 (Finkelstein and Lynch 2000). ABI5 positively

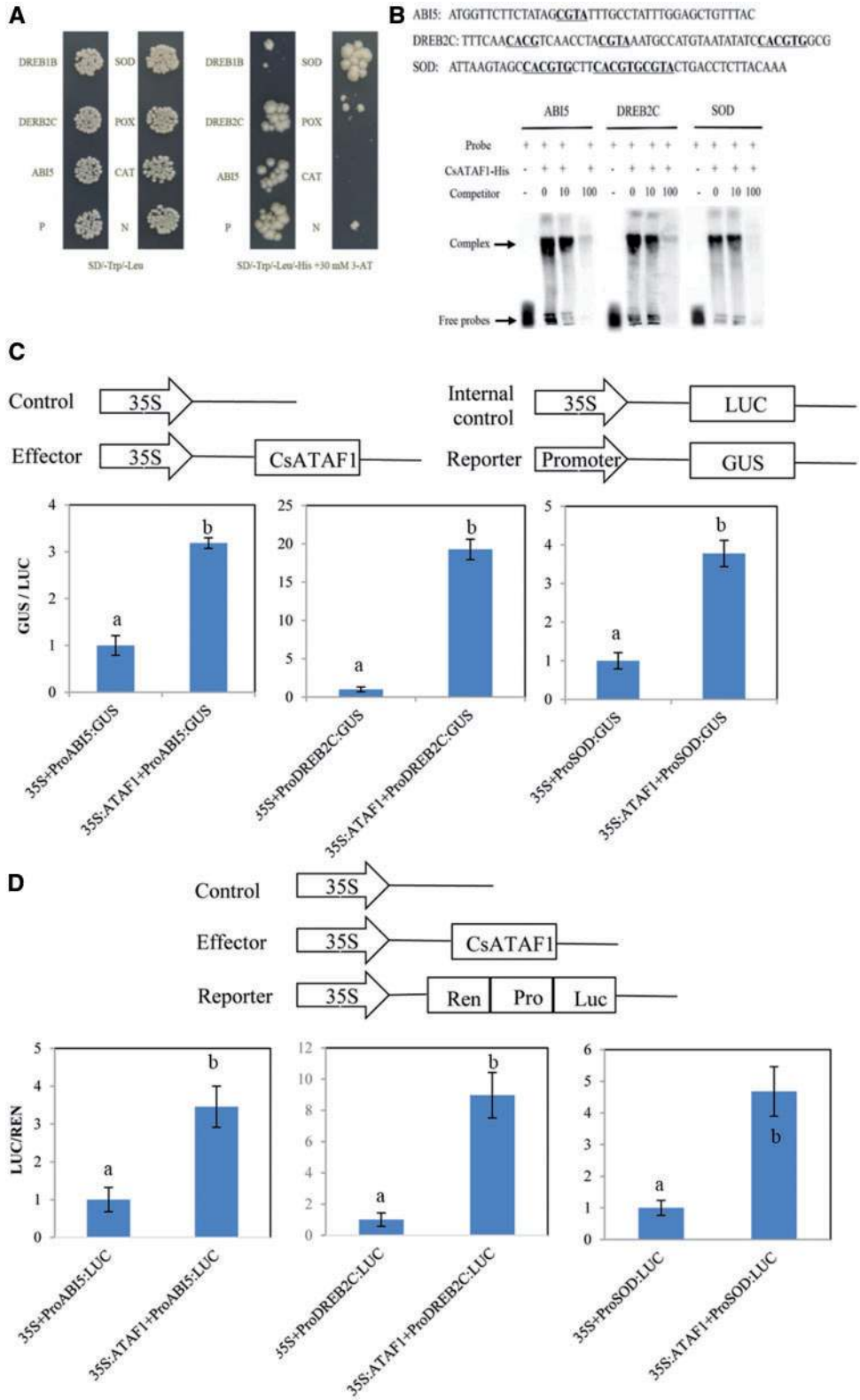

Fig. 7 *CsATAF1* directly regulates *CsDREB2C*, *CsCu-ZnSOD* and *CsABI5*. (A) Growth of pGADT7-*CsATAF1* and each of the reporter constructs on SD/-Leu/-Trp medium and on SD/-Leu/-Trp/-His medium containing 30 mmol l^{-1} 3-AT. pGADT7-53 was co-transformed with pHIS2-P53 as a positive control (P) and pGADT7-*CsATAF1* was co-transformed with pHIS2-P53 as a negative control (N). (B) EMSA of CsATAF1 binding to *CsABI5*, *CsDREB2C* and *CsCu-ZnSOD* promoters. Biotin-labeled probes incubated with His protein served as negative controls. (C and D) Transient expression assay of the promoter activity in tobacco leaves (C) and cucumber protoplasts (D) co-transformed with the effector and the reporter. The GUS/LUC ratio of tobacco leaves and the LUC/REN ratio of protoplasts transformed with the control vector and the reporter were set to 1. Different letters above the columns indicate significant differences ($P < 0.05$).

regulated the expression of the ABA-responsive genes *Cor6.6*, *Cor15a* and *Rab18* (Brocard et al. 2002). Therefore, *CsRAB18*, *CsEM1* and *CsCOR413* were selected for further research. The expression of *CsRAB18* and *CsCOR413* was significantly higher in *CsATAF1*-OE plants than in WT plants (Supplementary Fig. S5). These results showed that *CsATAF1* participated in the ABA signaling pathway by up-regulating ABA-responsive genes.

DREB transcription factors positively regulate the expression of some stress-responsive genes to increase the tolerance to drought stress via an ABA-dependent pathway (Liu et al. 1998, Lata and Prasad 2011, Mizoi et al. 2012). In this study, we selected six DREB genes, which were reported to respond to drought stress. Among these genes, *CsDREB2C* and *CsDREB1B* were induced by *CsATAF1* under normal conditions, and *CsATAF1* bound to the promoter of *CsDREB2C* but not to that of *CsDREB1B* (**Figs. 6, 7A**). *RD29A*, *RD29B*, *ERD15* and aquaporin genes were reported to be the target genes of DREB transcription factors (Jiang et al. 2017, Liao et al. 2017). The expression levels of *CsRD29A*, *CsRD29B*, *CsERD15* and *CsTIP41* were also induced in *CsATAF1*-OE plants (Supplementary Fig. S5). CsATAF1 functioned as a positive regulator in response to drought by regulating stress-responsive genes. NAC transcription factors function in drought tolerance via regulating ABA-dependent and ABA-independent genes (Xu et al. 2013). Whether CsATAF1 regulates ABA-independent genes remains unclear and needs further study.

Cucumber is an important horticultural crop, but the molecular mechanism of drought resistance has yet to be studied in depth. In the current study, *CsATAF1* of cucumber was isolated and characterized. *CsATAF1* was induced by drought stress, directly bound to the promoters of *CsABI5*, *CsCu-ZnSOD* and *CsDREB2C*, and regulated the expression of these genes to resist drought stress (**Fig. 8**). The expression of *CsPOD*, *CsCAT* and *CsDREB1B* was indirectly up-regulated by CsATAF1. CsATAF1 probably interacts with other factors to regulate the expression of these genes. Further studies are required to unravel the mechanism of the response of NAC transcription factors to drought stress in cucumber.

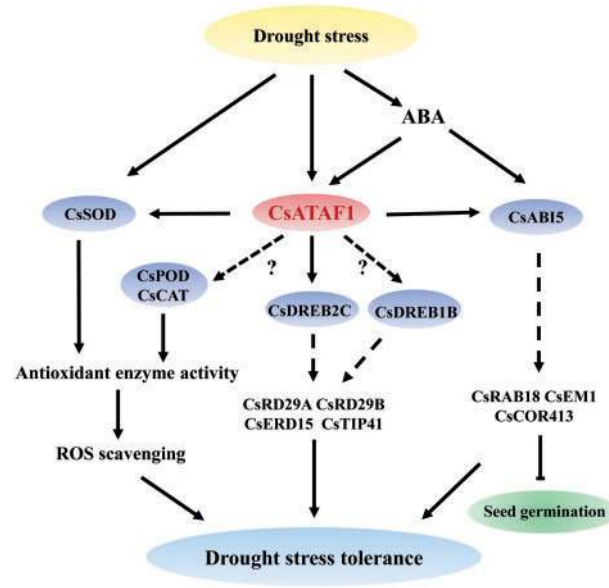

Fig. 8 A working model for CsATAF1 functions in drought response. CsATAF1 directly binds to the promoters of *CsDREB2C*, *CsABI5* and *CsCu-ZnSOD*, and induces the expression of *CsDREB2C*, *CsABI5* and *CsCu-ZnSOD*, as a response to drought stress. The way in which *CsATAF1* regulates *CsDREB1B*, *CsPOD* and *CsCAT* is unknown. A straight line indicates that the pathway was identified in the present study, whereas a dotted line indicates an unknown pathway. The arrows indicate positive regulation and the bar indicates negative regulation.

Materials and Methods

Cloning and sequence analysis of *CsATAF1*

We obtained the full-length ORFs of *CsATAF1* via RT-PCR; the primer pair was designed via the cDNA sequence of *CsATAF1* (Supplementary Table S1). Alignment of CsATAF1 and NAC sequences in other species was performed with DNAman. The MEGA program (ver 5.0) was used to construct the phylogenetic tree via the Neighbor–Joining (NJ) algorithm.

Plant material and stress treatments

For expression analysis of *CsATAF1* in cucumber, seedlings were planted in Hoagland nutrient solution with a 16 h light/8 h dark cycle at 28°C/18°C. Four-week-old seedlings were subjected to stress treatments including dehydration (nutrient solution containing 10% PEG), salt (nutrient solution containing 150 mM NaCl), H_2O_2 (nutrient solution containing 10% H_2O_2) and hormone treatments, spraying 100 μM of the following on the leaves: ABA, MeJA, SA, gibberellin (GA_3) and 2,4-D. Leaves were collected after different stress treatments at the designated time (0, 1, 3, 6, 9, 12, and 24 h).

For evaluation of drought stress tolerance, T_2 transgenic and WT plants were used to perform the experiments. These seedlings were placed in a growth chamber under a 16 h/8 h light/dark cycle at 28°C/18°C. Water was withheld from 4-week-old cucumber plants for 10 d. Cucumber seedlings treated with water were used as a mock control. The drought treatment experiments were repeated three times for transgenic and WT plants. Approximately 60 seeds of homozygous CsATAF1 transgenic lines and WT lines were germinated on filter paper containing PEG and ABA (10, 20 and 50 μM) under dark conditions at 28°C/18°C. Cucumber seeds of WT and transgenic lines germinated with water were used as a mock control. Root length was measured on the third day. Each experiment was performed in triplicate.

Quantitative real-time PCR

The first-strand cDNA was synthesized using Prime Script™ RT reagent Kit (TAKARA). qPCR was conducted on a 7500 Real-time PCR System (Applied Biosystems) using the KAPA SYBR fast Universal qPCR Kit (KAPA) according to the protocol. The $2^{-\Delta\Delta CT}$ method was used for qPCR analysis. The sequences of the primer utilized are listed in Supplementary Table S1. The transcript level was normalized to *CsActin*. Three biological replicates were performed for each sample.

Subcellular localization of CsATAF1 protein in epidermal cells of onion

The coding sequence of *CsATAF1* was cloned into pCAMBIA 1302 (Addgene) (Supplementary Table S1). Both fusion constructs [*CsATAF1*–green fluorescent protein (GFP)] and control vectors (GFP) were transformed into onion epidermal cells. These cells were analyzed by confocal microscopy with bright field and fluorescence imaging after incubation for 26 h in the dark at 25°C (Nikon Inc.). The experiment was performed in three technical replicates.

Transactivation assay of *CsATAF1*

The PCR products of *CsATAF1* (pGBKT7-*CsATAF1*-FL$^{1-296}$), the N-terminus (pGBKT7-*CsATAF1*-N$^{1-147}$) and the C-terminus (pGBKT7-*CsATAF1*-C$^{147-296}$) were inserted into the pGBKT7 vector (Supplementary Table S1). These above constructs were transferred into the yeast strain AH109 (**Fig. 1C**). The transferred strains were streaked on SD/-Trp and SD/-Trp/-His plates. The transcriptional activation activity and quantification of transformants were performed according to the yeast protocols handbook (Clontech). The pGBKT7 vector was used as a negative control. Each experiment was performed in triplicate.

Plant transformation

To construct *CsATAF1*-OE transgenic cucumber lines, the full-length *CsATAF1* cDNA was inserted into pCAMBIA1302 (Supplementary Table S1). To construct the *CsATAF1*-RNAi transgenic cucumber plants, the target sequence 441–888 bp of *CsATAF1* was used; two segments of *CsATAF1* were amplified using specific primers (Supplementary Table S1). Both segments were inversely inserted into the pFGC1008 vector (Addgene). Then we transferred these constructs into *Agrobacterium tumefaciens* strain GV3101 via the heat shock method described by the manufacturer (Huayueyang). The cucumber line 'Jinyan-4' was used for the transformation method. Briefly, after 3 d of seed germination, the cotyledons were cut into two halves, the upper halves of cotyledons were removed, and the other halves without growing points were dipped in Murashige and Skoog (MS) liquid medium containing *A. tumefaciens* for 15 min. These explants were then dried on filter paper and placed on medium containing 1 mg l^{-1} ABA, 0.5 mg l^{-1} 6-benzylamimnopurine (6-BA) and co-cultured for 2 d at 25°C in the dark. These explants were next moved to resistance differentiation medium containing 1 mg l^{-1} ABA, 0.5 mg l^{-1} 6-BA, 500 mg l^{-1} carbenicillin (Sigma) and 5 mg l^{-1} hygromycin and then cultured for 2–3 weeks at 25°C, with a 16 h light/8 h dark cycle in an artificial climate chamber (Cheng et al. 2015). The hygromycin-resistant buds were cut and moved into rooting medium containing 10 mg l^{-1} hygromycin and 600 mg l^{-1} carbenicillin for rooting. The procedures for generation of RNAi and OE plants are the same. PCR and qPCR were used to screen the positive plants in regenerated plants. PCRs with the *GFP* primer pair were used to evaluate the overexpressing transgenic plants; the length of the *GFP* fragment is 521 bp (Supplementary Table S1). To detect the RNAi plants, the *HPT* (hygromycin B phosphotransferase) primer pair was designed; the length of the *HPT* fragment is 667 bp (Supplementary Table S1).

Analysis of water loss rate, MDA and ROS accumulation, antioxidant enzyme activity and stomatal aperture

The WT and the transgenic cucumber plants under drought stress conditions were used for analysis of water loss rate, MDA, the content of H_2O_2 and $O_2\cdot^-$ and antioxidant enzyme activity. The water loss rate was measured according to Mao et al. (2012). Chl *a* fluorescence was measured with a Chl fluorescence imaging system (IMAGING-PAM, Walz and Effeltrich). The MDA content in leaves was measured according to Zhang et al. (2009). The content of H_2O_2 and $O_2\cdot^-$, and the activities of SOD, POD and CAT in leaves were measured with a previously described method (Ranieri et al. 2000, Alexieva et al. 2001, Li et al. 2017). H_2O_2 and $O_2\cdot^-$ were examined by histochemical staining with DAB and NBT, respectively, according to Fryer et al. (2002).

Stomatal aperture measurement

The epidermis strips of cucumber cotyledon were used to measure stomatal aperture. For ABA sensitivity analysis, the epidermis strips were floated in a solution of 30 mM KCl and 10 mM MES-KOH (pH 6.15) for 2.5 h at 22°C in a light incubator to open the stomata fully (Ramirez et al. 2009). ABA was added in the same solution under light for another 2.5 h. More than 130 stomata of the WT and transgenic plants were individually measured using IMAGEJ 1.36 b software (Broken Symmetry Software). Epidermis strips of cucumber cotyledon without ABA treatment were used as a mock control. Each experiment was performed in triplicate.

Yeast one-hybrid assay

The yeast one-hybrid assay followed the manufacturer's protocols (Clontech). The PCR products of *CsATAF1* were fused to the pGADT7-Rec2 vector (Clontech) and the promoters of the target genes were fused to the pHIS2 vector. The growth performance of yeast strain Y187 containing the two constructs was observed on SD/-Leu/-Trp and SD/-Leu/-Trp/-His medium in the presence of 30 mM 3-AT. pGAD-53 was co-transformed with pHIS2-P53 as a positive control and pGADT7-CsATAF1 was co-transformed with pHIS2-P53 as a negative control. The experiment was performed in triplicate.

Protein expression and EMSAs

The coding sequence (CDS) of *CsATAF1* was cloned into the prokaryotic expression vector pET30a (Supplementary Table S1). The construct was transformed into *Escherichia coli* strain BL21. The CsATAF1-His protein was induced overnight with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16°C and

purified with Ni-NTA resin (Qiagen). For EMSA, probes were the biotin-labeled segments of the promoters of the target genes containing NACRS *cis*-elements and the competitors were the non-biotin-labeled segments of the same sequences.

EMSAs were performed according to the protocol of the Light Shift Chemiluminescent EMSA Kit (Pierce). Biotin-labeled probes served as negative controls.

Transient expression assay in *Nicotiana benthamiana*

To construct pro*CsABI5*, pro*CsCu-ZnSOD* and pro*CsDREB2C*, the 1.5 kb promoters of *CsABI5*, *CsCu-ZnSOD* and *CsDREB2C* were cloned into the reporter vector pCAMBIA1391 (Addgene) and *CsATAF1* was cloned into the effector vector pCAMBIA1302 (Supplementary Table S1). The empty vector pCAMBIA1302 was used as the control. The effector, reporter and *35S:LUC* vectors were co-transferred into tobacco leaves by transient transformation (Huang et al. 2016). The GUS and LUC activity of the infiltrated leaves were measured respectively. The GUS/LUC ratio of tobacco leaves transformed with the control vector and the reporter was set to 1. Each experiment was performed in triplicate.

Transient expression assay in cucumber protoplasts

To construct pro*CsABI5*, pro*CsCu-ZnSOD* and pro*CsDREB2C*, the 1.5 kb promoters of *CsABI5*, *CsCu-ZnSOD* and *CsDREB2C* were cloned into the reporter vector pGreen II 0800-LUC (Addgene) and *CsATAF1* was cloned into the effector vector pGreenII 62-SK (Supplementary Table S1). The empty vector pGreenII 62-SK was used as control. The effector and reporter constructs were co-transferred into tobacco leaves by transient transformation. The LUC/REN ratio of tobacco leaves transformed with the control vector and the reporter was set to 1. Each experiment was performed in triplicate. The isolation and transformation of cucumber protoplasts were performed according to a previous method (Huang et al. 2013). The dual-luciferase activity of protoplasts was measured via the Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

The data were analyzed by Duncan's multiple range tests ($P < 0.05$) in SPSS 18.0 software (IBM).

Accession numbers

Sequence data for the cucumber genes described in this study can be found in the Cucurbit Genomics Database under the following accession numbers: Csa4M361820.1 for *CsATAF1*, Csa7M336510.1 for *CsABI3*, Csa6M011730.1 for *CsABI4*, Csa6M152920.1 for *CsABI5*, Csa3M180260.1 for *CsDREB1B*, Csa6M124180.1 for *CsDREB2A*, Csa6M012810.1 for *CsDREB2C*, Csa2M363010.1 for *CsDREB2D*, Csa5M155570.1 for *CsDREB1E*, Csa5M174570.1 for *CsDREB1A* Csa1M573600.2 for *CsCu-ZnSOD*, Csa7M419570.1 for *CsPOD*, Csa4M658600.1 for *CsCAT*, Csa3M914030.2 for *CsRD29A*, Csa3M914030.1 for *CsRD29B*, Csa4M045040.1 for *CsRAB18*, Csa6M013350.1 for *CsERD15*, Csa6M445100.1 for *CsPIP2; 2*, Csa7M071610.1 for *CsTIP41*, Csa4M311730.1 for *CsEM1*, and Csa1M459500.1 for *CsCOR413*.

Supplementary Data

Supplementary data are available at PCP online.

Acknowledgments

We thank Prof. Yan Guo (China Agricultural University) for helpful discussion and critical reading of the manuscript.

Funding

This work was partly supported by the National Key Research and Development Program of China (2016YFD0101007) to Y.-D.G., the Beijing Key Research and Development Program (BNSF6152015 and BLVT-03) to Y.-D.G. and the National Key Research and Development Program of China (2016YFD0100204) to A. M.

Disclosures

The authors have no conflicts of interest to declare.

References

Alexieva, V., Sergiev, I., Mapelli, S. and Karanov, E. (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant. Cell Environ.* 24: 1337–1344.

Brocard, I.M., Lynch, T.J. and Finkelstein, R.R. (2002) Regulation and role of the Arabidopsis Abscisic Acid-Insensitive 5 gene in abscisic acid, sugar, and stress response. *Plant Physiol.* 129: 1533–1543.

Chen, H.-Y., Hsieh, E.-J., Cheng, M.-C., Chen, C.-Y., Hwang, S.-Y. and Lin, T.-P. (2016) ORA47 (octadecanoid-responsive AP2/ERF-domain transcription factor 47) regulates jasmonic acid and abscisic acid biosynthesis and signaling through binding to a novel cis-element. *New Phytol*. 211: 599–613.

Chen, X., Wang, Y., Lv, B., Li, J., Luo, L., Lu, S., et al. (2014) The NAC family transcription factor OsNAP confers abiotic stress response through the ABA pathway. *Plant Cell Physiol.* 55: 604–619.

Cheng, J., Wang, Z., Yao, F., Gao, L., Ma, S., Sui, X., et al. (2015) Down-regulating CsHT1, a cucumber pollen-specific hexose transporter, inhibits pollen germination, tube growth, and seed development. *Plant Physiol.* 168: 635–647.

Choudhury, S., Panda, P., Sahoo, L. and Panda, S.K. (2013) Reactive oxygen species signaling in plants under abiotic stress. *Plant Signal. Behav*. 8: e23681.

Choudhury, F.K., Rivero, R.M., Blumwald, E. and Mittler, R. (2017) Reactive oxygen species, abiotic stress and stress combination. *Plant J.* 90: 856–867.

Fang, Y., Liao, K., Du, H., Xu, Y., Song, H., Li, X., et al. (2015) A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species in rice. *J. Exp. Bot.* 66: 6803–6817.

Fang, Y., You, J., Xie, K., Xie, W. and 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: 547–563.

Finkelstein, R.R. and Lynch, T.J. (2000) The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. *Plant Cell* 12: 599–609.

Finkelstein, R.R., Wang, M.L., Lynch, T.J., Rao, S. and Goodman, H.M. (1998) The Arabidopsis abscisic acid response locus ABI4 encodes an APETALA2 domain protein. *Plant Cell* 10: 1043–1054.

Fryer, M.J., Oxborough, K., Mullineaux, P.M. and Baker, N.R. (2002) Imaging of photo-oxidative stress responses in leaves. *J. Exp. Bot.* 53: 1249–1254.

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

Fujita, Y., Fujita, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2011) ABA-mediated transcriptional regulation in response to osmotic stress in plants. *J. Plant Res.* 124: 509–525.

Garapati, P., Xue, G.-P., Munne-Bosch, S. and Balazadeh, S. (2015) Transcription factor ATAF1 in Arabidopsis promotes senescence by direct regulation of key chloroplast maintenance and senescence transcriptional cascades. *Plant Physiol.* 168: 1122–1139.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F. and Goodman, H.M. (1992) Isolation of the Arabidopsis ABI3 gene by positional cloning. *Plant Cell* 4: 1251–1261.

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

Hanin, M., Brini, F., Ebel, C., Toda, Y., Takeda, S. and Masmoudi, K. (2011) Plant dehydrins and stress tolerance. Versatile proteins for complex mechanisms. *Plant Signal. Behav.* 6: 1503–1509.

He, X.J., Mu, R.L., Cao, W.H., Zhang, Z.G., Zhang, J.S. and Chen, S.Y. (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.

He, X., Zhu, L.F., Xu, L., Guo, W.F. and Zhang, X.L. (2016) GhATAF1, a NAC transcription factor, confers abiotic and biotic stress responses by regulating phytohormonal signaling networks. *Plant Cell Rep.* 35: 2167–2179.

Hong, Y., Zhang, H., Huang, L., Li, D. and Song, F. (2016) Overexpression of a stress-responsive NAC transcription factor gene ONACO22 improves drought and salt tolerance in rice. *Front. Plant Sci.* 7: 4.

Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q., et al. (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Natl. Acad. Sci. USA* 103: 12987–12992.

Hu, L., Li, H., Pang, H. and Fu, J. (2012) Responses of antioxidant gene, protein and enzymes to salinity stress in two genotypes of perennial ryegrass (*Lolium perenne*) differing in salt tolerance. *J. Plant Physiol.* 169: 146–156.

Huang, H., Wang, Z., Cheng, J., Zhao, W., Li, X., Wang, H., et al. (2013) An efficient cucumber (*Cucumis sativus* L.) protoplast isolation and transient expression system. *Sci. Hortic.* 150: 206–212.

Huang, S., Li, R., Zhang, Z., Li, L., Gu, X., Fan, W., et al. (2009) The genome of the cucumber, *Cucumis sativus* L. *Nat. Genet.* 41: 1275–U1229.

Huang, Y., Feng, C.-Z., Ye, Q., Wu, W.-H. and Chen, Y.-F. (2016) Arabidopsis WRKY6 transcription factor acts as a positive regulator of abscisic acid signaling during seed germination and early seedling development. *PLoS Genet.* 12: e1005833.

Jensen, M.K., Hagedorn, P.H., de Torres-Zabala, M., Grant, M.R., Rung, J.H., Collinge, D.B., et al. (2008) Transcriptional regulation by an NAC (NAM-ATAF1,2-CUC2) transcription factor attenuates ABA signalling for efficient basal defence towards *Blumeria graminis* f. sp *hordei* in Arabidopsis. *Plant J.* 56: 867–880.

Jensen, M.K., Lindemose, S., de Masi, F., Reimer, J.J., Nielsen, M., Perera, V., et al. (2013) ATAF1 transcription factor directly regulates abscisic acid biosynthetic gene NCED3 in *Arabidopsis thaliana*. *FEBS Open Bio* 3: 321–327.

Jiang, L., Wang, Y., Zhang, S., He, R., Li, W., Han, J., et al. (2017) Tomato SlDREB1 gene conferred the transcriptional activation of drought-induced gene and an enhanced tolerance of the transgenic Arabidopsis to drought stress. *Plant Growth Regul.* 81: 131–145.

Jin, H., Huang, F., Cheng, H., Song, H. and Yu, D. (2013) Overexpression of the GmNAC2 gene, an NAC transcription factor, reduces abiotic stress tolerance in tobacco. *Plant Mol. Biol. Rep.* 31: 435–442.

Lata, C. and Prasad, M. (2011) Role of DREBs in regulation of abiotic stress responses in plants. *J. Exp. Bot.* 62: 4731–4748.

Li, K., Xing, C., Yao, Z. and Huang, X. (2017) PbrMYB21, a novel MYB protein of *Pyrus betulaefolia*, functions in drought tolerance and modulates polyamine levels by regulating arginine decarboxylase gene. *Plant Biotechnol. J.* 15: 1186–1203.

Liao, X., Guo, X., Wang, Q., Wang, Y., Zhao, D., Yao, L., et al. (2017) Overexpression of MsDREB6.2 results in cytokinin-deficient developmental phenotypes and enhances drought tolerance in transgenic apple plants. *Plant J.* 89: 510–526.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., et al. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell* 10: 1391–1406.

Liu, Y.C., Sun, J. and Wu, Y.R. (2016) Arabidopsis ATAF1 enhances the tolerance to salt stress and ABA in transgenic rice. *J. Plant Res.* 129: 955–962.

Lu, P.-L., Chen, N.-Z., An, R., Su, Z., Qi, B.-S., Ren, F., et al. (2007) A novel drought-inducible gene, ATAF1, encodes a NAC family protein that negatively regulates the expression of stress-responsive genes in Arabidopsis. *Plant Mol. Biol.* 63: 289–305.

Mao, X., Zhang, H., Qian, X., Li, A., Zhao, G. and Jing, R. (2012) TaNAC2, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in Arabidopsis. *J. Exp. Bot.* 63: 2933–2946.

Mauch-Mani, B. and Flors, V. (2009) The ATAF1 transcription factor: at the convergence point of ABA-dependent plant defense against biotic and abiotic stresses. *Cell Res.* 19: 1322–1323.

Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2012) AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta* 1819: 86–96.

Nakashima, K., Tran, L.-S.P., Van Nguyen, D., Fujita, M., Maruyama, K., Todaka, D., et al. (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.

Olsen, A.N., Ernst, H.A., Lo Leggio, L. and Skriver, K. (2005) NAC transcription factors: structurally distinct, functionally diverse. *Trends Plant Sci.* 10: 79–87.

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

Pruthvi, V., Narasimhan, R. and Nataraja, K.N. (2014) Simultaneous expression of abiotic stress responsive transcription factors, AtDREB2A, AtHB7 and AtABF3 improves salinity and drought tolerance in peanut (*Arachis hypogaea* L.). *PLoS One* 9: e111152.

Ramirez, V., Coego, A., Lopez, A., Agorio, A., Flors, V. and Vera, P. (2009) Drought tolerance in Arabidopsis is controlled by the OCP3 disease resistance regulator. *Plant J.* 58: 578–591.

Ranieri, A., Petacco, F., Castagna, A. and Soldatini, G.F. (2000) Redox state and peroxidase system in sunflower plants exposed to ozone. *Plant Sci.* 159: 159–167.

Simpson, S.D., Nakashima, K., Narusaka, Y., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) Two different novel cis-acting elements of erd1, a clpA homologous Arabidopsis gene function in induction by dehydration stress and dark-induced senescence. *Plant J.* 33: 259–270.

Tran, L.S.P., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K., et al. (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.

Wang, H., Sui, X., Guo, J., Wang, Z., Cheng, J., Ma, S., et al. (2014) Antisense suppression of cucumber (*Cucumis sativus* L.) sucrose synthase 3 (CsSUS3) reduces hypoxic stress tolerance. *Plant. Cell Environ.* 37: 795–810.

Wei, T., Deng, K., Liu, D., Gao, Y., Liu, Y., Yang, M., et al. (2016) Ectopic expression of DREB transcription factor, AtDREB1A, confers tolerance to drought in transgenic *Salvia miltiorrhiza*. *Plant Cell Physiol.* 57: 1593–1609.

Wu, Y., Deng, Z., Lai, J., Zhang, Y., Yang, C., Yin, B., et al. (2009) Dual function of Arabidopsis ATAF1 in abiotic and biotic stress responses. *Cell Res.* 19: 1279–1290.

Xu, Z.-Y., Kim, S.Y., Hyeon, D.Y., Kim, D.H., Dong, T., Park, Y., et al. (2013) The Arabidopsis NAC transcription factor ANAC096 cooperates with bZIP-type transcription factors in dehydration and osmotic stress responses. *Plant Cell* 25: 4708–4724.

Yang, X., Bai, Y., Shang, J., Xin, R. and Tang, W. (2016) The antagonistic regulation of abscisic acid-inhibited root growth by brassinosteroids is partially mediated via direct suppression of ABSCISIC ACID INSENSITIVE 5 expression by BRASSINAZOLE RESISTANT 1. *Plant Cell Environ.* 39: 1994–2003.

Yao, D., Zhang, X., Zhao, X., Liu, C., Wang, C., Zhang, Z., et al. (2011) Transcriptome analysis reveals salt-stress-regulated biological processes and key pathways in roots of cotton (*Gossypium hirsutum* L.). *Genomics* 98: 47–55.

Zhang, L., Tian, L.H., Zhao, J.F., Song, Y., Zhang, C.J. and Guo, Y. (2009) Identification of an apoplastic protein involved in the initial phase of salt stress response in rice root by two-dimensional electrophoresis. *Plant Physiol.* 149: 916–928.

Zhang, X.M., Yu, H.J., Sun, C., Deng, J., Zhang, X., Liu, P., et al. (2017) Genome-wide characterization and expression profiling of the NAC genes under abiotic stresses in *Cucumis sativus*. *Plant Physiol. Biochem.* 113: 98–109.

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

Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* 53: 247–273.

Zhu, J.-K. (2016) Abiotic stress signaling and responses in plants. *Cell* 167: 313–324.

Zhu, M., Chen, G., Zhou, S., Tu, Y., Wang, Y., Dong, T., et al. (2014) A new tomato NAC (NAM/ATAF1/2/CUC2) transcription factor, SlNAC4, functions as a positive regulator of fruit ripening and carotenoid accumulation. *Plant Cell Physiol*. 55: 119–135.