The AP2 transcription factor NtERF172 confers drought resistance by modifying NtCAT

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Summary

Drought stress often limits plant growth and global crop yields. Catalase (CAT)-mediated hydrogen peroxide (H2O2) scavenging plays an important role in the adaptation of plant stress responses, but the transcriptional regulation of the CAT gene in response to drought stress is not well understood. Here, we isolated an APETALA2/EThylenE-RESPONSIVE FACTOR (AP2/ERF) domain-containing transcription factor (TF), NtERF172, which was strongly induced by drought, abscisic acid (ABA) and H2O2, from tobacco (Nicotiana tabacum) by yeast one-hybrid screening. NtERF172 localized to the nucleus and acted as a transcriptional activator. Chromatin immunoprecipitation, yeast one-hybrid assays, electrophoretic mobility shift assays and transient expression analysis assays showed that NtERF172 directly bound to the promoter region of the NtCAT gene and positively regulated its expression. Transgenic plants overexpressing NtERF172 displayed enhanced tolerance to drought stress, whereas suppression of NtERF172 decreased drought tolerance. Under drought stress conditions, the NtERF172-overexpressed lines showed higher catalase activity and lower accumulation of H2O2 compared with wild-type (WT) plants, while the NtERF172-silenced plants showed the inverse correlation. Exogenous application of amino-1,2,4-triazole (3-AT), an irreversible CAT inhibitor, to the NtERF172-overexpression lines showed decreased catalase activity and drought tolerance, and increased levels of cellular H2O2. Knockdown of NtCAT in the NtERF172-overexpression lines displayed a more drought stress-sensitive phenotype than NtERF172-overexpression lines. We propose that NtERF172 acts as a positive factor in drought stress tolerance, at least in part through the regulation of CAT-mediated H2O2 homeostasis.

Introduction

Drought is a major abiotic stressor with several adverse effects on plants, including stomatal closure, metabolic and osmotic damage, repression of cell growth and photosynthesis (Flexas and Medrano, 2002; Tardieu et al., 2014). It limits the growth, productivity and quality of crops (Faroq et al., 2009; Thirumalaikumar et al., 2017). Plants have evolved a variety of complex mechanisms to respond and adapt to drought stress through biochemical and physiological processes, such as reducing water loss and increasing tolerance (Basu et al., 2016; Osakabe et al., 2014; Rasheed et al., 2016).

Previous studies in Arabidopsis, rice and other plants have shown that drought stress increases the production of reactive oxygen species (ROS), including the singlet oxygen (1O2), superoxide anion (O2−), hydrogen peroxide (H2O2) and hydroxyl radicals (OH). ROS overaccumulation in cells results in oxidative damage to DNA, RNA, proteins and membranes (Choudhury et al., 2016; Miller et al., 2010; Mittler, 2017). Therefore, plants have evolved both enzymatic and nonenzymatic defense systems for maintaining ROS at non-damaging levels. In enzymatic systems, enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase, ascorbate peroxidase (APX) and glutathione peroxidase (GPX) are the major ROS scavengers for maintaining overall plant homeostasis (Mittler et al., 2004). In contrast to other peroxidases, catalase (EC 1.11.1.6), which requires a cofactor to catalyse the dismutation of H2O2 into water (H2O) and O2, plays important role in plant growth and response to environment factors (Mhamdi et al., 2010). Catalases are highly expressed and have a very rapid turnover rate because the affinity (Km) for H2O2 (40–600 μM) is much lower than that of APX and peroxiredoxin (PRX) (Km 100 μM) for H2O2 (Chelikani et al., 2004; König et al., 2002).

In animals, CAT is encoded by a single gene. However, in most other organisms, catalase enzymes are encoded in multiple genes. In plants, CATs are encoded by a small gene family and have been reported in Arabidopsis, tobacco, maize, rice and tomato (Du et al., 2008; Mhamdi et al., 2010; Wang et al., 2019). Studies have demonstrated that the various forms of CAT genes can exhibit different expression patterns, functions and cellular localization in some plants. In Arabidopsis, there are three gene members, CAT1 (At1g20630), CAT2 (At4g35090) and CAT3 (At1g20620), which share high sequence similarity and are involved in catalysing H2O2 (Du et al., 2008; Frugoli et al., 1996). CAT1 is mainly found in rosette leaves and siliques, CAT2 is primarily expressed in young leaves, siliques and flowers, and...
CAT3 is expressed in all tissues, with a stronger expression in roots and young leaves (Du et al., 2008; Mhamdi et al., 2010; Zimmermann et al., 2006). Catalase activity or expression is induced by various stresses (including salinity, drought, cold and H₂O₂) (Du et al., 2008; Leung, 2018). In potatoes, the CAT gene was induced in roots by nematodes and bacteria (Nielbel et al., 1995). NtCAT2 mRNA expression in tobacco was induced by treatment with tobacco mosaic virus or fungal elicitor (Dorey et al., 1998). In Arabidopsis, no obvious phenotype was detected in cat1 and cat2 knockout mutants, while cat2 had a dwarf phenotype (Mhamdi et al., 2010). However, ectopic expression of the maize CAT2 gene in tobacco enhanced the resistance to pathogen infection (Polidoros et al., 2001). In rice, overexpressed Cat4 and Catc improved drought stress tolerance in transgenic rice (Luo et al., 2014). The cat2 mutant showed sensitivity to drought stress and increased CAT3 expression leading to enhanced tolerance to drought stress in Arabidopsis (Zou et al., 2015). Knockout of CAT2 reduced catalase activity by 80% and cat2 mutant plants were hypersensitive to H₂O₂, NaCl and cold (Bueso et al., 2007; Juul et al., 2010).

The regulation mechanisms of CAT have been investigated at the transcriptional and translational levels under different stresses. Several proteins have been reported to interact with CAT protein or regulate CAT expression. For instance, receptor-like cytoplasmic kinase 1 (STRK1) interacted with and phosphorylated Catc, improving the salt tolerance in rice (Zhou et al., 2018). CALCIUM-DEPENDENT PROTEIN KINASE8 (CPK8) could phosphorylate CatC, improving the salt tolerance in rice (Zhou et al., 2008). In Arabidopsis, no obvious phenotype was detected in cat1 and cat3 knockout mutants, while cat2 had a dwarf phenotype (Mhamdi et al., 2010). However, ectopic expression of the maize CAT2 gene in tobacco enhanced the resistance to pathogen infection (Polidoros et al., 2001).

In this study, we report on an AP2 TF, NtERF172, which binds to the NtCAT promoter and regulate seed germination by affecting ROS homeostasis in Arabidopsis (Bi et al., 2017). G-BOX-BINDING FACTOR 1 (GBF1) directly regulated CAT2 transcription to promote pathogen defence in Arabidopsis (Giri et al., 2017). However, it is not clear whether or how the TF proteins are involved in enhancing drought tolerance by binding the promoter of CATs and regulating CAT-mediated ROS scavenging. In this study, we report on an AP2 TF, NtERF172, which binds to the NtCAT promoter and play an important role in maintaining H₂O₂ homeostasis in response to drought stress.

**Results**

**Involvement of the catalase gene NtCAT in drought tolerance**

To confirm whether NtCAT functioned in response to drought stress (see Figure S1a in Supporting Information), we examined the expression of NtCAT mRNA in response to drought treatment using quantitative real-time reverse transcription PCR (qRT-PCR). The NtCAT transcript level was slightly induced within 1 h and peaked at 3 day, suggesting that the expression of NtCAT was induced by drought (Figure 1a). The tissue-expression patterns of NtCAT were first detected by qRT-PCR. NtCAT was expressed in all tissues including root, stem, leaf, flower and seed, with higher expression in stem, leaf and seed (Figure 2a). To assess the expression of NtERF172 in response to drought stress, we performed qRT-PCR using RNA isolated from drought-treated tobacco. NtERF172 was significantly induced by drought stress (Figure 2b).

**Identification of the key region(s) and TF(s) binding sites in the NtCAT promoter**

In order to identify the cis-regulatory sequences required for drought response, we performed a series of 350 bp-deleted promoter constructs fused to the β-glucuronidase (GUS) reporter gene in transgenic tobacco (namely, Δ1, Δ2, Δ3, Δ4 and ΔS, Figure S1b, c). As shown in Figure S1d, the GUS activities were enhanced under drought stress treatment in the full-length (FL) (2700 to 1), Δ1 (2350 to 1) and Δ2 (2000 to 1) plants. However, the deletion from position Δ3 (1650 to 1), Δ4 (1300 to 1) and ΔS (2700 to 2000 and 1650 to 1) lost the response to drought stress treatment.

To identify the TF(s) that regulated the expression of NtCAT and the activity of catalase under drought stress, a yeast one-hybrid (Y1H) system was carried out against a cDNA library generated from tobacco using the P sequence as a bait. The clones were screened by SD/-Leu/-Trp/-His with 150 mM AbA. Among several positive clones, an AP2 domain protein, NtERF, was eventually identified as a binding protein. The open reading frame (ORF) of NtERF was 1035 nucleotides and encoded a protein of 344 amino acid. Sequence analysis showed that NtERF contained the highly conserved AP2/ERF domain putatively involved in DNA binding (Figure S2a). Phylogenetic tree analysis of NtERF in relation to AP2 members from tobacco and other plant species showed NtERF with Arabidopsis ERF17, but sharing an approximately 100% sequence identity with NtERF172 (the gene named by Rushton et al., 2008), so the gene encoding this TF was named NtERF172 (Figure S2b).

NtERF172 expression is significantly induced by drought stress

The tissue-expression patterns of NtERF172 were first detected by qRT-PCR. NtERF172 was expressed in all tissues including root, stem, leaf, flower and seed, with higher expression in stem, leaf and seed (Figure 2a). To assess the expression of NtERF172 in response to drought stress, we performed qRT-PCR using RNA isolated from drought-treated tobacco. NtERF172 was significantly induced by drought stress (Figure 2b). Drought stress is known to induce the accumulation of various signal molecules, such as ABA and H₂O₂. Therefore, we examined the expression of NtERF172 following treatment with 100 μM ABA and 10 mM H₂O₂. qRT-PCR analysis revealed that ABA and H₂O₂ treatment significantly up-regulated NtERF172 expression, with the highest increases (sixfold and 13.8-fold, respectively) at 3 h after treatment (Figure 2c, d).

To further analyse the expression of NtERF172, a 2.1-kb fragment upstream from the initiation codon was cloned into pCXGUS-P vector and transformed into tobacco plants (Figure S2c). GUS staining and GUS activity measurements consistently showed increased GUS activity in response to drought, ABA and H₂O₂ treatments in both the leaves and roots of plants expressing pNtERF172::GUS (Figure 2e, f). These results suggest that the NtERF172 transcript was strongly induced by drought, ABA and H₂O₂.

**Subcellular localization and transcriptional activity analysis of NtERF172**

To verify the subcellular localization of the NtERF172 protein, we generated a construct (35S::NtERF172-GFP) by fusing the GFP reporter protein in the C-terminal under the cauliflower mosaic virus 35S promoter (CaMV 35S). The constructs were expressed in
N. benthamiana epidermal cells by Agrobacterium tumefaciens-mediated transformation. The vector 35S::GFP was used as a control. A strong green fluorescence signal was detected only in the nucleus, which was confirmed by staining with DAPI, indicating that NtERF172 was a nuclear protein (Figure S3a).

The transcriptional activity of TFs has been reported to be an important feature. To investigate whether this was also true for NtERF172, a yeast two-hybrid assay was used. We fused the full-length NtERF172 with a GAL4 DNA-binding domain in the pGBKT7 vector to form recombinant vector pGBKT7-NtERF172, using pGBKT7 as a negative control. The constructs were transformed into the yeast strain Y2H, which were then screened on the selection medium (SD/-Trp/-His/-Ade). The yeast cells grew well on SD/-Trp medium, whereas only the cells transformed with the recombinant vector pGBKT7-NtERF172 survived on the selection medium alone (Figure S3b), suggesting that NtERF172 possesses transcriptional activity.

NtERF172 binds to the promoter of NtCAT

One DRE motif (P1) was found in the promoter of NtCAT (from -2000 to -1650, Figure 3a). To confirm that NtERF172 could directly bind to the DRE motif sequence in the promoter of NtCAT, we performed chromatin immunoprecipitation (ChiP)-PCR, Y1H assays and an electrophoretic mobility shift assay (EMSA). In the ChiP-PCR assay, the 35S::GFP and 35S::NtERF172-GFP fusion proteins were expressed in tobacco plants and immunoprecipitated using an anti-GFP antibody. The NtCAT bands were amplified when 35S::NtERF172-GFP was precipitated, but the corresponding DNA fragments in the 35S::GFP control were not (Figure 3b). This demonstrated that NtERF172 could bind to the NtCAT promoter regions around the P1 elements in vivo. In the Y1H assay, all the yeast cells grew normally on the SD/-Leu medium without 150 ng/mL AbA. However, when 150 ng/mL AbA was added, growth continued in the positive control and bait-prey, whereas the mP1 and AD-NtERF172 were completely inhibited (Figure 3c). This indicated that NtERF172 interacted with the promoter fragment in yeast. In the EMSA, a band was detected for His-NtERF172, but not for the His protein. The band was reduced when increasing amounts of the unlabelled P1 competitor probe with the same sequence were added. This competition was not observed when the mutated version was used (Figure 3d).

To examine whether NtERF172 directly activated the NtCAT promoter, we performed transient expression assays using a double reporter plasmid containing firefly luciferase (LUC) driven by promoter fragments containing P1 or mP1, and Renilla luciferase (REN) driven by the 35S promoter, together with an effector plasmid expressing NtERF172. The LUC/REN ratio was significantly lower in the controls than in the presence of NtERF172 (Figure 3e), indicating that NtERF172 directly activated the promoter fragment. However, when the motifs were mutated, the LUC/REN ratios of the samples were similar to
Figure 2  The expression profiles of NtERF172 in response to drought stress, abscisic acid (ABA) and hydrogen peroxide (H$_2$O$_2$). (a) Expression of NtERF172 in the different tissues. Values are means ± SD of three biological replicates. Asterisks indicate a significant difference relative to root (*P < 0.01). (b-d). Expression analyses of NtERF172 by quantitative real-time PCR (qRT-PCR). Tobacco plants were treated with drought, 100 μM ABA and 10 mM H$_2$O$_2$ for the indicated times. Data represent means ± SD from three biological replicates. (d, e) Analyses of NtERF172 expression in response to drought, ABA and H$_2$O$_2$ treatment for 4h using β-glucuronidase (GUS) histochemical staining (d) and GUS activity measurement (e). Three independent replicates were performed, and data represent means ± SD of at least three biological replicates. Asterisks indicate a significant difference relative to control (*P < 0.01).

Figure 3  NtERF172 binds to the promoter regions of NtCAT gene. (a) Schematic structures of the motif in NtCAT promoter (P1 indicates the putative DRE motif). (b) Chromatin immunoprecipitation (ChIP)-PCR shows that NtERF172 binds to the DRE motif of the NtCAT promoter in vivo. (c) Y1H Gold yeast cells of bait-prey co-transformation on SD/-Leu medium with or without 150 ng/mL ABA. P1 (GCCGACGT) indicates the fragment with the normal motif sequence. mP1 (GCTTTTGT) indicates the mutant motif sequence. (d) His-NtERF172 bound to the promoter regions of NtCAT as determined by EMSA analysis. Arrows indicate protein–DNA complexes (upper arrows) or free probe (lower arrows). WT, unlabelled probes. Mut, DRE-mutated probes. His was used as the control. ‘+’ indicates presence, and ‘−’ indicates absence. (e) Schematic of the reporters and effectors used in the transient expression assays. (f) Transient expression assays of the promoter activities co-transformed with effector and reporter constructs using P1 or mP1. Error bars indicate ± SD of at least three biological replicates. Asterisks indicate a significant difference relative to WT (*P < 0.01).
those of the controls, indicating that no activation was observed (Figure 3f).

**Overexpression of NtERF172 increases drought resistance**

To further understand the function of NIERF172, we transformed the 35S::NtERF172 construct into tobacco (Nicotiana tabacum L. ‘NC89’) by A. tumefaciens-mediated leaf disc transformation. Three T3 representative NtERF172-ox lines (designated L1, L2 and L3) were selected using qRT-PCR for further analysis (Figure S4a). The effects of PEG6000 treatment on seed germination were examined. Under normal conditions, transgenic lines and WT plants exhibited similar germination rates (Figure S4b). When the medium was supplemented with 10% PEG6000, the seed germination ratios of WT and the three transgenic lines significantly decreased. However, the three transgenic lines exhibited higher seed germination ratios than WT. Furthermore, over 85% of the WT seeds failed to germinate, whereas approximately 50%–65% of the seeds of the three transgenic lines germinated under 15% PEG6000 treatment (Figure S4b).

The transgenic tobacco lines were morphologically indistinguishable from WT plants under normal growing conditions. To investigate the potential role of NtERF172 in drought resistance, water was withheld from 30-day-old WT and 35S::NtERF172 transgenic lines for 15 days. WT plants exhibited wilting symptoms, whereas the transgenic lines showed little damage. After 7 days of recovery watering, WT plants wilted and their leaves turned dry, whereas most 35S::NtERF172 transgenic plants remained turgid and their leaves remained green (Figure 4a). Approximately half of the transgenic plants survived (40.5%–62.3%), whereas the survival rate of the WT plants was only 18.3% (Figure 4b). These results suggested that the 35S::NtERF172 transgenic plants may have reduced their daily transpiration rate.

We next compared the stomatal apertures of leaves of 35S::NtERF172 transgenic lines and WT plants. Under normal conditions, there were no differences in the stomatal aperture index between WT and 35S::NtERF172 transgenic lines. However, following drought treatment, the stomatal apertures of 35S::NtERF172 transgenic lines were significantly smaller than those of WT plants (Figure 4c, d). Consistent with these results, the rate of water loss from the detached leaves of WT plants was much faster than that of 35S::NtERF172 transgenic lines (Figure 4e). This suggests that the differences in drought tolerance between WT and 35S::NtERF172-overexpressing lines are at least partly attributable to the inability of WT plants to efficiently close their stomata and reduce transpiration.

We then measured the relative water content (RWC) of the leaves. Under normal conditions, the RWC was similar between the transgenic lines and WT. Following drought treatment, the RWC in transgenic lines decreased to about 58%–67%, but it decreased to approximately 38% in WT (Figure S5a), demonstrating that transgenic lines had higher RWC compared with WT. Additionally, we measured levels of ion leakage (IL) and malondialdehyde (MDA, as a lipid peroxidation marker), which are important indicators of membrane damage (Moore and Roberts, 1998). Under drought stress, transgenic lines had significantly lower MDA and IL accumulation compared to WT (Figure S5b, c), suggesting that the transgenic plants suffered less membrane damage than WT plants during drought stress. The fresh weight (FW) did not differ significantly between WT and transgenic plants under control conditions, but transgenic lines had significantly higher FW than WT under drought stress conditions (Figure S5d). These physiological parameters demonstrate that the transgenic lines were more resistant to drought stress.

**NtERF172-silenced plants are more sensitive to drought stress**

To further elucidate the role of NIERF172 in drought tolerance, we created NIERF172-silenced plants using a virus-induced gene silencing (VIGS) system in tobacco. In silencing construct-infected plants (2mDNA1::NIERF172), NIERF172 transcripts were significantly reduced compared with controls infected with empty vector (2mDNA1) (Figure S6a). After a 10-day drought period, the NIERF172-silenced plants suffered more severe injury than the 2mDNA1 plants. Of the NIERF172-silenced plants, only 11.6% survived, whereas the survival rate of the empty vector plants was 53.2% (Figure 4f, g). The 2mDNA1 plants exhibited a significantly lower MDA content relative to the NIERF172-silenced plants (Figure 4h). The rate of water loss from detached leaves under drought conditions was lower in 2mDNA1 plants than in NIERF172-silenced plants (Figure 4i). Additionally, the RWC and FW in NIERF172-silenced plants were significantly lower compared to 2mDNA1 plants (Figure 4j, k). The stomatal apertures of NIERF172-silenced plants were more open than those of 2mDNA1 plants after drought treatment (Figure 4l, m). These results suggest that silencing NIERF172 reduced drought tolerance in tobacco seedlings.

**NtERF172 regulates H2O2 accumulation and catalase enzyme activities**

Since NIERF172 could bind to the NtCAT promoter and activate its transcription, it was possible that the increased drought tolerance of the transgenic line might be associated with NtCAT-mediated ROS-scavenging ability. As shown in Figure 5a, the activities of the catalase enzyme, a product of NtCAT, were higher in the 35S::NtERF172 (the representative line L3 was selected for catalase enzyme activity) transgenic line than in the WT, but the NIERF172-silenced plants had a decrease in catalase activity of 22.3% compared with WT plants.

We, therefore, examined the accumulation of H2O2 and ROS. Drought stress increased H2O2 levels in WT and transgenic, but with an accelerated accumulation in WT, as indicated by 3,3′-diaminobenzidine (DAB) staining and measurement (Figure 5b, c). H2O2 accumulation was higher in NIERF172-silenced plants than in 2mDNA1 plants under drought treatment (Figure 5d, e). The ROS production in leaves of NIERF172-ox, NIERF172-silenced, 2mDNA1 and WT plants was further analysed using the dye 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) (Bao et al., 2015). After treatment with drought stress, the pixel intensity of fluorescence emission was lower in the leaves of NIERF172-ox plants and higher in NIERF172-silenced plants than those of WT plants and 2mDNA1, respectively (Figure 5f, g). All these data indicate that NIERF172 can mediate the ROS accumulation and catalase activity under drought stress conditions.

**Decreased catalase activity suppresses the drought tolerance phenotype of NtERF172-ox plants**

Based on the above data, we hypothesized that CAT-mediated ROS scavenging is important for the drought tolerance of the 35S::NtERF172 overexpression plants. To confirm the regulatory
pathway, we used 10 mM 3-amino-1,2,4-triazole (3-AT) as an inhibitor to suppress catalase activity. In the WT, CAT activity was reduced from 36.8 to 18.4 U/mg protein following 3-AT treatment (Figure 6a). Next, the WT and transgenic seedlings were treated with water or inhibitors and then exposed to 15% PEG6000. When the seedlings were treated with water, the transgenic plants exhibited much better growth than the WT plants, and more than 75.4%–85.7% of the transgenic seedlings survived 15% PEG6000 treatment compared with only 45.2% of the WT seedlings (Figure 6b, c). However, when 3-AT was used, the survival rate of the transgenic lines was sharply decreased, being only slightly higher than the WT plants (Figure 6b, c).

We then generated NtERF172-ox/2mDNA1:NtCAT plants by transforming 2mDNA1:NtCAT into NtERF172-ox (Figure S6b). We found that knockdown of NtCAT partly reduced the tolerance of the drought stress phenotype of NtERF172-ox, including increasing the MDA content and H2O2 content. Consistent with the visible phenotype, the RWC and FW in NtERF172-ox plants were decreased by knockdown of NtCAT (Figure 6d-h).

Figure 4 Analysis of the drought tolerance in 35S::NtERF172 and NtERF172-silenced plants. (a) Drought resistance in 35S::NtERF172 transgenic plants (L1, L2 and L3). Wild-type (WT) and 35S::NtERF172 plants were grown in soil with sufficient water for 2 weeks before water was withheld for 15 days, followed by recovery for 7 days. (b) Survival rates of WT and 35S::NtERF172 transgenic plants were investigated during recovery watering for 7 days. Average survival rates and standard errors were calculated from at least three biological replicates. (c, d) Stomatal closure in WT and 35S::NtERF172 transgenic plants under drought conditions. Values are means of width to length. Error bars represent the SE of at least three biological replicates. (e) Water loss from detached leaves of WT and 35S::NtERF172 transgenic plants. Water loss is expressed as the percentage of initial fresh weight (FW). Values are means of six leaves each from at least three biological replicates. Asterisks indicate a significant difference relative to WT (*P < 0.01). (f) Phenotypic comparison of tobacco plants subjected to drought stress. 2mDNA1 and NtERF172-silenced plants (2mDNA1:NtERF172) were grown in soil with sufficient water before water was withheld for 10 days. (g-k) Survival rates (g), malondialdehyde (MDA) content (h), water loss (i), RWC (j) and FW (k) in tobacco plants measured after drought stress treatment. RWC, relative water content. FW, fresh weight. Each bar represents the mean of at least three biological replicates. (l, m) Stomatal closure of 2mDNA1 and NtERF172-silenced plants under control or drought stress conditions. Data represent means ± SD (n ≥ 30). Asterisks indicate a significant difference relative to WT (*P < 0.01).

NtERF172-ox plants enhanced oxidative stress tolerance
To assess the role of NtERF172 in regulating oxidative stress, we investigated the tolerance of NtERF172 caused by H2O2. Leaves were cut into small pieces and treated for 24 h with distilled water or 5% H2O2. Under the distilled water treatment conditions, there were no differences between leaf pieces from transgenic lines or WT. However, under 5% H2O2 treatment, most of the WT pieces turned brown and necrotic, whereas those from transgenic lines maintained their green colour (Figure 7a). The chlorophyll (Chl) content was higher in the transgenic lines than in the WT (Figure 7b, c). Interestingly, the DAB staining and quantitative measurements showed that upon exogenous H2O2 treatment, the NtERF172-ox line leaves displayed less H2O2 accumulation than the WT plants (Figure 7d, e), implying that NtERF172-ox leaves had a higher H2O2-scavenging capacity. However, the leaf discs from 2mDNA1:NtERF172 transgenic plants had a lower Chl content and higher H2O2 accumulation compared with 2mDNA1 after 5% H2O2 treatment (Figure S7).
Discussion

Drought stress affects physiological metabolic reactions, including oxidative stress and membrane lipids damage through excess ROS accumulation (Fedoroff et al., 2010; Schieber and Chandel, 2014; Zhu, 2016). To protect the cells against damage because of ROS, plants have evolved a broad range of adaptive responses, involving enzymatic and nonenzymatic antioxidants (Miller et al., 2010; Mittler et al., 2004). As an important H$_2$O$_2$-scavenging enzyme, the expression or activity of catalase plays a vital role in plant response to stresses (Bueso et al., 2007; Du et al., 2008; Xing et al., 2007; Zimmermann et al., 2006). For example, the transcription of CAT3 could be induced by drought and overexpression lines were insensitive to drought stress (Zou et al., 2015). In tobacco, biochemical and transcriptomic analyses showed that CAT transcript level and catalase enzyme activity were up-regulated by drought stress treatment (Yang et al., 2017; Yin et al., 2015). The results we obtained are consistent with the view that NICAT expression was induced by drought, and overexpression lines had high survival rates under drought stress conditions (Figure 1), suggesting that NICAT acts as a positive regulator of drought tolerance.

In plants, several TFs have been shown to bind to the promoter regions of CATs to activate or suppress their expression and catalase activity involved in seed germination, leaf senescence and disease resistance (Bi et al., 2017; Giri et al., 2017; Smykowski et al., 2010). However, the TFs involved in regulating CAT-mediated H$_2$O$_2$ scavenging under drought stress conditions are not well understood. Here, we identified a TF, NtERF172, with a potential role as a transcriptional activator in regulating NICAT expression, by screening a cDNA library using the Y1H screening method (Figure 3d). The AP2/ERF family is a large group of plant-specific TFs that play important roles in plant response to abiotic stresses, including drought, salinity and temperature (Mizoi et al., 2012; Nakano et al., 2006; Sakuma et al., 2002; Xie et al., 2019). Phylogenetic tree analysis indicated that the NtERF172 protein was clustered within the same clade as ERF17 of Arabidopsis and belonged to the DREBs subgroup A5 (Figure S2b). The function of AtERF17 is unclear in terms of stress response, but its homologs from citrus (CitERF13) and tomatoes (SIERF16) are involved in fruit
degreening (Li et al., 2019). From this, we conclude that the NtERF172 of different plant species may exhibit diverse functions. Increasing evidence has shown that ERF transcription levels are regulated by abiotic stress responses (Licausi et al., 2013; Mizoi et al., 2012). For example, the DREB1/CBFs subfamily is rapidly induced in response to cold stress but not to dehydration or high salinity (Jaglo-Ottosen et al., 1998; Liu et al., 1998). The DREB2 subgroup has eight members which are induced by dehydration, high salinity and heat in an ABA-independent manner (Mizoi et al., 2012; Xie et al., 2019). The transcript levels of NtERF172 were also induced by drought treatment (Figure 2), indicating that it may play a role in regulating response to drought stresses.

Figure 6 Silenced NtCAT represses the drought tolerance of NtERF172-ox plants. (a) Catalase activity in plants treated with water or 10 mM 3-AT for 5 h. (b) Phenotypes of PEG6000-treated transgenic and WT plants pretreated with water or 10 mM 3-AT. The treatment was repeated five times with ten replicates for different lines at each repetition. (c) Survival rates of transgenic and WT plants were analysed following PEG6000 treatment. The data represent the means ± SD of at least three biological replicates. Asterisks indicate a significant difference relative to WT (*P < 0.01). (d) The drought phenotypes of 2mDNA1, 3SS::NtERF172/2mDNA1 and 3SS::NtERF172/2mDNA1:NtCAT plants. MDA levels (e), H2O2 contents (f), RWC (g) and FW (h) of the 2mDNA1, 3SS::NtERF172/2mDNA1 and 3SS::NtERF172/2mDNA1:NtCAT plants after drought treatment. RWC, relative water content. FW, fresh weight.

Figure 7 Oxidative stress tolerance assays in WT and 3SS::NtERF172 transgenic plants treated with 5% H2O2. (a) Representative photographs showing leaf pieces of WT and transgenic plants after 2% H2O2 treatment. (b, c) Chl extraction solutions and Chl content in leaf pieces of WT and 3SS::NtERF172 transgenic plants. FW, fresh weight. (d, e) DAB staining and H2O2 contents of leaves from WT and transgenic plants pretreated with water or 5% H2O2. Data represent means ± SD calculated from three biological replicates. Three biological experiments produced similar results. Asterisks indicate a significant difference relative to WT (*P < 0.01).
ERFs are reported to bind to the DRE/CRT or ERE (known as GCC-box) motifs on stress-responsive genes to confer resistance to abiotic and biotic stresses (Shinozaki and Yamaguchi-Shinozaki, 2007). For instance, NtERF53 overexpression in Arabidopsis increases drought tolerance by binding to the GCC-box and/or the DRE element in the promoters of downstream genes, such as COR15A and P5CS1 (Cheng et al., 2012). RAP2.4-ox Arabidopsis enhances drought tolerance by regulating a number of genes containing DRE or a similar cis-element named the C-repeat (CRT, core sequence TGCCCGAC), including RD29A, COR47 and COR15A (Lin et al., 2008). Our work shows that NtERF172, which binds to the DRE motif, is an upstream regulator of NtCAT, confirmed by Y1H assay, ChIP-PCR assay and EMSA (Figure 3). The phenotype analysis indicates that NtERF172 is a positive regulatory factor in drought stress tolerance in plants (Figure 4). It is well established that antioxidant enzymes, which are regulated by TFs, play a predominant role in eliminating ROS accumulation under abiotic stresses (Choudhury et al., 2016; Mittler, 2017). For example, PtrbHLH binds to the promoter region of a POD gene and positively regulates POD-mediated ROS removal under cold stress conditions (Huang et al., 2013). AtbHLH112 binds to GCC- and E-box motifs in the promoter regions of SOD or POD genes and mediates ROS scavenging and proline biosynthesis under stress (Liu et al., 2015). ERF74 binds specifically to the GCC-box element in the promoter of RbohD and activates RbohD-mediated ROS bursts in the early stages of different stresses (Yao et al., 2017). Consistent with these results, NtERF172 regulated catalase activity and functions in conferring drought tolerance through CAT-mediated ROS scavenging in the transgenic plants (Figures 5 and 6).

Plants have evolved a variety of mechanisms to improve their drought tolerance (Miller et al., 2010). ERF TFs are involved in several processes in response to drought stress, including stomatal development, root hair formation, root meristem size and hormone metabolism (Shinozaki et al., 2003 Shinozaki and Yamaguchi-Shinozaki, 2007). Stomatal apertures are a key determinant of transpirational water loss, and stomatal closure reduces water loss. This property is critical for maintaining high water potential under drought stress conditions in plants (Nilson and Assmann, 2007). TF-mediated stomatal closure is an important process in drought stress responses (Castilhos et al., 2014). bHLH112 enhances drought tolerance by decreasing stomatal apertures in Arabidopsis (Liu et al., 2014). The Arabidopsis GTL1 TF regulates water use efficiency by modulating stomatal density under drought stress conditions (Yoo et al., 2010). In this study, NtERF172 transgenic lines regulated stomatal aperture and transpirational water loss (Figure 4), indicating that NtERF172 functions as a positive regulator of drought stress responses by regulating stomatal aperture and transpiration.

Based on the results of this study, we propose a model of NtERF172 function in response to drought stress in tobacco plants. Under drought stress conditions, NtERF172 is induced and acts upstream of NtCAT, directly regulating its expression by binding to the DRE motif in the promoter region. Activated NtCAT then promotes ROS scavenging under drought stress. It is possible that NtERF172 regulates stress via other stress tolerance pathways under drought stress. Additional research is needed to determine whether NtERF172 directly regulates other stress-related genes to enhance drought tolerance. We conclude that NtERF172 exhibits important physiological functions in the drought stress response through the regulation of CAT-mediated ROS scavenging and other pathways, thus protecting plants against oxidative damage.

**Materials and methods**

**Plant materials and treatments**

Tobacco cultivars (N. tabacum L. ‘NC89’) were used for expression analyses and genetic transformation. The seeds were surface-sterilized with 3% NaClO for 8 min and then germinated on 1/2 MS medium containing 2.5% sucrose and 1.0% agar at 25°C. The seedlings were transferred to soil and cultivated at 25±1°C with a 16-h light/8-h dark photoperiod.

For drought treatment, uniform and healthy 10-day-old seedlings were transferred to filter paper and dried at 23°C for 0, 1, 3, 6, 12 and 24 h. For 100 μM ABA, and 10 mM H2O2 treatments, 10-day-old seedlings were removed from the agar medium and submersed in a solution containing 100 μM ABA or 10 mM H2O2 for the durations indicated. Seedlings were independently harvested and immediately frozen in liquid nitrogen and stored at –80°C until RNA extraction.

**Vector construction and plant transformation**

The full-length sequences of NtCAT and NtERF172 were amplified from tobacco genomic cDNA by PCR and cloned into the binary vector pRI under the control of the CaMV 35S promoter region and a green fluorescent protein (GFP) coding region. For the deletion analysis of the NtCAT promoter, the FL (2.7 Kb) and the consecutively truncated regions Δ1 (−2350 to 0 kb), Δ2 (−2000 to 0 kb), A3 (−1650 to 0 kb) and Δ4 (−1300 to 0 kb) and Δ5 (−2700 to −2000 kb and −1650 to 0 kb) were amplified by PCR and cloned into pCXGUS-P to drive the GUS reporter gene. Then, the recombinant plasmids were introduced into the A. tumefaciens strain EHA105. The primer sequences are listed in Table S1.

Tobacco transformation was performed by leaf disc transformation using the A. tumefaciens method described by Horsch et al. (1985). Tobacco leaves were cut into small pieces and immersed in A. tumefaciens suspension cultures. Seedlings from three independent T3 homozygous transgenic lines were used in further investigations.

**qRT-PCR**

Total RNAs were isolated from roots, stems, leaves, flowers, seeds and whole tobacco samples using the TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. The first-strand cDNA was generated with the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer’s protocol. qRT-PCR was performed using the SYBR Premix Ex Taq (TaKaRa) in a 20-µL volume and an Applied Biosystems 7500 Real-Time System (Applied Biosystems, Foster City, CA). The PCR conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. NtActin was used as an internal control. The expression analysis at each time point was replicated at least three times. The primer sequences are listed in Table S2.

**Virus-induced gene silencing assays**

The tobacco curly shoot virus-associated alphasatellite vector (2mDNA1) was used in N. tabacum as described by Huang et al. (2009). PCR was used to amplify 329-bp and 220-bp fragments which were inserted into the plasmid 2mDNA1 to produce 2mDNA1:NtERF172 and 2mDNA1:NtCAT, respectively. Then, the 2mDNA1:NtERF172, 2mDNA1:NtCAT and empty vector (2mDNA1) constructs were transformed into A. tumefaciens EHA105. The transformed A. tumefaciens was individually co-
infiltrated with tomato yellow leaf curl China virus (TYLCCNV) into N. tabacam plants as previously described (Huang et al., 2009). NtERF172 silencing or NtCAT silencing was confirmed by qRT-PCR.

Germination analysis and stress tolerance assays

The seeds of both WT and transgenic lines were sterilized by 3% NaClO and planted on MS medium or PEG-infused MS medium (described by Verslues et al., 2006) for tolerance assays and were incubated at 25 ± 1°C with a 16-h light/8-h dark photoperiod. The germination rates were measured daily.

For drought tolerance assays, 30-day-old soil-grown WT and transgenic lines were deprived of water for 15 days and then allowed to recover for 7 days. Survival rates were then scored, and physiological indices were measured. Water loss measurements were performed according to Liu et al. (2014). Water loss was determined as a percentage of the FW at the beginning of the experiment and weighed at the designated time intervals. IL was measured based on the procedures described by Huang et al. (2013). RWC was measured according to Munne-Bosch et al. (1999) and evaluated via the equation RWC (%) = (FW – dry weight)/(turgid weight – dry weight) × 100. The MDA content was detected by the thiobarbituric acid-based colorimetric method as described by Heath and Packer (1968).

For the oxidative stress test, leaf pieces from WT and transgenic lines were incubated in 5% H2O2 for 48 h. After treatment, photographs were taken and total Chl content was measured.

Stomatal aperture analysis

Stomatal apertures were measured as described previously (Liu et al., 2014). The leaves, which were chosen from uniform and healthy 30-day-old WT and transgenic lines, were exposed to drought conditions for 15 or 10 days. The stomata on epidermal strips obtained from the leaves were then measured under a microscope (Olympus ix71, Tokyo, Japan). More than 40 guard cells from each sample were imaged. The width and length of stomatal pores were determined using ImageJ software (National Institutes of Health, Bethesda, MD), and the stomatal apertures were calculated as the ratio of width to length.

Histochemical staining and catalase activity assays

H2O2 accumulation in leaves was examined visually using histochemical staining with DAB. The H2O2 content was quantified according to the method of Zhao et al. (2016a). For enzyme assays, catalase activities were measured according to the methods of Zou et al. (2015). Each assay was replicated at least three times.

GUS analysis

For histochemical staining, the transgenic lines and WT were immersed in GUS staining buffer as previously described (Zhao et al., 2016a).

GUS activity analysis was conducted as previously described (Jefferson et al., 1987). The proteins from whole tobacco plants were extracted with extraction buffer and reacted with 4-MUG (Sigma-Aldrich, St. Louis, MO) at 37°C. The GUS activity was determined using a VersaFluor spectrofluorometer (excitation 365 nm and emission 450 nm).

ChIP-PCR, Y1H and EMSA

ChIP-PCR assays were performed as described by Zhao et al. (2016b). After immunoprecipitation, recovered chromatin fragments were subjected to qRT-PCR. The primers are shown in Table S1.

Y1H assays were performed according to the manufacturer’s instructions. The full-length NtERF172 was cloned into the pGADT7 vector to generate the construct AD-NtERF172. The promoter fragments of the NtCAT gene containing the binding cis-elements were inserted into the pAbAi vector. Different combinations were co-transformed into the yeast strain Y1H Gold, and the interactions were examined on SD/-Leu medium with or without 150 ng/mL ABA.

For EMSAs, the NtERF172 open reading frame was amplified by PCR and cloned into the pET32a vector. The construct was introduced into E. coli BL21 (DE3) to induce recombinant His-NtERF172 protein. The protein was produced and purified with a nickel-nitrilotriacetic acid (NINNTA) agarose column. EMSAs were carried out using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocol. Reaction mixtures containing the protein and probes were then incubated for 30 min at room temperature and separated on 6% polyacrylamide gels in 0.5 × TBE buffer. Finally, the signals were detected using the EMSA Kit and ChemiDoc XRS + (Bio-Rad).

Transient expression assays

The NtCAT promoter fragments containing the binding motifs were cloned into pGreenII 0800-LUC to act as the reporter gene. Full-length NtERF172 was cloned into the pGreenII 62-5K to serve as the effector.

The constructs and the pSoup helper were transformed into A. tumefaciens EHA105 and then infiltrated into tobacco (N. benthamiana) leaves. Three days later, the infiltrated leaves were collected and the activities of firefly and REN luciferase were measured using dual luciferase assay reagents (Beyotime Institute of Biotechnology, Haimen, China). Eight independent biological samples were used.

Statistical analysis

Data analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL). Experimental data were analysed with Student’s t-test with significance at \( P < 0.01 \) (*). Sample variability is given as the standard deviation (SD) of the mean.

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Conflicts of interest

The authors declare no conflict of interest.
Author contributions

QZ, XHX and YYL conceived and designed the research. QZ performed most of the experiments. QZ, RSH, DL, XL, JW, XHX and YYL performed the research. QZ, XHX and YYL analysed the data and wrote the paper.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.