

Phosphorylation of a NAC Transcription Factor by a Calcium/Calmodulin-Dependent Protein Kinase Regulates Abscisic Acid-Induced Antioxidant Defense in Maize^{1[OPEN]}

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Calcium/calmodulin-dependent protein kinase (CCaMK) has been shown to play an important role in abscisic acid (ABA)-induced antioxidant defense and enhance the tolerance of plants to drought stress. However, its downstream molecular events are poorly understood. Here, we identify a NAC transcription factor, *ZmNAC84*, in maize (*Zea mays*), which physically interacts with *ZmCCaMK* in vitro and in vivo. *ZmNAC84* displays a partially overlapping expression pattern with *ZmCCaMK* after ABA treatment, and H₂O₂ is required for ABA-induced *ZmNAC84* expression. Functional analysis reveals that *ZmNAC84* is essential for ABA-induced antioxidant defense in a *ZmCCaMK*-dependent manner. Furthermore, *ZmCCaMK* directly phosphorylates Ser-113 of *ZmNAC84* in vitro, and Ser-113 is essential for the ABA-induced stimulation of antioxidant defense by *ZmCCaMK*. Moreover, overexpression of *ZmNAC84* in tobacco (*Nicotiana tabacum*) can improve drought tolerance and alleviate drought-induced oxidative damage of transgenic plants. These results define a mechanism for *ZmCCaMK* function in ABA-induced antioxidant defense, where ABA-produced H₂O₂ first induces expression of *ZmCCaMK* and *ZmNAC84* and activates *ZmCCaMK*. Subsequently, the activated *ZmCCaMK* phosphorylates *ZmNAC84* at Ser-113, thereby inducing antioxidant defense by activating downstream genes.

Drought stress limits plant productivity worldwide. The phytohormone abscisic acid (ABA) plays important roles in regulating the adaptive response of plants to drought. One mode of ABA-enhanced drought tolerance is associated with the induction of antioxidant defense system. ABA can cause the generation of reactive oxygen species (ROS), which at low concentration are important signaling molecules that activate antioxidant defense to scavenge excess ROS, whereby plants can maintain ROS at a balanced level to avoid unwanted damage. Many signaling molecules, such as calcium ion (Ca²⁺) and nitric oxide, and protein kinases, such as mitogen-activated protein kinase (MAPK), calcium-dependent protein kinase, and calcium/calmodulin-dependent protein kinase (CCaMK), have been found to be involved in ROS response to ABA signaling (Ding et al., 2013; Ma et al., 2012; Sang et al., 2008; Zhang et al., 2006). However, the mechanism of ABA-induced antioxidant defense remains unclear.

CCaMK was first identified in flowers of lily (*Lilium longiflorum*) and subsequently isolated from tobacco (*Nicotiana tabacum*), maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), *Lotus japonicus*, *Medicago truncatula*, and *Sesbania rostrata* (Capoen et al., 2009; Chen et al., 2007; DeFalco et al., 2009; Godfroy et al., 2006; Harper et al., 2004; Hayashi et al., 2010; Lévy et al.,

2004; Mitra et al., 2004; Patil et al., 1995; Tirichine et al., 2006; Yang and Poovaiah, 2003; Yang et al., 2011a; Zhang and Lu, 2003). CCaMK has a Ser/Thr kinase domain, a CaM-binding domain, and three EF-hand motifs. A model for the activation of CCaMK has been proposed based on work with CCaMK from lily (Sathyanarayanan et al., 2000): Ca²⁺ binding to the EF-hands of CCaMK promotes autophosphorylation, which in turn promotes CaM binding, relieving autoinhibition of the kinase and thus promoting substrate phosphorylation. This model includes a two-step activation of CCaMK by Ca²⁺ (Miller et al., 2013; Poovaiah et al., 2013). CCaMK is a central decoder of Ca²⁺-spiking in the common symbiosis pathway for regulating root nodules and arbuscular mycorrhizae (Gobbato, 2015; Miller et al., 2013; Shimoda et al., 2012). However, CCaMK is highly expressed outside of roots, indicating other roles besides the regulation of symbiosis. CCaMK has been shown to be involved in ABA signaling during abiotic stress response (Yang et al., 2011a) and ABA-induced antioxidant defense (Ma et al., 2012; Shi et al., 2012). ABA-induced H₂O₂ production activates CCaMK, which in turn induces antioxidant defense system by a largely unknown mechanism.

The NAC superfamily is one of the largest families of plant transcription factors (TFs) and widely distributed

in plants (Olsen et al., 2005). The characteristic feature of this group of TFs is the presence of a highly conserved NAC domain at the N terminus, which serves as a platform for DNA binding and for homo- or heterodimerization with other NAC proteins. The C-terminal region, in contrast, is variable in sequence and length and serves as a transcriptional activator or repressor (Nuruzzaman et al., 2010), which reflects their numerous functions. NAC TFs regulate a diverse range of processes in plants, such as nutrient acquisition (He et al., 2015), root growth (Guo et al., 2005), plant height (Chen et al., 2015), leaf senescence (Guo and Gan, 2006), formation of secondary walls (Mitsuda et al., 2007), and hormone signaling (Fujita et al., 2004). Moreover, many members of the NAC TF family coordinate the response to biotic and abiotic stress including fungal infection (Wang et al., 2009), salt (Hu et al., 2006a), temperature (Fang et al., 2015), and drought (Huang et al., 2015; Mao et al., 2015). The diverse functions of NAC TFs possibly come from their multiple regulatory modes. They are regulated at the transcriptional level through numerous TF recognition sequences in their promoters, at the posttranscriptional level by microRNA-mediated cleavage of transcripts and at the posttranslational level by protein degradation, dimerization, interaction with other non-NAC proteins, and phosphorylation (Puranik et al., 2012).

In this study, using the full-length *ZmCCaMK* (GenBank ID DQ403196) from maize as bait in a yeast two-hybrid interaction screening approach, we identified a NAC transcription factor, *ZmNAC84*. We analyzed the

interactions between *ZmCCaMK* and *ZmNAC84*, investigated the roles of *ZmNAC84* in ABA-induced antioxidant defense, and identified the phosphorylation site of *ZmNAC84* by *ZmCCaMK* in this process.

RESULTS

Identification of *ZmNAC84* as a *ZmCCaMK*-Interacting Protein

To elucidate the molecular basis by which *ZmCCaMK* functions in ABA-induced antioxidant defense, we used a yeast two-hybrid screen of a maize leaf cDNA library with the full-length *ZmCCaMK* as bait. A total of 29 positive clones were isolated from the screen, corresponding to nine genes. Seven of 29 positive clones containing genes encoded a NAC-type transcription factor (GRMZM2G166721) belonging to a family with more than 100 members in the maize genome. GRMZM2G166721 (GenBank ID AFU81568.1) has previously been designated as *ZmNAC84* (Fan et al., 2014). The interaction of *ZmNAC84* with *ZmCCaMK* in the yeast two-hybrid system is shown in Figure 1A.

To validate the yeast two-hybrid data, both *in vitro* and *in vivo* experiments were performed to test the *ZmNAC84*-*ZmCCaMK* interaction. Recombinant full-length *ZmCCaMK* and glutathione S-transferase (GST) fusion protein and the full-length *ZmNAC84* tagged with poly-His were produced in *Escherichia coli* and purified. The His-tagged *ZmNAC84* was retained on beads with immobilized GST-*ZmCCaMK* but not with immobilized GST alone (Fig. 1B). The *in vitro* interaction between *ZmCCaMK* and *ZmNAC84* was further confirmed by immunocomplex kinase assay using His-tagged *ZmNAC84* as substrate. *ZmCCaMK* was found to phosphorylate His-*ZmNAC84* *in vitro* (Fig. 1C).

To further confirm the interaction, bimolecular fluorescence complementation (BiFC) assays were performed in onion (*Allium cepa*) epidermal cells. YFP was reconstituted when the coding sequences of *ZmCCaMK* and *ZmNAC84* were coexpressed (Fig. 1D, a–c). In contrast, coexpression of the YFP-N terminus and *ZmNAC84*-YFP C terminus, or the *ZmCCaMK*-YFP N terminus and YFP-C terminus, did not result in fluorescence (Fig. 1D, d–i), which confirmed that the *ZmCCaMK*-*ZmNAC84* interaction is specific. Moreover, the BiFC experiments revealed that *ZmCCaMK*-*ZmNAC84* interacted in nucleus (Fig. 1D, a–c; Supplemental Fig. S1).

Coimmunoprecipitation (Co-IP) assays were performed to confirm the interaction between *ZmCCaMK* and *ZmNAC84* in maize mesophyll protoplasts. *ZmCCaMK-myc* and *ZmNAC84-His* were coexpressed or *ZmNAC84-His* was expressed alone transiently in maize protoplasts. Crude protein extracts of protoplasts were immunoprecipitated by anti-myc antibody and then analyzed by immunoblotting with anti-His antibody. As shown in Figure 1E, *ZmNAC84-His* was detected, further confirming the *in vivo* interaction between the two proteins.

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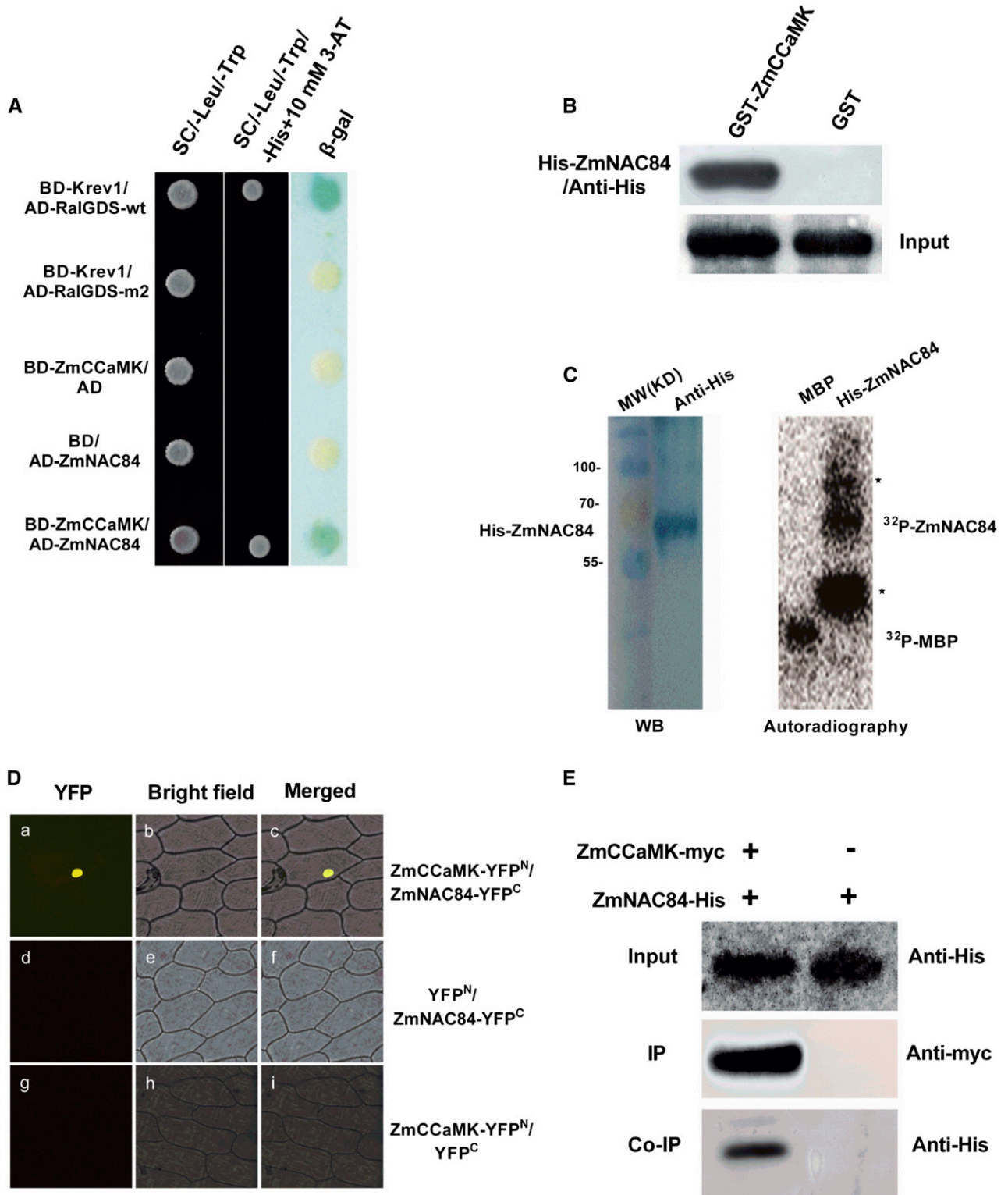
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A.Z. conceived the project and designed the experiment; Y.Z., J.Y., and W.L. did most of the experiments and analysis presented in this study and wrote the manuscript; L.L. was involved in identification of phosphorylation site of *ZmNAC84*; Y.Sh. participated in analysis of physical interaction between *ZmNAC84* and *ZmCCaMK*; Y.S. analyzed data and generated Figures 3 and 4; Y.L. screened *ZmNAC84* as an interacting protein of *ZmCCaMK*; H.V.S. helped to revise the manuscript; M.J. and X.H. gave advice on manuscript writing; L.N. provided technical assistance to W.L. and L.L.; all authors approved the final manuscript.

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ZmNAC84 Displays a Partially Overlapping Expression Pattern with *ZmCCaMK* after ABA Treatment and H₂O₂ Is Required for ABA-Induced *ZmNAC84* Expression

Using quantitative RT-PCR (qRT-PCR), we first examined *ZmNAC84* mRNA accumulation in various organs/tissues at different developmental stages. *ZmNAC84* was broadly expressed with the highest level in female flower and a higher level in leaf compared with male flower, root, and stem (Supplemental Fig. S2). To gain insight into the functional significance of the interaction between *ZmNAC84* and *ZmCCaMK*, we further explored the expression pattern of *ZmNAC84* after ABA treatment in maize leaves. A significant response in the expression of *ZmNAC84* was observed after 15 min ABA treatment, was maximal at 45 min, and then decreased (Fig. 2A). This expression pattern overlapped with that of *ZmCCaMK* but displayed some differences, as *ZmCCaMK* expression peaked 30 min after ABA treatment (Supplemental Fig. S3).

H₂O₂ is required for ABA-induced *ZmCCaMK* activation and gene expression (Ma et al., 2012). To study the role of H₂O₂ in ABA-induced *ZmNAC84* expression, we treated maize plants with exogenous H₂O₂. qRT-PCR results showed that H₂O₂ treatment also induced a significant increase in the expression of *ZmNAC84* at 30 min, i.e. faster than after ABA treatment (Fig. 2A), which suggests that ABA-induced *ZmNAC84* expression might be via H₂O₂. To test this hypothesis, the NADPH oxidase inhibitor, diphenylene iodonium (DPI), which blocks the ABA-induced H₂O₂ production (Hu et al., 2005, 2006b), was used. We treated maize plants with DPI before ABA treatment. Results showed that pretreatment with DPI significantly blocked ABA-induced *ZmNAC84* expression (Fig. 2B). These data suggest that H₂O₂ is required for ABA-induced *ZmNAC84* expression.

ZmNAC84 Is Involved in ABA-Induced Antioxidant Defense

To explore the possible function of *ZmNAC84* in ABA-induced antioxidant defense, we transiently expressed or silenced *ZmNAC84* in maize mesophyll protoplasts (Sheen, 2001; Zhai et al., 2009) and determined the effect on activities of ascorbate peroxidase (APX) and superoxide dismutase (SOD), which are key enzymes in antioxidant defense. As shown in Figure 3A, transient expression of *ZmNAC84* resulted in significant increases in the activities of APX and SOD when compared with control, which were further enhanced by ABA treatment. Conversely, RNAi-mediated transient silencing

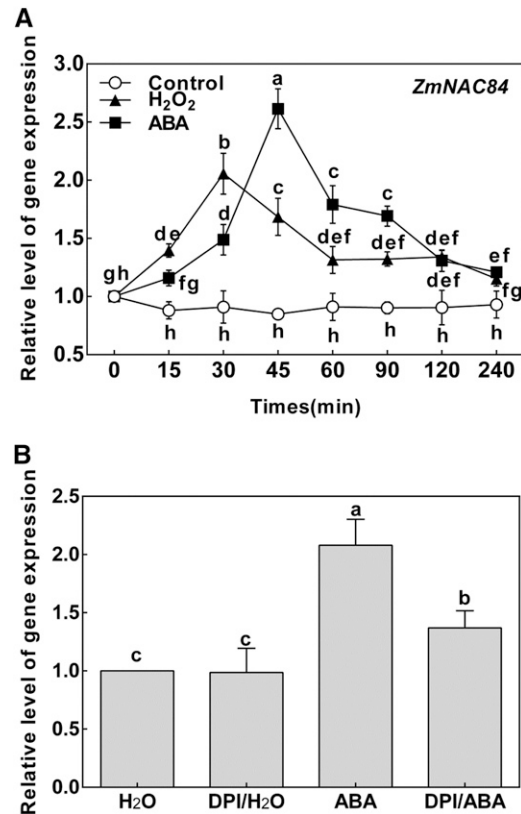


Figure 2. H₂O₂ is required for ABA-induced expression of *ZmNAC84* in maize leaves. A, Expression analysis of *ZmNAC84* in leaves of maize plants exposed to ABA or H₂O₂ treatment. The maize seedlings were treated with ABA (100 μM) or H₂O₂ (10 mM) for various times as indicated. B, Effects of pretreatment with the NADPH oxidase inhibitor DPI on the expression of *ZmNAC84*. The maize seedlings were treated with 100 μM DPI for 4 h and then exposed to 100 μM ABA for 45 min. Relative expression level of *ZmNAC84* was analyzed by qRT-PCR. Values are means ± SE of three different experiments. Means denoted by the same letter did not differ significantly at *P* < 0.05 according to Duncan's multiple range test.

of *ZmNAC84* decreased the activities of APX and SOD, which could not be restored to the level of controls by ABA treatment (Fig. 3B). Given the functional redundancy of NAC family, we used the chimeric repressor silencing technology to confirm the function of *ZmNAC84* (Hiratsu et al., 2003; Lin et al., 2013; Mitsuda et al., 2005). We fused the exogenous EAR motif repression domain SRDX to the C terminus of *ZmNAC84* (*ubi:ZmNAC84-SRDX-mCherry*) and then transfected it into protoplasts. Transient expression of *ZmNAC84-SRDX* decreased the activities of APX and SOD in ABA-treated and -untreated

Figure 1. (Continued.)

in-gel kinase assay. Asterisks indicate phosphorylated contaminants. WB, western blot. D, Interaction of *ZmNAC84* and *ZmCCaMK* in onion epidermal cells. YFP signals were detected by confocal laser scanning microscope. E, In vivo Co-IP assay of *ZmCCaMK* and *ZmNAC84* interaction. Maize mesophyll protoplasts were transfected with *ubi:ZmCCaMK-myc* and *ubi:ZmNAC84-His* simultaneously or *ubi:ZmNAC84-His* alone. Crude protein was immunoprecipitated with anti-myc and anti-His antibodies.

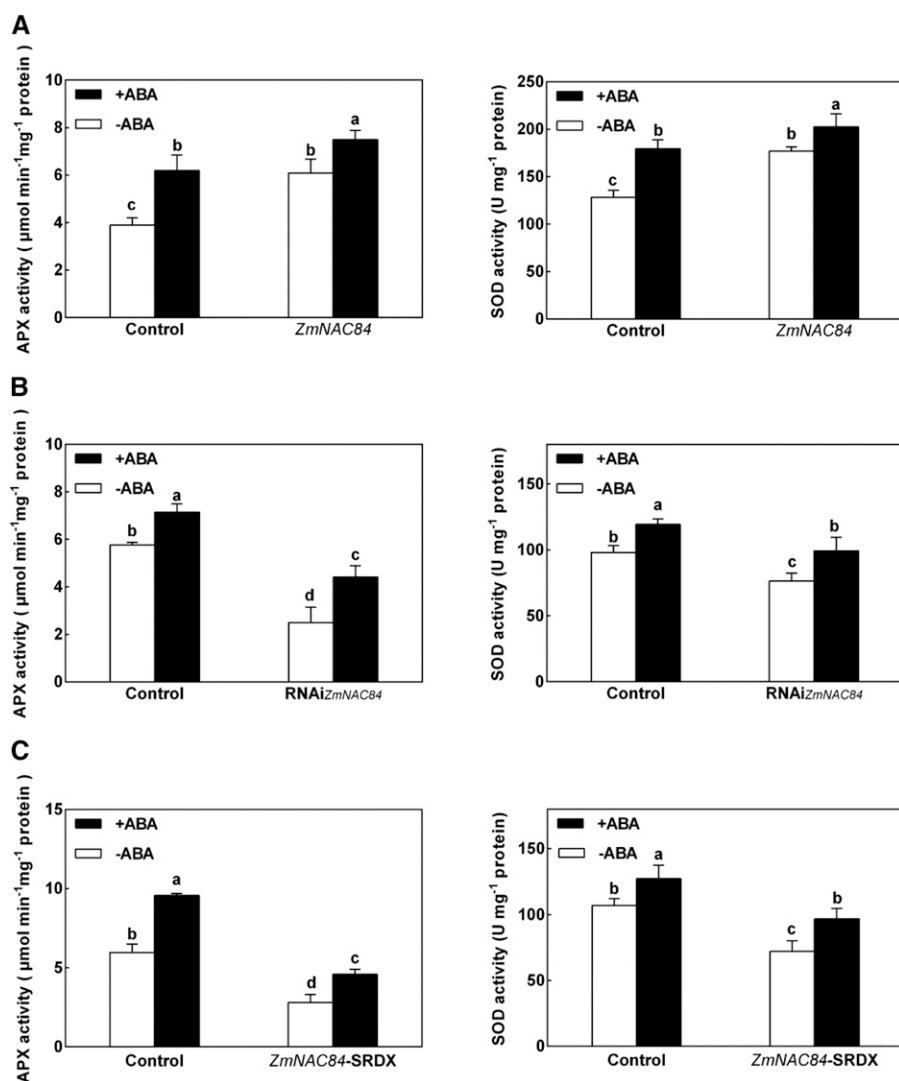


Figure 3. Role of ZmNAC84 in ABA-induced increases in activities of APX and SOD in maize protoplasts. A, The activities of APX and SOD in protoplasts transiently expressing *ZmNAC84*. The protoplasts were transfected with constructs carrying *ubi:ZmNAC84-mCherry* (*ZmNAC84*) or empty vector (Control). B, The activities of APX and SOD in protoplasts transiently silencing *ZmNAC84*. The protoplasts were transfected with dsRNA against *ZmNAC84* (RNAi_{ZmNAC84}) or distilled water (Control). C, The activities of APX and SOD in protoplasts transiently expressing *ZmNAC84-SRDX*. The protoplasts were transfected with constructs carrying *ubi:ZmNAC84-SRDX-mCherry* (*ZmNAC84-SRDX*) or empty vector (Control). The protoplasts (A–C) were treated with culture medium (–ABA) or 10 μM ABA (+ABA) for 10 min. The activities of APX and SOD were measured as described in “Materials and Methods.” Values are means \pm SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan’s multiple range test.

protoplasts (Fig. 3C), similarly to that of *ZmNAC84* transiently silencing analysis. These results clearly indicated that *ZmNAC84* is involved in ABA-induced antioxidant defense.

ZmNAC84 Interacting with ZmCCaMK Affects ABA-Induced Antioxidant Defense

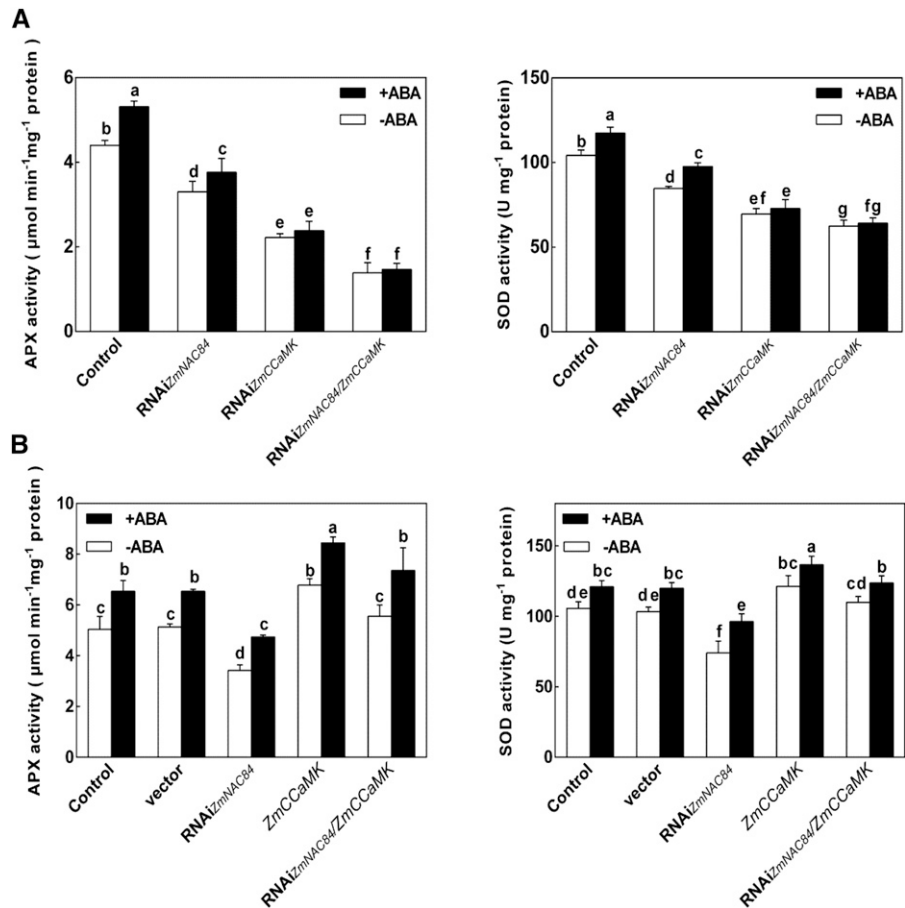
Both *ZmNAC84* (Fig. 3) and *ZmCCaMK* (Ma et al., 2012) were involved in ABA-induced antioxidant defense. To further dissect the biological relationship of *ZmNAC84* and *ZmCCaMK* in regulating ABA-induced antioxidant defense, we then transiently silenced both of them in maize mesophyll protoplasts. Silencing both *ZmNAC84* and *ZmCCaMK* (RNAi_{ZmNAC84/ZmCCaMK}) resulted in a more severe inhibition of the activities of APX and SOD compared with silencing *ZmNAC84* (RNAi_{ZmNAC84}) or *ZmCCaMK* (RNAi_{ZmCCaMK}) alone (Fig. 4A), suggesting that the two genes could act together during ABA-induced antioxidant defense. To further examine

this interaction hypothesis, we transiently silenced *ZmNAC84* and expressed *ZmCCaMK* (RNAi_{ZmNAC84/ZmCCaMK}) simultaneously. As shown in Figure 4B, RNAi_{ZmNAC84/ZmCCaMK} protoplasts exhibited higher activities of APX and SOD than *ZmNAC84* transiently silencing (RNAi_{ZmNAC84}) and lower activities than *ZmCCaMK* transiently expressing (*ZmCCaMK*) protoplasts, suggesting that increased expression of *ZmCCaMK* could partially complement the *ZmNAC84* silencing. These data provide further evidence that *ZmNAC84* is a functional partner of *ZmCCaMK* in ABA-induced antioxidant defense.

Phosphorylation of Ser-113 of ZmNAC84 by ZmCCaMK Is Required for the Effect on Antioxidant Defense

To identify the phosphorylation sites of *ZmNAC84* in regulating antioxidant enzyme activity, we predicted eight Ser/Thr residues as potential phosphorylation sites (Fig. 5A) and generated multiple versions of *ZmNAC84* by site-directed mutagenesis (S113A, T120A,

Figure 4. Effects of ZmNAC84 interacting with ZmCCaMK on regulating ABA-induced antioxidant enzyme activity. A, The activities of APX and SOD in protoplasts transiently silencing *ZmNAC84* alone (RNAi_{ZmNAC84}), *ZmCCaMK* alone (RNAi_{ZmCCaMK}), or *ZmNAC84* and *ZmCCaMK* simultaneously (RNAi_{ZmNAC84/ZmCCaMK}). B, The activities of APX and SOD in protoplasts transiently silencing *ZmNAC84* alone (RNAi_{ZmNAC84}), transiently expressing *ZmCCaMK* alone (*ZmCCaMK*), or transiently silencing *ZmNAC84* and transiently expressing *ZmCCaMK* simultaneously (RNAi_{ZmNAC84/ZmCCaMK}). The protoplasts were treated with culture medium (–ABA) or 10 μM ABA (+ABA) for 10 min. The activities of APX and SOD were measured as described in “Materials and Methods.” Values are means ± SE of three different experiments. Means denoted by the same letter did not differ significantly at *P* < 0.05 according to Duncan’s multiple range test.



S211A, T226A, S240A, S281A, T328A, and T420A) to eliminate phosphorylation at these residues. These mutant versions of *ZmNAC84* were coexpressed with *ZmCCaMK* in maize mesophyll protoplasts and examined for their ability to affect antioxidant enzyme activity. Remarkably, only the S113A mutation blocked the increases in APX and SOD activities (Fig. 5B). These results suggest that Ser-113 is an important site of *ZmNAC84* phosphorylation by *ZmCCaMK* in regulating antioxidant enzyme activity. To confirm that Ser-113 is the phosphorylation site of *ZmNAC84* by *ZmCCaMK*, we performed mass spectrometry (liquid chromatography-tandem mass spectrometry [LC-MS/MS]) analysis. His-tagged truncations of *ZmNAC84* containing the Ser-113 site, Asn-186 (1–186 amino acids) and Asn-118 (1–118 amino acids), were generated, and GST pull-down analysis showed that both Asn-186 and Asn-118 could interact with *ZmCCaMK* (Fig. 6A). Further in vitro kinase assay showed that *ZmCCaMK* phosphorylated Asn-118 (Fig. 6B). Then, we enriched Asn-118 peptides and analyzed them by LC-MS/MS. LC-MS/MS analysis revealed that Ser-113 is the phosphorylation site of *ZmNAC84* by *ZmCCaMK* (Fig. 6C). To further confirm the phosphorylation of Ser-113 in *ZmNAC84* by *ZmCCaMK*, we mutated Ser-113 to Ala or Asp and generated *ZmNAC84*^{S113A} (phosphor-ablative mutant) or *ZmNAC84*^{S113D} (phosphor-

mimicking mutant). The mutation of S113A and S113D did not affect the subcellular localization of *ZmNAC84* (Supplemental Fig. S4) but substantially blocked the phosphorylation of *ZmNAC84* by *ZmCCaMK* (Fig. 6D). Taken together, these data indicate that Ser-113 is a crucial phosphorylation site of *ZmNAC84* by *ZmCCaMK* in regulating antioxidant enzyme activity.

Ser-113 Is a Crucial Phosphorylation Site of *ZmNAC84* Functioning in ABA-Induced Antioxidant Defense

In order to determine the significance of *ZmNAC84* Ser-113 phosphorylation in ABA-induced antioxidant defense, we transiently expressed *ZmNAC84* mutant (*ZmNAC84*^{S113A} or *ZmNAC84*^{S113D}) alone or expressed it with *ZmCCaMK* simultaneously in maize mesophyll protoplasts. The results showed that mutation of S113A (*ZmNAC84*^{S113A}) led to complete loss of the *ZmNAC84*-dependent increases of APX and SOD activities in response to ABA (Fig. 7). Moreover, ABA treatment significantly enhanced the activities of APX and SOD in coexpressed *ZmNAC84* and *ZmCCaMK* protoplasts, whereas the activities in coexpressed *ZmNAC84*^{S113A} and *ZmCCaMK* were the same as with expression of *ZmCCaMK* alone (Fig. 7). Expressed *ZmNAC84*^{S113D} alone and coexpressed with *ZmCCaMK* increased the activities of APX and SOD

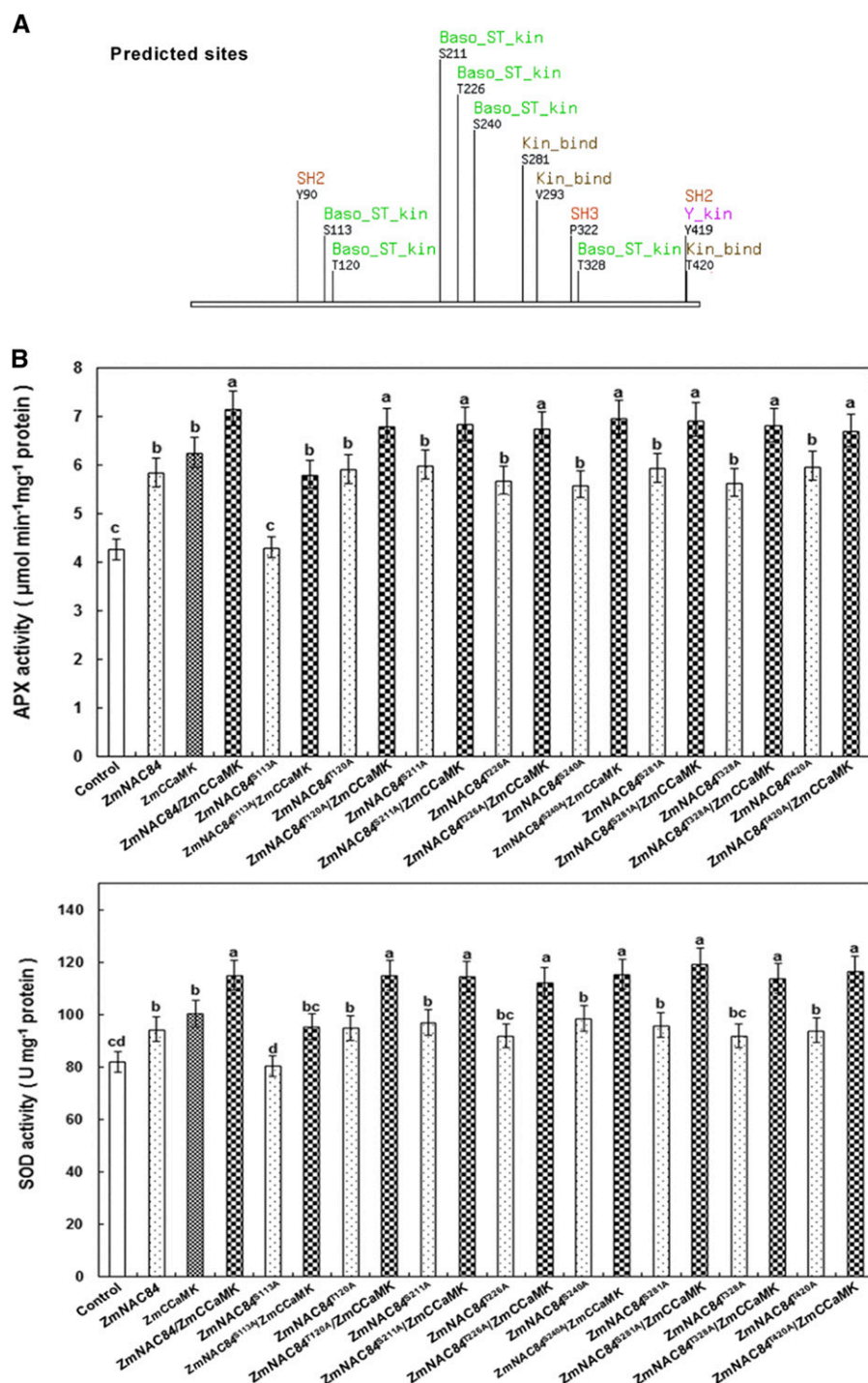


Figure 5. Prediction of phosphorylation sites of ZmNAC84 and functional analysis of these sites in regulating antioxidant enzyme activity. A, Prediction of phosphorylation sites of ZmNAC84. Potential phosphorylation residues of ZmNAC84 were predicted according to common calcium-dependent kinase phosphorylation motifs: [MVLIF]-x-R-x(2)-[ST]-x(3)-[MVLIF]. B, The activities of APX and SOD in protoplasts transiently expressing various mutants of *ZmNAC84* and *ZmCCaMK*. In B, values are means \pm SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

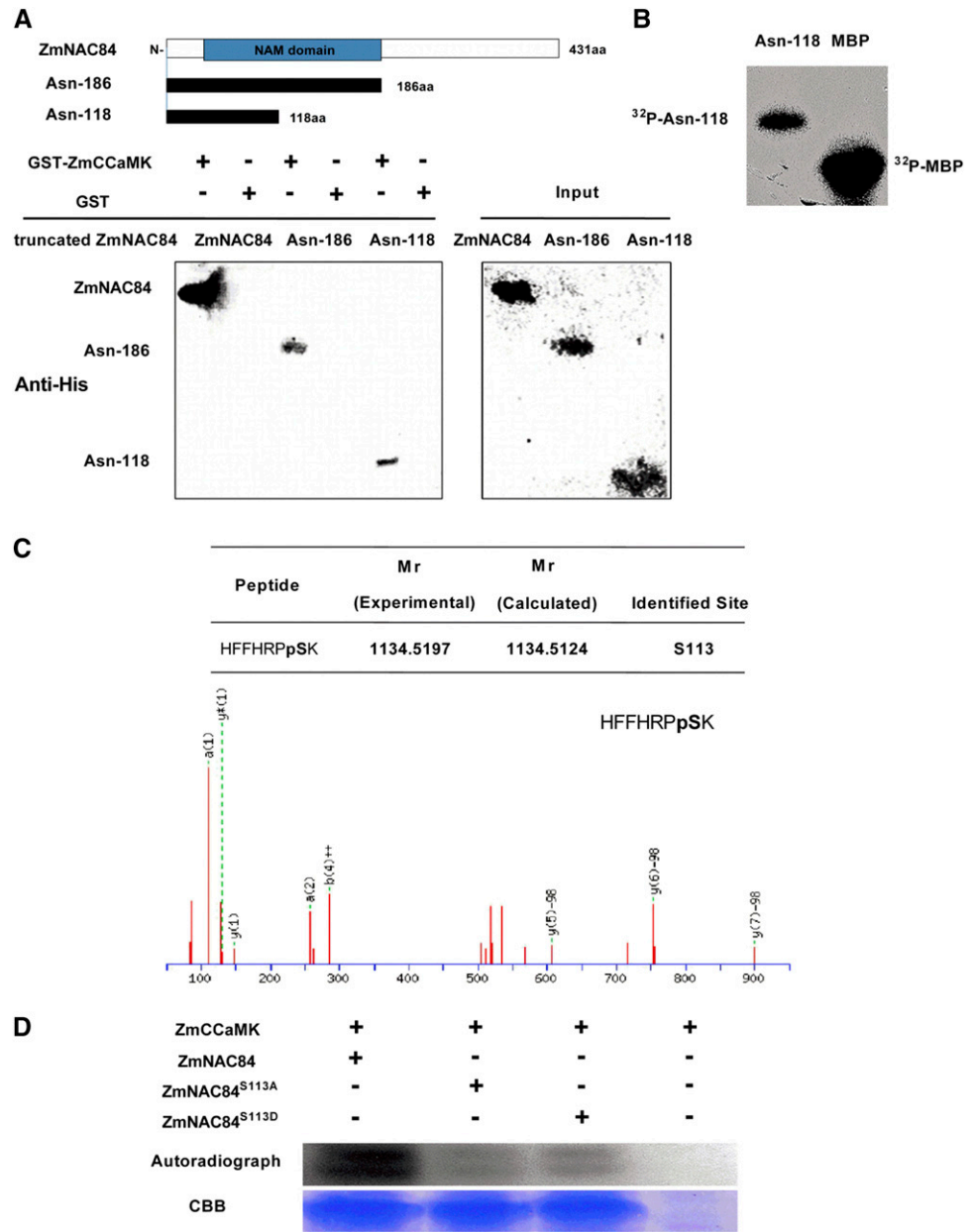
compared with control (Fig. 7). These data clearly suggested that ZmCCaMK phosphorylating ZmNAC84 at Ser-113 regulates ABA-induced antioxidant defense.

ZmNAC84 Overexpression in Tobacco Confers Seedling Drought Tolerance

To further confirm the role of ZmNAC84 in ABA-induced antioxidant defense, we generated transgenic

tobacco overexpressing the coding sequence of *ZmNAC84* under control of the cauliflower mosaic virus 35S promoter. The recombinant vector and empty vector were transferred to *Agrobacterium tumefaciens* and then were used to transform tobacco. Three independent lines (#7, #9, and #12) with *ZmNAC84* expression (Supplemental Fig. S5) were selected for analysis. As shown in Figure 8A, the *ZmNAC84* transgenic tobacco (*ZmNAC84*) displayed higher activities of APX and SOD

Figure 6. Identification of Ser-113 as a phosphorylation site of ZmNAC84. A, GST pull-down analysis of ZmCCaMK interaction with truncations of ZmNAC84. GST-ZmCCaMK fusion protein or GST alone was incubated with His-tagged full-length (ZmNAC84) or its truncations (Asn-186, N-terminal amino acids 1–186; Asn-118, N-terminal amino acids 1–118) in GST beads. ZmNAC84 or truncations with His-tag were then detected by western blot using anti-His antibody. B, Phosphorylation of the Asn-118 polypeptide by ZmCCaMK in vitro. Protein extract from maize leaves was immunoprecipitated with ZmCCaMK antibody. Recombinant His-Asn-118 or MBP was used as substrates and subjected to in-gel kinase assay. C, Phosphorylation analysis of Asn-118 by LC-MS/MS. D, Phosphorylation of ZmNAC84 mutant versions (ZmNAC84^{S113A} and ZmNAC84^{S113D}) by ZmCCaMK in vitro. Protein extract from maize leaves was immunoprecipitated with ZmCCaMK antibody. Recombinant His-ZmNAC84, His-ZmNAC84^{S113A}, or His-ZmNAC84^{S113D} was used as substrates and subjected to in-gel kinase assay. CBB, Coomassie Brilliant Blue.



compared with the empty vector control plants (vector). One mode of plant tolerance to drought stress is inducing antioxidant defense system, so we next investigate the response of ZmNAC84 to drought stress. We first analyzed the expression of *ZmNAC84* under polyethylene glycol (PEG) treatment. Results showed that PEG treatment strongly induced *ZmNAC84* expression (Supplemental Fig. S6). Moreover, compared with the control plants, the *ZmNAC84* transgenic tobacco displayed enhanced drought tolerance, whereas no morphological changes were observed under normal growth conditions (Fig. 8B). Fresh weight and relative water content (RWC) of the transgenic plants were significantly higher than those of control after drought treatment (Fig. 8C). The transgenic tobacco also showed less oxidative damage

in response to drought as indicated by the content of malondialdehyde (MDA) and the percentage of electrolyte leakage compared with control (Fig. 8C). These data clearly indicated that ZmNAC84 could enhance tobacco seedling tolerance to drought stress.

DISCUSSION

CCaMK is a central regulator of nodule organogenesis, rhizobia infection, and arbuscular mycorrhizal signaling (Gobbato, 2015; Miller et al., 2013; Shimoda et al., 2012). Recently, a growing body of evidence reveals additional functions of CCaMK, such as response to ABA signaling and stress tolerance in wheat, maize,

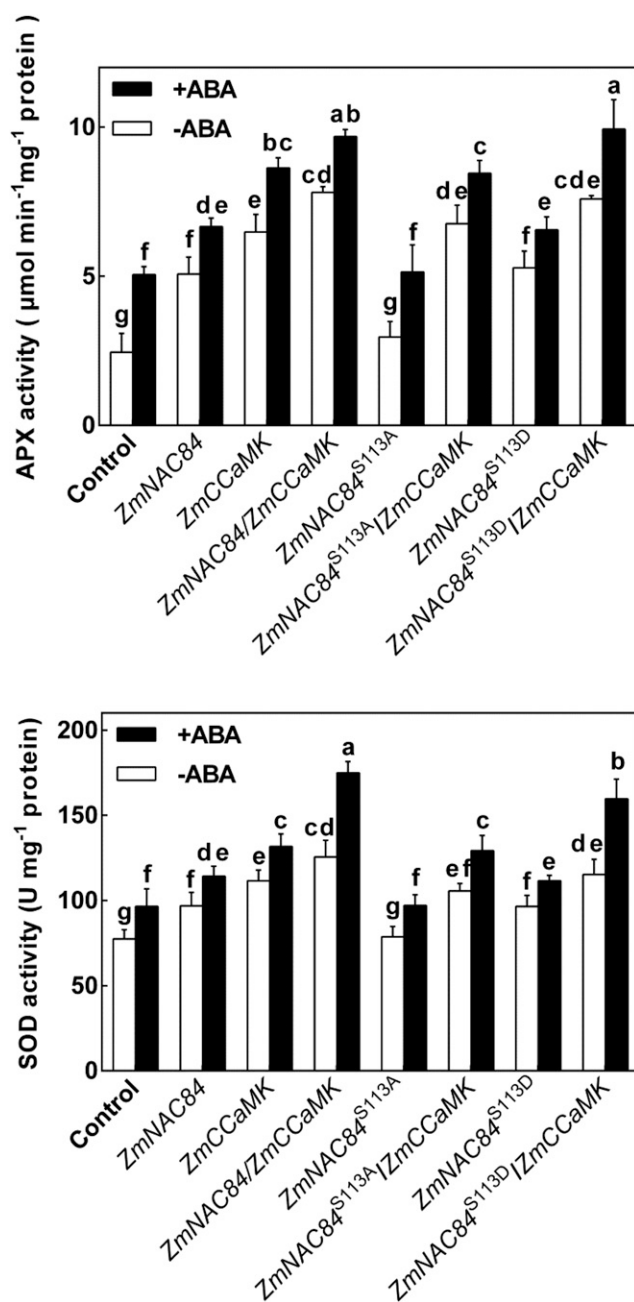


Figure 7. The effects of Ser-113 mutation on ABA-induced antioxidant defense. The protoplasts transiently expressing *ZmNAC84* alone, *ZmCCaMK* alone, *ZmNAC84*^{S113A} alone, *ZmNAC84*^{S113D} alone, *ZmNAC84* and *ZmCCaMK* simultaneously, *ZmNAC84*^{S113A} and *ZmCCaMK* simultaneously, or *ZmNAC84*^{S113D} and *ZmCCaMK* simultaneously were treated with culture medium (-ABA) or 10 µM ABA (+ABA) for 10 min. The activities of APX and SOD were measured as described in "Materials and Methods." Values are means ± SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

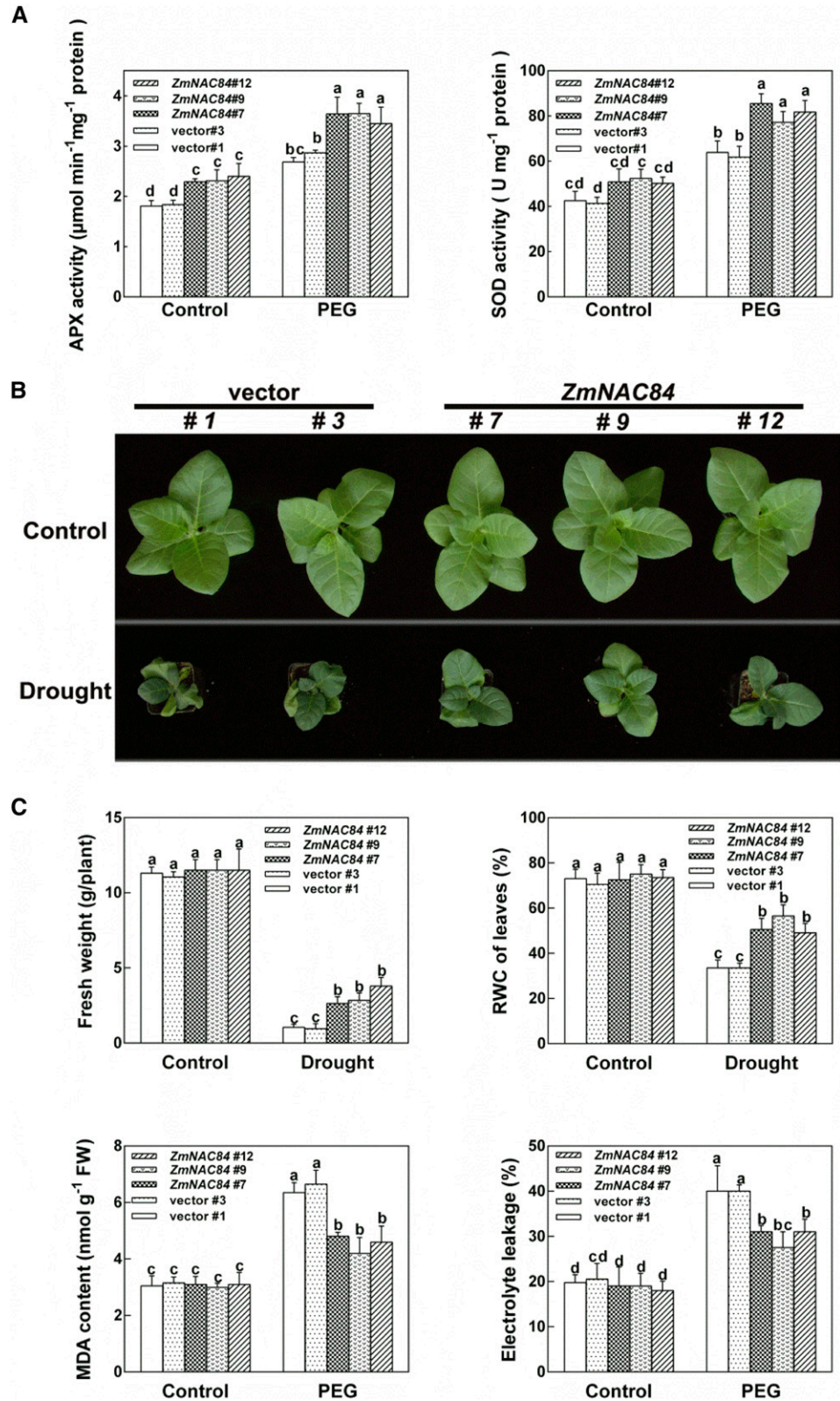
and rice (Ma et al., 2012; Shi et al., 2012; Yang et al., 2011a). Hence, it is getting increasingly important to identify the downstream targets of CCaMK in order to understand the physiological role of CCaMK in

different biological processes. So far, only two directly interacting proteins of CCaMK, IPD3/CYCLOPS and CIP73 in *L. japonicus*, *M. truncatula*, and rice, were identified (Chen et al., 2008; Horváth et al., 2011; Kang et al., 2011; Messinese et al., 2007; Yano et al., 2008). IPD3 is a member of the common symbiotic signaling pathway and is necessary for the invasion of the host cell by both rhizobial and fungal symbiotic partners (Horváth et al., 2011). CIP73 has also been shown to be essential for nodule formation (Kang et al., 2011). However, neither IPD3 nor CIP73 was involved in ABA-induced increases in the activities of antioxidant enzymes (Shi et al., 2014), and the mechanism and phosphorylation targets through which CCaMK mediates this process have been completely unclear. In this report, we identified a physically interacting protein of ZmCCaMK, ZmNAC84, which functions in ABA-induced antioxidant defense.

Using the full-length *ZmCCaMK* as bait in a yeast two-hybrid screen, we identified a NAC transcription factor, ZmNAC84, and we could confirm physical interaction between the two proteins in vitro and in vivo (Fig. 1). Therefore, ZmNAC84 is a new interaction protein of ZmCCaMK besides IPD3 and CIP73. Our previous study showed localization of ZmCCaMK in the nucleus (Ma et al., 2012), and ZmNAC84 was also located in the nucleus in maize protoplasts (Supplemental Fig. S1). The molecular mechanism by which ZmCCaMK and ZmNAC84 mediate biological processes presumably involves the activation of downstream genes, where ZmNAC84 functions either as a downstream effector, tethering ZmCCaMK to transcription regulatory complexes of target genes, as a substrate of this protein kinase, or both. The ZmNAC84 regulated downstream genes remain to be identified.

ZmNAC84 belongs to the NAC superfamily of TFs (Fan et al., 2014). The typical NAC proteins share a conserved N-terminal DNA binding domain but vary greatly in other regions, resulting in distinct functions of different proteins. The NAC family has been found to play pivotal roles in response to abiotic stress (Fang et al., 2015; Garapati et al., 2015; Hu et al., 2006a; Huang et al., 2015; Mao et al., 2015; Wu et al., 2012) and ABA signaling (Du et al., 2014; Fujita et al., 2004; Garapati et al., 2015; Hu et al., 2006a; Yang et al., 2011b). NAC TFs regulate abiotic stress in both ABA-dependent and ABA-independent manner (Xu et al., 2013). In this study, we demonstrated that ZmNAC84 is a positive component of ABA-induced antioxidant defense involved in tolerance to drought stress. This conclusion was based on the following results: First, expression analysis of *ZmNAC84* after PEG treatment indicates that *ZmNAC84* expression was positively correlated with adaptation to seedling drought stress (Supplemental Fig. S6), which suggested a positive regulatory role of *ZmNAC84* in maize seedlings exposed to drought stress. Second, ABA treatment also up-regulated the expression of *ZmNAC84* (Fig. 2). Third, ZmNAC84 was essential for ABA-induced antioxidant defense (Fig. 3). Finally, over-expression of the *ZmNAC84* in transgenic tobacco could

Figure 8. Drought tolerance of *ZmNAC84* transgenic tobacco. **A**, The activities of APX and SOD in transgenic tobacco of *ZmNAC84*. Plants were exposed to 20% PEG for 6 h. **B**, Phenotype of *ZmNAC84* transgenic tobacco. Photographs were taken under well-watered conditions (Control) and drought treatment for 14 d. Vector-transformed (vector #1 and #3) and *ZmNAC84*-transformed (*ZmNAC84*) transgenic plants are shown **C**, Fresh weight, RWC, the content of MDA, and the percentage of leakage of electrolyte in *ZmNAC84* transgenic tobacco. Plants were exposed to drought treatment (fresh weight, RWC) for 14 d or 20% PEG (the content of MDA and the percentage of leakage of electrolyte) for 12 h. The experiments were repeated at least three times. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.



improve drought tolerance and alleviated oxidative damage in response to drought (Fig. 8).

Both exogenous H_2O_2 and ABA-produced endogenous H_2O_2 affected *ZmNAC84* expression (Fig. 2).

Hence, the increased expression of *ZmNAC84* in response to ABA may be indirect via the ABA-induced increase in H_2O_2 . Our previous studies demonstrated that ABA first induced initial H_2O_2 accumulation, the

initially accumulated H₂O₂ activated CCaMK, and the activated CCaMK enhanced H₂O₂ production, forming a positive amplification loop of H₂O₂ in ABA signaling (Shi et al., 2012). In this report, ZmNAC84 interacting with ZmCCaMK performed an essential role in ABA-induced antioxidant defense (Fig. 4). Therefore, it is highly possible that initial produced H₂O₂-induced ZmNAC84, interacting with H₂O₂-activated ZmCCaMK, promotes H₂O₂ amplification, and thus activates antioxidant defense in ABA signaling. Moreover, silenced *ZmNAC84* in overexpressed *ZmCCaMK* protoplasts could not completely block the effects of ZmCCaMK on ABA-induced antioxidant enzyme activities (Fig. 4B). Possibly, yet to be identified phosphorylation targets of CCaMK are able to, at least partially, substitute for the loss of ZmNAC84 in this process.

To further explain the mechanism of ZmCCaMK interaction with ZmNAC84 in ABA-induced antioxidant defense, we predicted the possible phosphorylation sites of ZmNAC84 and mutated them from Ser/Thr to Ala to eliminate phosphorylation of these sites. Only S113A mutagenesis of ZmNAC84 blocked the ZmCCaMK-enhanced and ABA-induced antioxidant enzyme activity (Figs. 5 and 7). LC-MS/MS analysis confirmed Ser-113 in ZmNAC84 as the phosphorylation site by ZmCCaMK (Fig. 6). Future studies will be required to determine if and how the Ser-113 phosphorylation affects DNA binding of this NAC TF. Phosphorylation is a prerequisite for nuclear localization of OsNAC4 (Kaneda et al., 2009) and phosphorylation of AFAF1 by SnRK1 kinase could modulate its subcellular colocalization (Kleinow et al., 2009). However, the nuclear localization of ZmNAC84 was not altered by the phosphorylation state of Ser-113 site (Supplemental Fig. S4).

In summary, our data showed that ZmNAC84 interacts with ZmCCaMK in modulating ABA-induced antioxidant defense, and the phosphorylation at Ser-113 of ZmNAC84 by ZmCCaMK is essential for its role in this process.

MATERIALS AND METHODS

Plant Materials and Treatments

Seeds of maize (*Zea mays* cv Nongda 108; from Nanjing Agricultural University, China) were sown in trays of sand in a growth chamber at a temperature of 22 to 28°C, photosynthetic active radiation of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and a photoperiod of 14/10 h (day/night) and watered daily. For protoplast isolation, maize plants were grown at 25°C under dark conditions. When the second leaves were fully expanded, they were collected and used for investigations.

The plants were excised at the base of stem and placed in distilled water for 2 h to eliminate wound stress. Maize seedlings were placed in beakers wrapped with aluminum foil containing 100 μM ABA, 10 mM H₂O₂, or 10% (w/v) polyethylene glycol (PEG6000) solutions, respectively. In order to study the effects of inhibitor of ROS production, the detached plants were pretreated with 100 μM DPI for 4 h, then subjected to 100 μM ABA treatment for 45 min under the same conditions as described above. Detached plants were treated with distilled water under the same conditions for the whole period and served as controls for the above. After treatments of detached plants, the second leaves were sampled and immediately frozen under liquid N₂ for further analysis.

Yeast Two-Hybrid Assay

Yeast two-hybrid assays were performed using the ProQuest two-hybrid system with Gateway technology according to the manufacturer's instructions (Invitrogen). *Saccharomyces cerevisiae* strain MaV203 cotransformed with indicated constructs was grown on the SC/-Leu/-Trp or SC/-Leu/-Trp/-His +10 mM 3-amino-triazole media, and the transformants were used for LacZ assay using X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside).

GST Pull-Down Assay

GST or GST-ZmCCaMK fusion proteins were kept immobilized on glutathione Sepharose 4B beads and then were incubated with His-tagged ZmNAC84 or its truncations (Asn-118 and Asn-186) in binding buffer (140 mM NaCl, 4.2 mM Na₂HPO₄, 2 mM KH₂PO₄, 10 mM KCl, and 10% bovine serum albumin, pH 7.2) at 4°C for 2 h. Subsequently, the beads were washed at least five times with washing buffer (400 mM NaCl, 4.2 mM Na₂HPO₄, 2 mM KH₂PO₄, and 10 mM KCl, pH 7.2). After extensive washing, the pulled down proteins were eluted by boiling and separated by 12% SDS-PAGE. Proteins were probed with rat monoclonal anti-His antibody (Sigma-Aldrich) followed by incubation with goat anti-rat HRP-conjugated secondary antibody (Sigma-Aldrich). Signals were visualized by x-ray film.

BiFC Assay

For generation of the BiFC vectors, the *ZmNAC84* coding region without stop codon was amplified and cloned at the *Bam*HI-*Kpn*I sites in pSPYCE to form *ZmNAC84-YFP^C* construct, and *ZmCCaMK* without stop codon was cloned into the *Bam*HI-*Sall* sites of pSPYNE to generate *ZmCCaMK-YFP^N* plasmid. Onion (*Allium cepa*) tissues were prepared, and two fusion proteins were transiently transfected into onion epidermal cells as described previously (Lee et al., 2008). YFP fluorescence in transformed onion tissues was detected and imaged using a confocal laser scanning microscope (TCS-SP2; Leica) after incubation for 16 h.

Co-IP Assay

The coding sequences of *ZmNAC84* or *ZmCCaMK* were fused with His or myc tags and cloned into pXZP008 at *Bam*HI-*Kpn*I sites, respectively. Maize mesophyll protoplasts were transfected with *ubi:ZmCCaMK-myc* and *ubi:ZmNAC84-His* or *ubi:ZmNAC84-His* alone. After incubation for 16 h, protoplasts were harvested and homogenized in Co-IP buffer as described previously (Zhang et al., 2006). The solubilized proteins were incubated with anti-myc antibody bound to protein A beads for 2 h. The beads were washed three times with immunoprecipitation buffer, and the proteins were eluted by boiling in 1× SDS sample buffer for 5 min. After centrifugation, the supernatant fraction was analyzed by immunoblotting with anti-His antibody.

Immunoprecipitation Kinase Activity Assay

Protein were extracted and quantified as described previously (Ma et al., 2012). For immunocomplex kinase assay, protein extract (100 μg) was incubated with anti-ZmCCaMK antibody (7.5 μg) in immunoprecipitation buffer. Immunocomplex was incubated with 2 μg substrates in reaction buffer (25 mM Tris, pH 7.5, 5 mM MgCl₂, 1 mM DTT, 2.5 mM CaCl₂, 2 μM CaM with 200 nM ATP plus 1 μCi of [γ -³²P]ATP [3,000 Ci mM⁻¹]) for 30 min. The reaction was stopped by adding SDS sample buffer, and the reaction mix was separated on SDS-PAGE. The unincorporated [γ -³²P]ATP was removed by washing with 5% (w/v) trichloroacetic acid/1% (w/v) sodium pyrophosphate at least three times. The gel was dried onto Whatman 3 MM paper and exposed to Kodak XAR-5 film.

Isolation of Total RNA and qRT-PCR Analysis

Total RNA was isolated from leaves or protoplasts using an RNase-free DNase (TaKaRa) following the manufacturer's protocol and treated with RNase-free DNase to remove contaminating DNA (TaKaRa). Approximately 2 μg of total RNA was reversely transcribed using an oligo(dT)₁₆ primer and Moloney murine leukemia virus reverse transcriptase (TaKaRa). Transcript levels of several genes were measured by qRT-PCR using a DNA Engine Opticon 2 real-time PCR detection system (Bio-Rad) with SYBR Premix Ex Taq (TaKaRa)

according to the manufacturer's instructions. The expression level was normalized against that of *ZmActin* in maize or *NtActin* in tobacco (*Nicotiana tabacum*).

Vector Construction and in Vitro Synthesis of Double-Stranded RNA

The constructed vector pXZP008 with mCherry driven by ubiquitin promoter (the original 35S promoter was substituted by the ubiquitin promoter using *HindIII* and *BamHI*) was used for protoplast transient expression and subcellular localization assay. The full-length *ZmNAC84* was amplified by PCR and cloned into pXZP008 at *BamHI*-*KpnI* sites. The exogenous EAR motif repression domain *SRDX* (5'-CTGGATCTAGAACTCCGTTTG-3') was introduced into the C terminus of *ZmNAC84* by PCR and then cloned into pXZP008.

For in vitro synthesis of double-stranded RNA (dsRNA), DNA templates were produced by PCR using primers containing the T7 promoter sequence (5'-TTAATACGACTCACTATAGGGAGG-3') on both the 5' and 3' ends. The primers used to amplify DNA of *ZmCCaMK* or *ZmNAC84* are listed in Supplemental Table S1. dsRNA of *ZmCCaMK* or *ZmNAC84* was synthesized in vitro using the RiboMAX Large Scale RNA Production System-T7 (Promega) according to the manufacturer's instructions. The purity and concentration of synthesized dsRNA were checked by 2% agarose gel electrophoresis and spectrophotometry.

Protoplast Preparation and Transfection with DNA Constructs or dsRNAs

Protoplast isolation and transfection with DNA constructs or dsRNAs were based on the protocol for maize mesophyll protoplasts provided online by J. Sheen's laboratory (<http://genetics.mgh.harvard.edu/sheenweb>) with minor modifications. Maize protoplasts (1 mL, usually 5×10^5 cells mL⁻¹) were transfected with 100 µg of fusion constructs (empty vector as control) or 150 µg dsRNAs (water as control) using a PEG-calcium-mediated method. The transfected protoplasts were then incubated in incubation solution overnight in the dark at 25°C. After that, protoplasts were collected and used for further analysis.

Site-Directed Mutagenesis

To mutate *ZmNAC84*, the Multi-Directed Mutagenesis Kit (Agilent Technologies) was used according to the manufacturer's instructions. The DNA oligonucleotides used in mutagenesis were synthesized, and their sequences are listed in Supplemental Table S1. All of the mutated plasmids were confirmed by Sanger sequencing.

Localization

Protoplasts were transfected with *ubi:ZmNAC84-mCherry*, *ubi:ZmNAC84^{S113A}-mCherry*, or *ubi:ZmNAC84^{S113D}-mCherry* constructs and incubated for 16 h. The fluorescence was observed using a confocal laser scanning microscope (TCS-SP2; Leica). The nucleus was stained with 4',6-diamidino-2-phenylindole (1 µg µL⁻¹) dye.

Antioxidant Enzyme Assay

The protoplasts or tobacco leaves were homogenized in 0.6 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone, with the addition of 1 mM sodium ascorbate in the case of APX assay. The homogenate was centrifuged at 12,000g for 30 min at 4°C, and the supernatant was immediately used for the subsequent antioxidant enzyme assays. The total activities of antioxidant enzymes were determined as previously described (Zhu et al., 2013).

Mass Spectrometry Analysis

The truncated *ZmNAC84* (Asn-118) was reacted with *ZmCCaMK* in vitro in kinase assay buffer with ATP (200 nM). Phosphorylated His tagged Asn-118 was enriched and subjected to trypsin digestion followed by LC-MS/MS analysis as described previously (Gampala et al., 2007).

Generation of Transgenic Plants

The full-length cDNA of *ZmNAC84* was inserted into the *SacI*-*KpnI* sites of the binary vector super1300 driven by the cauliflower mosaic virus 35S promoter. The recombinant vector or empty super1300 (vector) was introduced into tobacco using *Agrobacterium tumefaciens* strain EHA105 via leaf disc transformation (Horsch et al., 1985). T0, T1, and T2 plants were grown in a greenhouse, and the presence of the transgene was determined in each generation by PCR analysis. The expression of *ZmNAC84* in transgenic plants was determined by qRT-PCR, and three independent T2 lines, *ZmNAC84#7*, *ZmNAC84#9*, and *ZmNAC84#12*, were selected for further analysis.

Phenotype and Oxidative Damage Analysis

For the phenotype, fresh weight, and the RWC assays, 4-week-old seedlings grown in pots with soil were treated by withholding water for 14 d. The phenotype of seedlings was photographed, and the shoot fresh weight and the RWC were measured as described by Jiang and Zhang (2001). For the oxidative damage analysis, 4-week-old seedlings were treated with 20% PEG for 12 h. The content of MDA and the percentage of electrolyte leakage were determined as described by Shi et al. (2012).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *ZmCCaMK* DQ403196; *ZmNAC84* AFU81568.1; *ZmActin* J01238; *NtActin* U60495.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Subcellular localization of *ZmNAC84* in maize protoplasts.

Supplemental Figure S2. Expression analysis of *ZmNAC84* in various tissues of maize.

Supplemental Figure S3. ABA-induced expression of *ZmCCaMK* in maize leaves.

Supplemental Figure S4. Subcellular localization of Ser-113 site-directed mutagenesis of *ZmNAC84* in maize protoplasts.

Supplemental Figure S5. *ZmNAC84* expression analysis in transgenic tobacco plants.

Supplemental Figure S6. PEG-induced expression of *ZmNAC84* in maize leaves.

Supplemental Table S1. PCR primers used.

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