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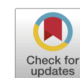
# Phosphorylation of ZmNAC84 at Ser-113 enhances the drought tolerance by directly modulating *ZmSOD2* expression in maize



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ABSTRACT

NAC (NAM, ATAF1/2, and CUC2) transcription factors play vital roles in response to multiple abiotic stresses. Our previous study has demonstrated that ZmNAC84, a maize NAC transcription factor, enhanced the drought tolerance by increasing abscisic acid (ABA)-induced antioxidant enzyme activities of APX and SOD, and Ser-113, a key phosphorylation site, of ZmNAC84 played an important role in this process. However, the target gene of ZmNAC84 in this process is still unknown. Here, we found that ZmNAC84 only regulated the luciferase activity driven by *ZmSOD2* promoter in tobacco. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assay showed that ZmNAC84 directly bound to the CACGTG motif of *ZmSOD2* promoter. Furthermore, phosphorylation of ZmNAC84 at Ser-113 up-regulated the *ZmSOD2* expression by enhancing the DNA binding ability of ZmNAC84 to *ZmSOD2* promoter and improved the drought tolerance. Taken together, our results demonstrate that ZmNAC84 directly regulates *ZmSOD2* expression to enhance drought tolerance and Ser-113 of ZmNAC84 is crucial in this process.

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## 1. Introduction

Drought stress is a major abiotic stress that affects plant development and causes reductions in crop yield [1]. Plants have evolved various strategies to cope with drought stress [2]. In general, plants sense the stimulus and transmit signals that regulate the downstream genes responsible for the adaptive response to drought stress. Numerous transcription factors, including bZIP, MYB, WRKY and NAC, have been identified in plants to function in improving drought tolerance [3–6].

The NAC (NAM, ATAF1/2, and CUC2) transcription factors, which is one of the largest transcription factor families, are plant-specific proteins [7]. To data, 106 NAC genes have been identified in *Arabidopsis*, 151 in *Oryza sativa* and 147 in *Zea mays* [8–10]. NACs play vital roles in various biological processes, such as the formation of plant meristem and organ boundaries, lateral root development, secondary cell wall synthesis and leaf senescence [11–14]. Besides that, NACs are also involved in the responses of plants to abiotic stresses [6,15–17]. AtNAC019, AtNAC055 and AtNAC072 play an important role in enhancing drought resistance in *Arabidopsis* [18]. OsSNAC1 and OsONAC022 positively regulate drought and salt tolerance in rice [16,17]. NAC binds to the promoter of its target genes to activate or repress these genes expressions. For instance, overexpression of *OsNAC6* in rice enhances tolerance to dehydration and salt stresses by activating the expression of at least two genes, including the gene encoding a peroxidase (*OsSPC4*) [19]. Overexpression of *OsONAC022* in rice enhances drought and salt tolerance by regulating the expression of ABA biosynthetic genes (*OsNCEDs* and *OsPSY*), signaling and regulatory genes (*OsPP2C02*, *OsPP2C49*, *OsPP2C68*, *OsbZIP23*, *OsAP37*, *OsDREB2a*, and *OsMYB2*) and late stress-responsive genes (*OsRAB21*, *OsLEA3*, and *OsP5CS1*) [17]. In *Arabidopsis*, overexpression of *GmNAC109* improves drought and salt tolerance by up-regulating the expression of stress response-related gene (*DREB1A*, *DREB2A*, *AREB1*, *AREB2*, *RD29A* and *COR15A*) [20]. Maize genome encodes 147 NACs [10]. Our previous study has found that overexpression of *ZmNAC84* in tobacco could enhance plant drought tolerance [21]. However, the target genes of ZmNAC84 in this process remain to be identified.

Here, we demonstrate that ZmNAC84 directly binds to the promoter of *ZmSOD2* in tobacco and directly up-regulates the *ZmSOD2* expression, then modulates the SOD activity, thus enhances the tolerance to drought stress.

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## 2. Materials and methods

### 2.1. Generation of maize transgenic plants

The coding region of Zm*NAC84* or its mutants were amplified by PCR using the specific primers (Supplemental Table 1) and inserted into plant expression vector pCUN-NHF driven by the *ubiquitin* promoter. The constructs were transformed into the maize (*Zea mays* L.) inbred line B73 using the *Agrobacterium*-mediated maize shoot-tip transformation method with minor changes [22]. The positive transformants were selected by glufosinate ammonium (Sigma-Aldrich, USA), and further confirmed by PCR amplification. $T_0$, $T_1$, and $T_2$ plants were grown in greenhouse and field, and the presence of the transgene was determined in each generation by PCR analysis. Resistant $T_2$ seedlings with 3:1 segregation of resistance were transferred to soil to obtain homozygous $T_3$ seeds from individual lines.

### 2.2. Isolation of total RNA and RT-qPCR analysis

Total RNA was isolated from maize leaves using RNAiso Plus Kit (TaKaRa, Japan) and RT-qPCR was performed using 2 × SYBR Green Fast qPCR Master Mix (YiFeiXue Bio Technology, China) according to the manufacturer's protocol. The relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method [23]. The primers are listed in Supplemental Table 1. The expression level was normalized against that of *ZmActin2* in maize.

### 2.3. Western blot analysis

Western blot was performed as described by Liu et al. [24]. The PVDF membrane was probed with primary antibody diluted in blocking solution at the following dilutions: anti-FLAG antibody (1:5000, Abmart, China), plant actin monoclonal antibody (1:8000, Biodragon, China). The secondary antibody, horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Abmart, China) was used at 1:8000 dilution. Chemiluminescent signal generated by ECL western blotting substrate (Beyotime, China) was detected by Tanon 5200 multi chemiluminescent imaging system (Tanon, China).

### 2.4. Dual luciferase reporter assay

The promoters of *ZmSODs* and *ZmAPXs* were separately cloned into p1381-LUC vector to act as the reporter. The full-length coding sequence of *ZmNAC84* was cloned into 1305-YFP vector to serve as the effector. Then, The *Pro::LUC-35S::REN* reporter constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and were infiltrated into 4-week-old *Nicotiana benthamiana* leaves with various combinations as indicated. The leaves were collected after infiltrated for 3 days. The LUC/REN was quantified using Dual Luciferase Reporter Gene Assay Kit (Yeasen Biotech, China) and detected with SpectraMax iD5 (Molecular Devices, USA) according to the manufacturer's instructions.

### 2.5. Eectrophoretic mobility shift assay (EMSA)

The EMSA assay was performed as described by Yan et al. [25]. The full-length coding sequence of *ZmNAC84* or its mutants were amplified and introduced into pET-30a for His fusions. These constructs were transformed into *Escherichia coli* BL21 (DE3). Reaction mixtures containing the His-ZmNAC84 or its mutant protein and probes were then incubated for 20 min at room temperature and separated on 4% polyacrylamide gels in 0.5 × TBE buffer. The signals were detected using the chemiluminescence EMSA kit (Beyotime, China).

### 2.6. Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assay

The ChIP-qPCR assay was performed as described by Yan et al. [26]. Transgenic maize seedlings of the OE-*ZmNAC84*#1, OE-*ZmNAC84*$^{S113A}$#1 and OE-*ZmNAC84*$^{S113D}$#1 plants were harvested and fixed in 1% formaldehyde with vacuum infiltration. Cross-linking was quenched by adding glycine (0.125 M). The chromatin was collected and sonicated, and immunoprecipitation using anti-DYKDDDDK (anti-FLAG, Abmart, China) antibody. The enrichment of DNA fragments was quantified by quantitative PCR using specific primers (Supplemental Table 1). A fragment of the *ZmActin2* coding region was used as a reference gene. Enriched values were normalized with the level of input DNA.

### 2.7. Phenotype and oxidative damage analysis

Maize seeds were sown on pots containing soil mixture (soil: vermiculite, 1: 1, v/v) in greenhouse at a temperature of 28 °C (day)/22 °C (night), photosynthetic active radiation of 200 μmol $m^{-2}$ $s^{-1}$ and a photoperiod of 14 h/10 h (day/night).

For drought treatment, 2-week-old seedlings of wild type and transgenic plants grown in soil pots were treated by withholding water for 10 days. The phenotype of seedlings was photographed.

The relative water content (RWC) was measured as described by Xiang et al. [27]. 2-week-old seedlings were subjected to drought stress by withholding water for 10 days, then the maize leaves were harvested. For the analysis of oxidative damage, 2-week-old seedlings were subjected to drought stress by withholding water for 2 days, then the content of malondialdehyde (MDA) and the percentage of electrolyte leakage were determined as described by Liu et al. [28].

### 2.8. Measurement of the superoxide dismutase (SOD) activity

2-week-old seedlings were subjected to drought by withholding water for 2 days, then the maize leaves were harvested. The leaves were homogenized in 0.6 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 g for 30 min at 4 °C, and the supernatant was immediately used to measure the SOD activity as described by Zhu et al. [21].

## 3. Results

### 3.1. ZmNAC84 only regulates the luciferase activity driven by the promoter of ZmSOD2

Our previous study has shown that ZmNAC84 up-regulated SOD and APX activities [21], but its target gene is still unknown. Maize genome encodes 5 *SOD* genes (*ZmSOD1*, *ZmSOD2*, *ZmSOD3*, *ZmSOD4* and *ZmSOD4A*) and 8 *APX* genes (*ZmAPX1.1*, *ZmAPX1.2*, *ZmAPX2*, *ZmAPX3*, *ZmAPX4*, *ZmAPX5*, *ZmAPX6* and *ZmAPX7*), then the effects of ZmNAC84 on the activity of *ZmSODs* and *ZmAPXs* promoters were analyzed using a dual luciferase reporter assay in tobacco leaves. The promoters of *ZmSODs* and *ZmAPXs* were amplified and fused with a *firefly luciferase* (LUC) gene. The *renilla luciferase* (REN) gene driven by cauliflower mosaic virus (CaMV) *35S* promoter was used as an internal control. The effector plasmids contain either *ZmNAC84-YFP* or *YFP* expression cassette. The ability of ZmNAC84 to transcriptionally activate its putative downstream genes was calculated through LUC/REN ratio. As shown in Fig. 1 and Supplemental Fig. 1, ZmNAC84 only activated the LUC activity

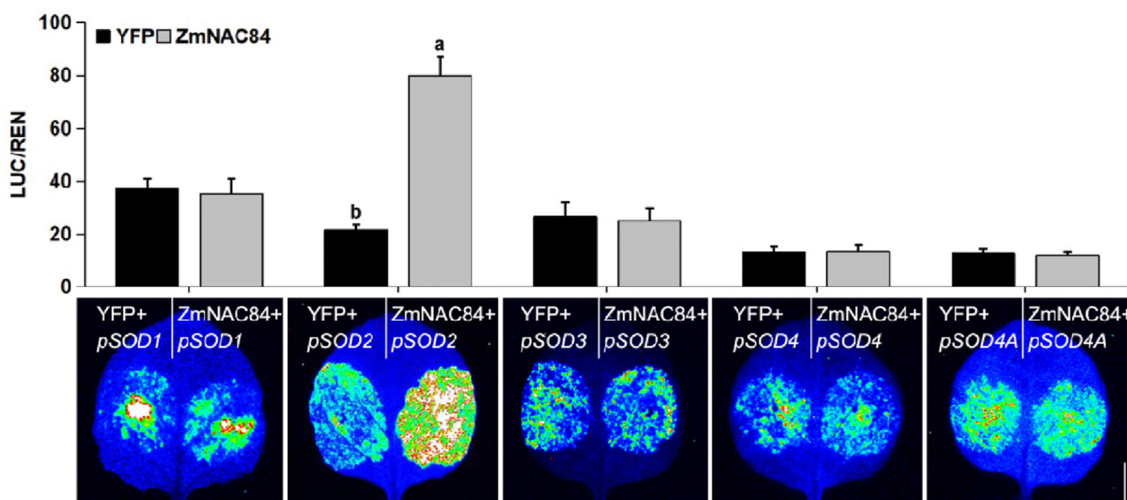


**Fig. 1.** ZmNAC84 only activates the LUC activity driven by *ZmSOD2* promoter. Images of LUC signal of *ZmSODs* promoters. The *pro:LUC-35S:REN* reporter constructs were transformed into tobacco leaves together with ZmNAC84 or YFP (Control). The bar indicates 1 cm. The LUC/REN ratio represented the relative activity of *ZmSODs* promoters. Data are means ± SE (n = 3). Different letters indicate significant differences at $P < 0.05$ according to one-way ANOVA.

driven by *ZmSOD2* promoter, but not affected the LUC activity driven by the other *ZmSODs* promoter or *ZmAPXs* promoter, suggesting that ZmNAC84 might regulate the *ZmSOD2* expression.

### 3.2. ZmNAC84 directly binds to the ZmSOD2 promoter in vitro and in vivo

Since ZmNAC84 could enhance the LUC activity driven by *ZmSOD2* promoter, we wondered whether ZmNAC84 could directly bind to the *ZmSOD2* promoter. NACs can regulate the expression of their target genes by recognizing CACG/CACGTA/CACGTG elements [15,18,29]. Then, we searched for putative ZmNAC84 binding elements in the promoter of *ZmSOD2* (−1459 bp to −1454 bp), and performed an electrophoretic mobility shift assay (EMSA). The unlabeled and mutated probes were used as competitors. The purified His-ZmNAC84 protein was incubated with the labeled probe, then the protein-DNA complex was detected. As shown in Fig. 2A, the band was reduced when the unlabeled competitor probe with the same sequence were added. Meanwhile, this competition was not observed when the mutated version was used. The assay shows that ZmNAC84 interacts with CACGTG motif in *ZmSOD2* promoter in vitro.

To determine whether ZmNAC84 directly binds to the promoter of *ZmSOD2* in vivo, the OE-*ZmNAC84* plants were constructed using *Agrobacterium*-mediated maize shoot-tip transformation, and the ZmNAC84-FLAG proteins were successfully detected in leaves of OE-*ZmNAC84*#1 plants (Supplemental Fig. 2). Then the OE-*ZmNAC84*#1 plant was used to perform a chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assay. The ChIP-qPCR assay showed that ZmNAC84 protein could bind to the P1, but not to the P2, which does not have a CACGTG motif (Fig. 2B). Together, these result clearly suggest that ZmNAC84 directly binds to a specific CACGTG motif within *ZmSOD2* promoter both in vitro and in vivo.

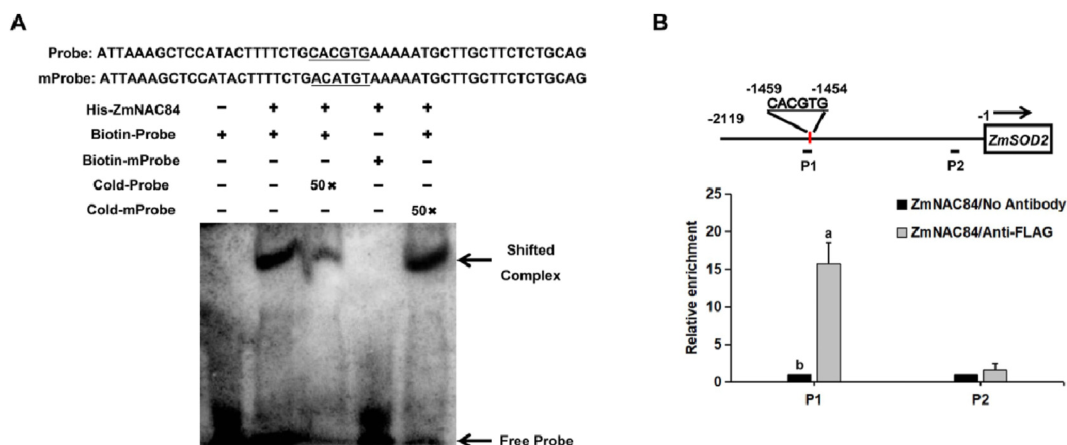


**Fig. 2.** ZmNAC84 directly binds to the *ZmSOD2* promoter in vitro and in vivo. (A) EMSA assay. Arrows indicate protein-DNA complexes (upper arrow) or free probe (lower arrow). Cold-Probe, unlabeled probes. Cold-mProbe, unlabeled mutated probes. Biotin-Probe, labeled probes. Biotin-mProbe, labeled mutated probes. '+' indicates presence, '−' indicates absence. (B) ChIP-qPCR assay. The P1 fragment contain the CACGTG motif (−1459 bp to −1454 bp) and P2 does not have the CACGTG motif. Chromatin was immunoprecipitated with an anti-FLAG antibody. Expression was determined by RT-qPCR and is expressed relative to the value in the control (no antibody), which was set as 1. *ZmActin2* was used as the internal reference. Data in (B) are means ± SE (n = 3). Different letters in (B) indicate significant differences at $P < 0.05$ according to one-way ANOVA.

### 3.3. Phosphorylation of ZmNAC84 at Ser-113 enhances the DNA binding ability of ZmNAC84 to ZmSOD2 promoter

Previous study has shown that Ser-113 was a crucial phosphorylation site of ZmNAC84, and Ser-113 located in the DNA binding domain of ZmNAC84. To investigate whether the phosphorylation of Ser-113 in ZmNAC84 affects its DNA binding ability, an EMSA assay was performed. The labeled probe was incubated with ZmNAC84, ZmNAC84$^{S113A}$ (substituted Ser-113 with Ala to mimic non-phosphorylation state) and ZmNAC84$^{S113D}$ (substituted Ser-113 with Asp to mimic phosphorylation state). As shown in Fig. 3A, the DNA binding ability of ZmNAC84$^{S113D}$ was higher than that of ZmNAC84 and ZmNAC84$^{S113A}$, suggesting that the phosphorylation of Ser-113 in ZmNAC84 significantly increases its DNA binding ability in vitro.

Next, the OE-*ZmNAC84*$^{S113A}$ and OE-*ZmNAC84*$^{S113D}$ plants were successfully constructed using *Agrobacterium*-mediated maize shoot-tip transformation (Supplemental Fig. 2). Then the OE-*ZmNAC84*#1, OE-*ZmNAC84*$^{S113A}$#1 and OE-*ZmNAC84*$^{S113D}$#1 plants were used to performed ChIP-qPCR assays. Also, the ChIP-qPCR assay showed that the phosphorylation of ZmNAC84 at Ser-113 enhanced the binding ability to P1, which has a specific binding motif (Fig. 3B). These results suggest that the phosphorylation of Ser-113 in ZmNAC84 plays crucial role in enhancing the DNA binding ability of ZmNAC84 to *ZmSOD2* promoter.

### 3.4. Phosphorylation of ZmNAC84 at Ser-113 enhances the ZmSOD2 expression to improve the drought tolerance

To explore whether the phosphorylation of ZmNAC84 at Ser-113 regulates the *ZmSOD2* expression in drought response, the independent lines of transgenic maize (OE-*ZmNAC84*#1, OE-*ZmNAC84*#2, OE-*ZmNAC84*$^{S113A}$#1, OE-*ZmNAC84*$^{S113A}$#2, OE-*ZmNAC84*$^{S113D}$#1 and OE-*ZmNAC84*$^{S113D}$#2) were chosen according to the RT-qPCR (Supplemental Fig. 3). Thus, the *ZmSOD2* expression and SOD activity in *ZmNAC84*, *ZmNAC84*$^{S113A}$, *ZmNAC84*$^{S113D}$ transgenic plants and wild type plants were analyzed after exposed to drought stress. Under drought stress, the *ZmSOD2* expression and SOD acitivity in *ZmNAC84*$^{S113D}$ transgenic plants was much higher compared to that of other genotypes (Fig. 4A and B). To explore the biological function of *ZmNAC84*$^{S113D}$ in maize, the drought tolerance test was performed. As shown in Fig. 4C, *ZmNAC84*$^{S113D}$ transgenic plants showed more tolerant to drought stress when compared with the other genotypes, whereas no significant difference was observed under normal growth conditions before natural drought. Additionally, *ZmNAC84*$^{S113D}$ transgenic plants had higher relative water content (RWC), lower malondialdehyde (MDA) content and lower percent leakage of electrolyte than those in other genotypes after drought treatment (Fig. 4D-F). These data demonstrate that the phosphorylation of ZmNAC84 at Ser-113 enhances the *ZmSOD2* expression to improve the drought tolerance.

## 4. Discussion

NAC family is one of the largest plant-specific transcription factor families. Many reports have demonstrated that NAC TFs could regulate drought tolerance in *Arabidopsis*, rice and other plants [15,17,30–32]. Our previous study has shown that overexpression of *ZmNAC84* in tobacco could improve drought tolerance by enhancing the activities of APX and SOD. However, the mechanism that how ZmNAC84 affect the activities of APX and SOD is still unknown.

NAC TFs responds to drought stress by regulating the expression of target genes. For example, a maize NAC, ZmNAC49, can directly bind to the promoter of *ZmMUTE* to represses its expression, thus reduces stomatal density in maize [27]. Here, we found that ZmNAC84 directly up-regulated the *ZmSOD2* expression to enhance the tolerance of maize to drought based on the following results: First, ZmNAC84 enhanced the LUC activity driven by *ZmSOD2* promoter in tobacco (Fig. 1). Secondly, ZmNAC84 directly bound to the CACGTG motif of *ZmSOD2* promoter in vitro and in vivo (Fig. 2). Finally, overexpression of *ZmNAC84* in maize significantly increased the *ZmSOD2* expression and SOD activity under drought stress (Fig. 4A and B). Previous study has shown that ZmNAC84 also regulated the APX activity [21]. However, ZmNAC84 did not affect the LUC activities driven by *ZmAPXs* promoter (Supplemental Fig. 1). One explanation is that ZmNAC84 could not directly regulate the *ZmAPXs* expression. Another possibility is that the effect of ZmNAC84 on APX activity does not depend on transcriptional regulation. The mechanism that how ZmNAC84 affects APX activity need to be explored in the future.

The phosphorylation modification on transcription factor might affect its localization, protein stability, DNA binding ability or transcriptional activation [33–36]. Our previous study has shown that Ser-113, a phosphorylation site, of ZmNAC84 was an important site in enhancing the activities of antioxidant enzymes and the mutation of Ser-113 in ZmNAC84 did not affect its sub-localization [21]. Indeed, Ser-113 locates in the DNA binding domain of ZmNAC84 (Supplemental Fig. 4). Phosphorylation of NAC transcription factor, RD26 or NST1, obviously affect its transcriptional activation [34,37]. In this study, we demonstrate that the phosphorylation of ZmNAC84 at Ser-113 enhances the DNA binding

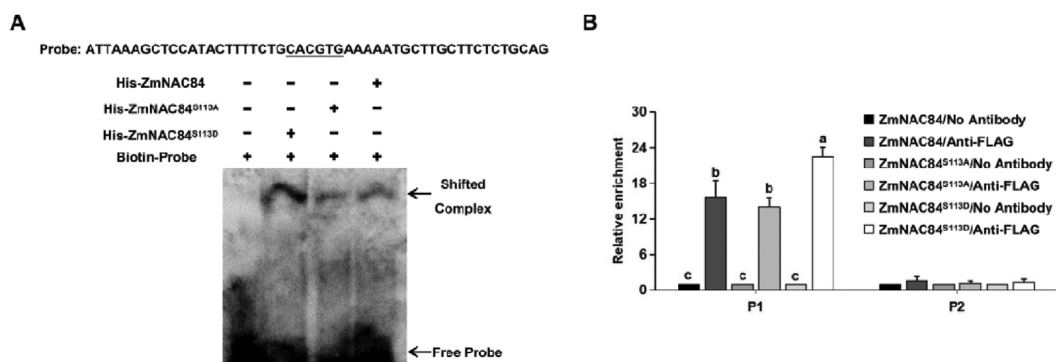


**Fig. 3.** Phosphorylation of ZmNAC84 at Ser-113 increases its ability to bind the *ZmSOD2* promoter. (A) EMSA assay. The labeled probe was incubated with ZmNAC84$^{S113A}$, ZmNAC84$^{S113D}$ or ZmNAC84. (B) ChIP-qPCR assay. The P1 contain the binding motif. Chromatin was immunoprecipitated with an anti-FLAG antibody. Expression was determined by RT-qPCR, and the value in the control (no antibody) was set as 1. *ZmActin2* was used as the internal reference. Data in (B) are means ± SE (n = 3). Different letters in (B) indicate significant differences at $P < 0.05$ according to one-way ANOVA.

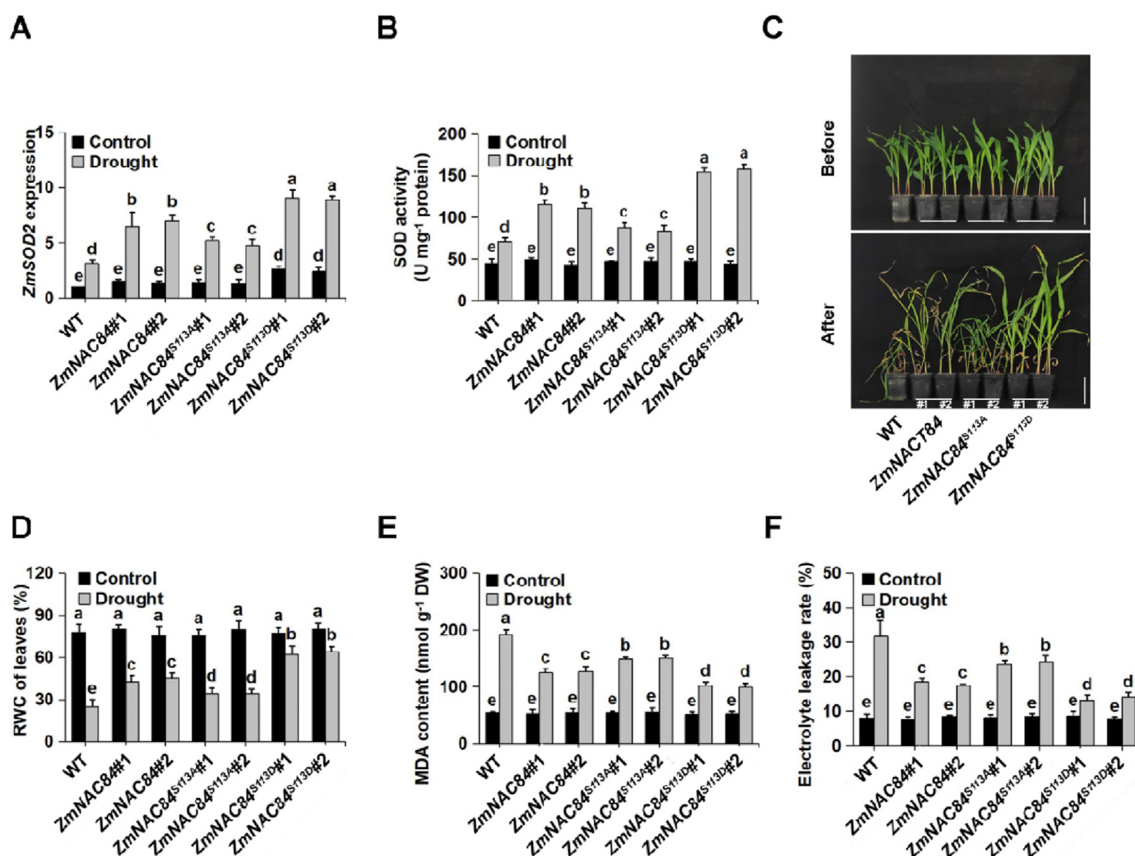


**Fig. 4.** Phosphorylation of ZmNAC84 at Ser-113 enhances the *ZmSOD2* expression and positively regulates the tolerance of transgenic maize to drought stress. (A) The expression of *ZmSOD2* in transgenic plants and wild type plants under drought stress. Expression was determined by RT-qPCR and *ZmActin2* was used as the internal reference. (B) The activity of SOD in transgenic maize. (C) Phenotype of transgenic maize under drought stress. (D–F) The RWC (D), the MDA content (E) and the percentage of electrolyte leakage (F) in transgenic maize after drought treatment. Data in (A, B, D-F) are means ± SE (n = 3). Different letters in (A, B, D-F) indicate significant differences at $P < 0.05$ according to two-way ANOVA.

ability of ZmNAC84 to *ZmSOD2* promoter, so we speculated that Ser-113 might affect the structure of ZmNAC84. However, more detailed structural analysis of ZmNAC84 need to be investigated in the future.

In conclusion, our results indicate that ZmNAC84 directly up-regulates the *ZmSOD2* expression to enhance drought tolerance in maize, and the phosphorylation of ZmNAC84 at Ser-113 plays crucial role in this process.

## Accession numbers

The sequence data in this article can be found in the following accession numbers:

*ZmNAC84* (GRMZM2G166721), *ZmActin2* (GRMZM2G030169), *ZmSOD1* (GRMZM5G864424), *ZmSOD2* (GRMZM2G025992), *ZmSOD3* (GRMZM2G059991), *ZmSOD4* (GRMZM2G169890), *ZmSOD4A* (GRMZM2G058522), *ZmAPX1.1* (GRMZM2G137839), *ZmAPX1.2* (GRMZM2G054300), *ZmAPX2* (GRMZM2G140667), *ZmAPX3* (GRMZM2G004211), *ZmAPX4* (GRMZM2G460406), *ZmAPX5* (GRMZM2G014397), *ZmAPX6* (GRMZM2G120517), *ZmAPX7* (GRMZM2G006791).

## Declaration of competing interest

The authors declare that there are no conflict of interests, we do not have any possible conflicts of interest.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.06.026.

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