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The transcription factor ZmNAC84 increases maize salt tolerance by regulating *ZmCAT1* expression

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ABSTRACT

Salt stress severely affects plant growth and yield. The transcription factor NAC plays a variety of important roles in plant abiotic stress, but we know relatively little about the specific molecular mechanisms of NAC in antioxidant defense. Here, our genetic studies reveal the positive regulation of salt tolerance in maize by the transcription factor ZmNAC84. Under salt stress, overexpression of *ZmNAC84* in maize increased the expression of *ZmCAT1*, enhanced CAT activity, and consequently reduced H_2O_2 accumulation, thereby improving salt stress tolerance in maize. Whereas RNA interference-mediated knockdown of *ZmNAC84* produced the opposite effect. Subsequently, we found that ZmNAC84 directly binds to and regulates the expression of the *ZmCAT1* promoter, and the hybridized material also demonstrated that *ZmCAT1* is a downstream target gene of *ZmNAC84*. In addition, phenotypic and biochemical analyses indicated that ZmCAT1 positively regulated salt tolerance by regulating H_2O_2 accumulation under salt stress. Taken together, these results reveal the function of ZmNAC84 in regulating ZmCAT1-mediated antioxidant defense in response to salt stress in plants.

1. Introduction

The plant is often subjected to various environmental stresses during growth and development. Among these, salt stress is one of the major abiotic stresses affecting plant growth, development, and yield. Salt stress leads to reactive oxygen species (ROS) accumulation and oxidative stress [1]. In response to this effect, plants have evolved complex strategies to cope with oxidative stress. Plants possess many transcription factors that can protect them by interacting with target genes to mitigate damage caused by abiotic stresses such as salt, drought, and temperature stress [2].

NAC transcription factors function in many biological processes. NAC (NAM) transcription factors are one of the largest plant-specific transcription factor families [3]. To date, 106 *NAC* genes have been identified in *Arabidopsis* (*A. thaliana* L.), 151 in rice (*Oryza sativa* L.), and 152 in maize (*Zea mays* L.) [4–6]. NACs are involved in the formation of plant meristems and organ boundaries [7], lateral root development [8,9], secondary cell wall synthesis [10,11], and leaf senescence [12,13]. NAC transcription factors function in plant abiotic stress responses. *Arabidopsis* NAC transcription factor *ANAC096* positively regulated tolerance to dehydration stress [14]. In rice, *ONAC106* regulates salt-responsive signaling genes to enable rice to acquire salt stress tolerance, and the expression of *ZmSNAC1* or *ZmNAC55* was strongly induced by salt stress in maize. [15,16].

To mitigate oxidative stress caused by ROS accumulation under high salinity conditions, plants have evolved ROS scavenging mechanisms, including non-enzymatic antioxidant metabolites and enzymes [17]. The enzyme systems in plants include catalase (CAT) and other enzymes [18,19]. Among them, CAT is a potent antioxidant enzyme, and the *CAT* gene is encoded by a small family of genes that have been reported in both monocotyledonous and dicotyledonous plants, including maize, rice, cotton, tobacco, and *Arabidopsis* [20–24]. CAT is a central antioxidant enzyme in most organisms that catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen, thereby controlling the abundance of this essential cell signaling molecule [25]. *CAT* genes respond to a variety of abiotic stresses. The MYB transcription factor TaODORANT1 in wheat promotes ROS scavenging and increases salt

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and drought tolerance in transgenic tobacco by upregulating *CAT* gene expression [26]. CAT proteins are phosphorylated by the receptor-like cytoplasmic kinase STRK1, which functions in salt stress response [27]. Salt stress induced the expression of *CAT* genes [28,29], suggesting that CAT may be transcriptionally regulated.

In this study, we investigated the roles of ZmNAC84 and ZmCAT1 in maize subjected to salt stress and found that ZmNAC84 enhanced the salt tolerance of maize by directly binding to the *ZmCAT1* promoter, increasing the expression of *ZmCAT1*, maintaining H_2O_2 homeostasis, and enhancing the antioxidant stress capacity of maize.

2. Materials and methods

2.1. Plant materials and growth conditions

Maize B73 and *Nicotiana benthamiana* seeds were sown in pots containing soil mixture (soil: vermiculite, 1:1, v/v) in the greenhouse at a temperature of 22 °C to 28 °C, photoperiod of 14 h/10 h (day/night) and watered daily.

2.2. Generation of maize transgenic plants

Our previous study showed that *ZmNAC84* is involved in ABA-induced antioxidant defense [30], and to further investigate the role of *ZmNAC84* under maize salt stress, we generated the *Ubi: ZmNAC84* and *Ubi: ZmCAT1* constructs for overexpression in maize. The coding regions of *ZmCAT1* and *ZmNAC84* were amplified by PCR using primers (Table S1) and inserted into plant expression vector pCUN-NHF driven by the *ubiquitin* promoter. To generate RNAi constructs of *ZmNAC84* and *ZmCAT1*, 270 bp *ZmNAC84*-specific fragment and 207 bp *ZmCAT1*-specific fragment were cloned in both sense and antisense directions using the specific primers (Table S1) and inserted into the vector pCUN-NHF-RNAi to knock down *ZmNAC84* and *ZmCAT1*. The constructs were transformed into B73 using the *Agrobacterium*-mediated maize shoot-tip transformation method [31]. Positive transformants were selected with glufosinate ammonium and then further confirmed by PCR amplification. T_0, T_1, and T_2 plants were grown in the greenhouse and field, and the presence of the transgene was determined in each generation by PCR. Resistant T_2 seedlings with 3:1 segregation of resistance were transferred to soil to obtain homozygous T_3 seeds from individual lines.

2.3. Isolation of total RNA and RT-qPCR

Total RNA was isolated from maize materials using FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, Nanjing, Jiangsu, China) following the manufacturer's protocol and the cDNA was synthesized using Hifair AdvanceFast One-step RT-gDNA Digestion SuperMix for qPCR (Cat No. 11,151 Yeasen Biotech, Shanghai, China). Reverse transcription-quantitative real-time PCR (RT-qPCR) was performed on a CFX96 Touch (Bio-Rad Laboratories, Hercules, CA, USA) system using ChamQ SYBR qPCR Master Mix (Vazyme) according to the manufacturer's protocol. Relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method [32]. The primers are listed in Table S1. Expression was normalized against that of maize *ZmActin2*.

2.4. Western blotting

Extracted proteins were separated by 12% SDS-PAGE. After electrophoresis, the gel was transferred to a PVDF membrane at 110 V for 60 min at 4 °C. The membrane was blocked with blocking solution containing PBST solution (137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 2 mmol L^{-1} KH_2PO_4, 10 mmol L^{-1} Na_2HPO_4, 0.1% [v/v] Tween-20, pH 7.5) and 5% [w/v] skimmed milk powder and then incubated for 2 h at room temperature with gentle shaking or overnight at 4 °C. Then, the membrane was probed with primary antibody diluted in blocking solution at the following dilutions: anti-ZmNAC84 antibody (The anti-ZmNAC84 antibody targeting the N-terminal 310 AA of ZmNAC84 were isolated from the serum of immunized rabbits. 1:2000, Kaijing Biotechnology, Shanghai, China), anti-FLAG antibody, and anti-YFP antibody (1:5,000, Abmart, Shanghai, China), plant actin monoclonal antibody (1:8000, Biodragon, Suzhou, Jiangsu, China). The secondary antibody, horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Abmart, China) was used at 1:8000 dilution. The chemiluminescent signal generated by the ECL western blotting substrate (Beyotime, Shanghai, China) was detected by the Tanon 5200 multi-chemiluminescent imaging system.

2.5. Sequence analysis and phylogenetic tree construction

Sequences of *NAC* genes were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) and MaizeGDB (https://www.maizegdb.org/) databases. A phylogenetic tree was generated by MEGA (https://www.megasoftware.net) software using the maximum parsimony algorithm with 1000 replications.

2.6. Phenotype of transgenic maize under salt stress treatment

For the hydroponic salt sensitivity assay, maize seedlings at the three-leaf stage were treated with Kimura B nutrient solution (Coolaber, Beijing, China) containing 200 mmol L^{-1} NaCl for an additional 5–8 d. After recovery in normal nutrient solution for 3 d. About 48 seedlings in each line per replicate were used for survival rate analysis.

2.7. Determination of salt stress-associated physiological indicators

Total chlorophyll content was measured according to the method described previously [33]. Maize seedlings at the three-leaf stage were treated with a nutrient solution containing 200 mmol L^{-1} NaCl for 4 d, and the second leaves were ground in liquid N_2. Then 2.5 mL of 80% acetone was added to 200 mg ground tissue, and the samples were mixed thoroughly and incubated in darkness overnight at 4 °C. The extract was centrifuged at 5000×*g* for 15 min at 4 °C. The supernatant was transferred to a colorimetric tube for absorbance measurement at 663 and 645 nm. The total chlorophyll content was calculated and expressed as mg g^{-1} FW.

For the analysis of oxidative damage and to evaluate the cell membrane permeability, maize seedlings at the three-leaf stage were treated with a nutrient solution containing 200 mmol L^{-1} NaCl for 2 d. The second leaves were sampled and the content of malondialdehyde (MDA) and the percent leakage of electrolytes were determined as described previously [34,35]. For MDA content, the maize leaves were homogenized in 2 mL of 10% trichloroacetic acid and centrifuged at 5000×*g* for 10 min at 4 °C. 1 mL of the supernatant was reacted with 1 mL of 0.6% (w/v) thiobarbituric acid. The mixture was boiled for 15 min and rapidly cooled. Then the mixture was centrifuged at 5000×*g* for 10 min at 4 °C. The absorbance of the supernatant was read at 450, 532, and 600 nm. For the percent leakage of electrolytes, after the initial electrical conductivity (EC_1) and the final electrical conductivity (EC_2) of the medium were measured, the percent leakage of electrolytes was calculated s $EC_1/EC_2 \times 100$.

2.8. Determination of H_2O_2 level

H_2O_2 was visually detected by staining the second leaf of the transgenic maize plants and the wild type (WT) plants with 3,3′-diaminobenzidine (DAB) as described previously [36]. Maize seedlings at the three-leaf stage were incubated in a nutrient solution containing 200 mmol L^{-1} NaCl for 2 d. The second leaves were sampled and immediately incubated in 1 mg mL^{-1} DAB solution (Solarbio, China) for 8 h in the dark at 28 °C. The leaves were immersed in boiling 95% (v/v) ethanol for 10 min to terminate the staining and to decolor the leaves. After cooling, the leaves were extracted with 75% (v/v) ethanol and preserved at 4 °C in 75% (v/v) ethanol before being photographed.

For quantification of H_2O_2, maize seedlings at the three-leaf stage were treated with nutrient solution containing 200 mmol L^{-1} NaCl for 2 d, and the second leaves were frozen in liquid N_2 and then ground. Then 1 mL lysis buffer was added to 50 mg ground tissue. The extract was centrifuged at 12,000×*g* for 5 min at 4 °C, and the supernatant was collected·H_2O_2 content was performed using the Hydrogen Peroxide Assay Kit (Beyotime, China) by monitoring the A_{560} of the Fe^{3+}-xylenol orange complex. Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2.

2.9. Catalase activity assays

The second leaves were sampled and homogenized in 0.6 mL of 50 mmol L^{-1} potassium phosphate buffer (pH 7.0) containing 1 mmol L^{-1} EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 12,000×*g* for 30 min at 4 °C, and the supernatant was immediately used for CAT activity assays. Protein content was determined according to Bradford's method [37] with BSA as the standard. CAT activity was measured following Aebi [38]. The CAT assay mixture of 3 mL consisted of 0.05 mL enzyme extract, 50 mmol L^{-1} potassium phosphate buffer (pH 7.0), 10 mmol L^{-1} H_2O_2, and distilled water. A decrease in absorbance was recorded at 240 nm. The enzyme activity was expressed as μmol min^{-1} mg^{-1} protein.

2.10. Dual luciferase reporter assay

The *ZmCATs* promoter fragments were cloned into p1381-LUC [39] to act as the reporter gene. Full-length *ZmNAC84* was cloned into the super1300 [40] to serve as the effector. The *renilla luciferase* (REN) under the control of the 35S promoter contained in the reporter construct was used as the endogenous control. Dual luciferase assay was performed as described previously [41]. Firefly and renilla luciferase activities were quantified using Dual Luciferase Reporter Gene Assay Kit (Yeasen Biotech, China) and detected with SpectraMax iD5 (Molecular Devices, San Jose, CA, USA) according to the manufacturer's instructions.

For the deletion analysis of the *ZmCAT1* promoter, the full-length (FL) (−2000 to −1) and the consecutively truncated regions Δ1 (−1597 to −1), Δ2 (−1109 to −1), Δ3 (−557 to −1), Δ4 (−207 to −1) and Δ5 (−2000 to −330 and −256 to −1) were amplified by PCR and cloned into p1381 −LUC to drive the *firefly luciferase* (LUC) reporter gene. Recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101. The primer sequences are listed in Table S1.

2.11. Protein expression and electrophoretic mobility shift assay (EMSA)

The full-length coding sequence of *ZmNAC84* was amplified and introduced into pET-30a for His fusions. This construct was transformed into *Escherichia coli* BL21 (DE3). When the bacteria were grown in Luria-Bertani broth with kanamycin (50 μg mL^{-1}) at 37 °C to OD_{600} at 0.6 to 0.8, recombinant protein expression was induced with 0.5 mmol L^{-1} isopropyl-β-D-thiogalactopyranoside for 6 h at 26 °C. The recombinant proteins were purified using the MagneHis Protein Purification System (Promega, Madison, WI, USA) according to the manufacturer's protocol. EMSA was carried out using the EMSA Probe Biotin Labeling Kit (Beyotime, China) according to the manufacturer's protocol. Reaction mixtures containing the protein and probes were then incubated for 20 min at room temperature and separated on 4% polyacrylamide gels in 0.5× TBE buffer. Finally, the signals were detected using the Chemiluminescence EMSA kit (Beyotime, China).

2.12. Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assay

The ChIP-qPCR assay was performed according to the method described previously [42]. Transgenic maize seedlings of the OE-*ZmNAC84*#3 line at the three-leaf stage were harvested and fixed in 1% formaldehyde with vacuum infiltration. Crosslinking was quenched by adding glycine (final concentration of 0.125 mol L^{-1}). The chromatin was collected and sonicated, and immunoprecipitation using anti-DYKDDDDK (anti-FLAG, Abmart, China). The enrichment of DNA fragments was quantified by quantitative PCR using specific primers (Table S1). A fragment of the *ZmActin2* coding region was used as a reference gene. Enriched values were normalized with the level of input DNA.

3. Results

3.1. ZmNAC84 positively regulates salt tolerance in maize

We examined the expression of ZmNAC84 in WT exposed to NaCl treatment. *ZmNAC84* transcript levels were slightly induced within 15 min of salt treatment and peaked at 30 min (Fig. S1A), suggesting that *ZmNAC84* responds to salt stress in maize. To elucidate the biological function of ZmNAC84 in maize salt response, the transgenic maize plants overexpressing *ZmNAC84* were generated and two independent lines (OE-*ZmNAC84*#3 and OE-*ZmNAC84*#6) were selected (Figs. S1B, S2A–C). *ZmNAC84* transgenic plants were also generated and two lines (Ri-*ZmNAC84*#5 and Ri-*ZmNAC84*#7) were confirmed (Fig. S2A, D, E). The expression of *ZmNAC84* homologous genes (*ZmNAC1*, *ZmNAC13*, *ZmNAC82*, and *ZmNAC87*) was detected, and only *ZmNAC84* was reduced in *ZmNAC84*-RNAi plants (Figs. S2D, S3). Thus, *ZmNAC84* was suppressed by RNAi.

To verify the role of ZmNAC84 in maize under salt stress, After NaCl treatment, *ZmNAC84*-overexpressing plants showed less severe wilting and growth inhibition than WT plants (Fig. 1A). After treatment with NaCl, the survival rate of *ZmNAC84*-overexpressing plants were all higher than WT plants (Fig. 1B). In contrast, after NaCl treatment, *ZmNAC84*-RNAi plants exhibited more severe wilting and growth inhibition compared to WT plants (Fig. 1C). After NaCl treatment, the survival rate of WT plants were higher than *ZmNAC84*-RNAi plants (Fig. 1D).

To further understand the physiological changes in transgenic plants under salt stress, the related physiological indexes were measured. Compared with WT plants, the chlorophyll content was higher in *ZmNAC84*-overexpressing plants but lower in *ZmNAC84*-RNAi plants (Fig. 1E). No difference in chlorophyll content was observed between the WT plants and all transgenic plants under normal conditions. Salt stress generally causes membrane lipid peroxidation and increases cell membrane permeability of plant cells. Therefore, MDA levels and electrolyte leakage were measured in transgenic and WT plants under salt stress and normal conditions. Under salt stress, both MDA levels and electrolyte

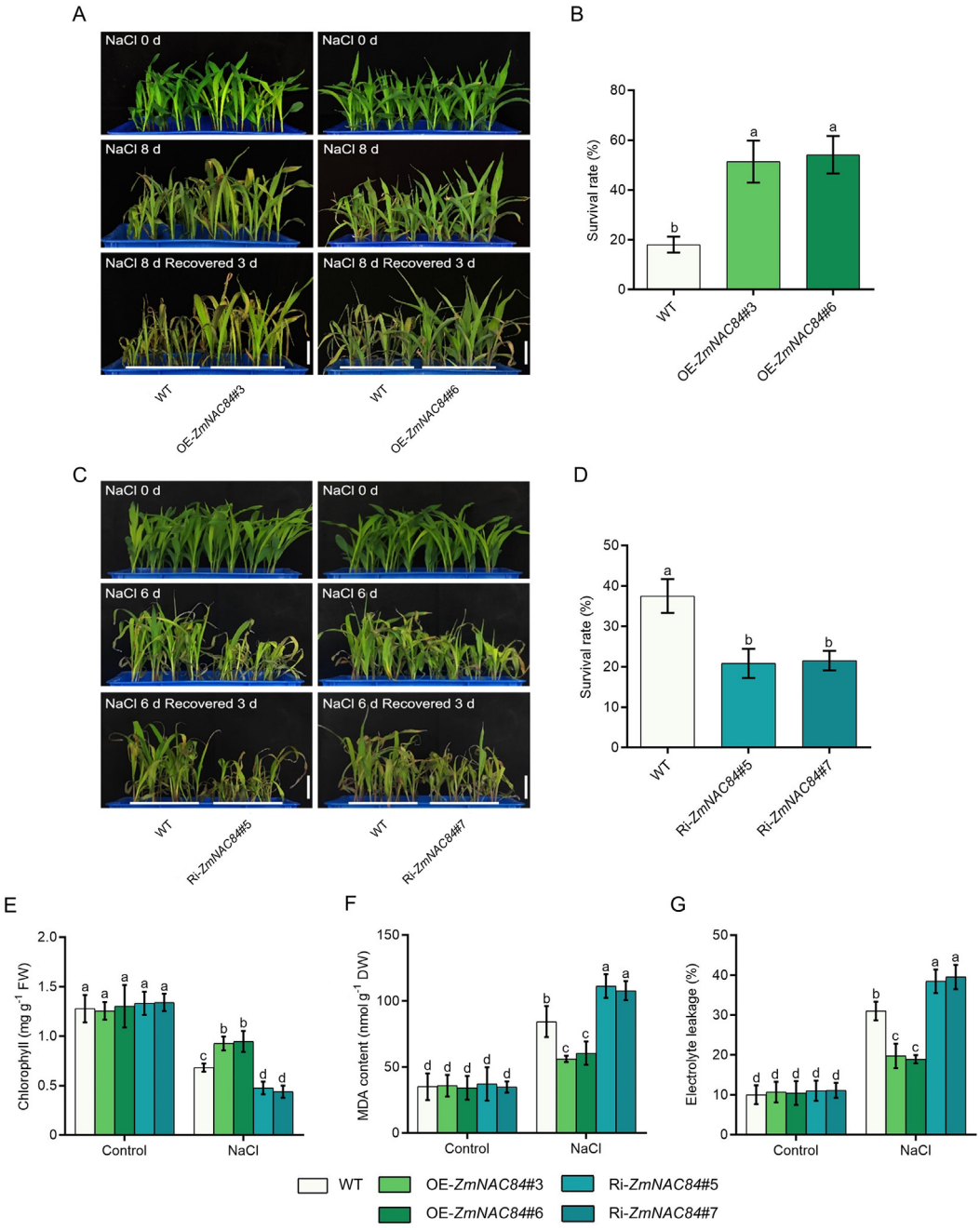

Fig. 1. Phenotype and physiological indicators of *ZmNAC84* transgenic plants under salt stress. (A) and (C) For OE-*ZmNAC84* and Ri-*ZmNAC84* transgenic plants were treated with NaCl and then recovered. Then the images were obtained (Scale bar, 5 cm). The experiments were repeated at least three times with similar results. (B) and (D) Survival rates of maize transgenic plants and WT plants in (A) and (C). (E) Chlorophyll content in maize leaves after NaCl treatment. (F) MDA content in maize leaves after treatment. (G) Electrolyte leakage in maize leaves after NaCl treatment. Values in (B), (D) and (E) to (G) are means ± SD ($n = 3$), and different letters indicate differences at $P < 0.05$ by one-way ANOVA with Duncan's multiple range test.

leakage were lower in *ZmNAC84*-overexpressing plants but higher in *ZmNAC84*-RNAi plants compared to WT plants (Fig. 1F, G). However, no differences in MDA levels and electrolyte leakage were observed between transgenic and WT plants under normal conditions (Fig. 1F, G). Taken together, these results suggest that ZmNAC84 enhances salt tolerance in maize by alleviating salt stress-induced oxidative damage.

3.2. *ZmNAC84* regulates H_2O_2 accumulation and CAT activity

To understand the role of ZmNAC84 in salt stress, we examined H_2O_2 accumulation in *ZmNAC84* transgenic plants and WT plants under salt stress. As expected, salt stress obviously increased H_2O_2 levels in both WT, *ZmNAC84*-overexpressing, and *ZmNAC84*-RNAi plants. However, less accumulation of H_2O_2 was observed in *ZmNAC84*-overexpressing plants and more accumulation of H_2O_2 was observed in *ZmNAC84*-RNAi plants compared to WT (Fig. 2A–C). ROS scavenging enzymes play a crucial role in ROS homeostasis and CAT is the major H_2O_2 scavenging enzyme among these enzymes. Therefore, we wondered whether ZmNAC84 regulates the activity of the CAT enzyme. As shown in Fig. 2D, there was no difference in CAT activity between WT plants and all transgenic plants under normal conditions. The CAT activity in *ZmNAC84*-overexpressing plants was higher than WT plants under salt stress, but the CAT activity of *ZmNAC84*-RNAi plants was lower than WT plants. Therefore, overexpression of *ZmNAC84* significantly activates

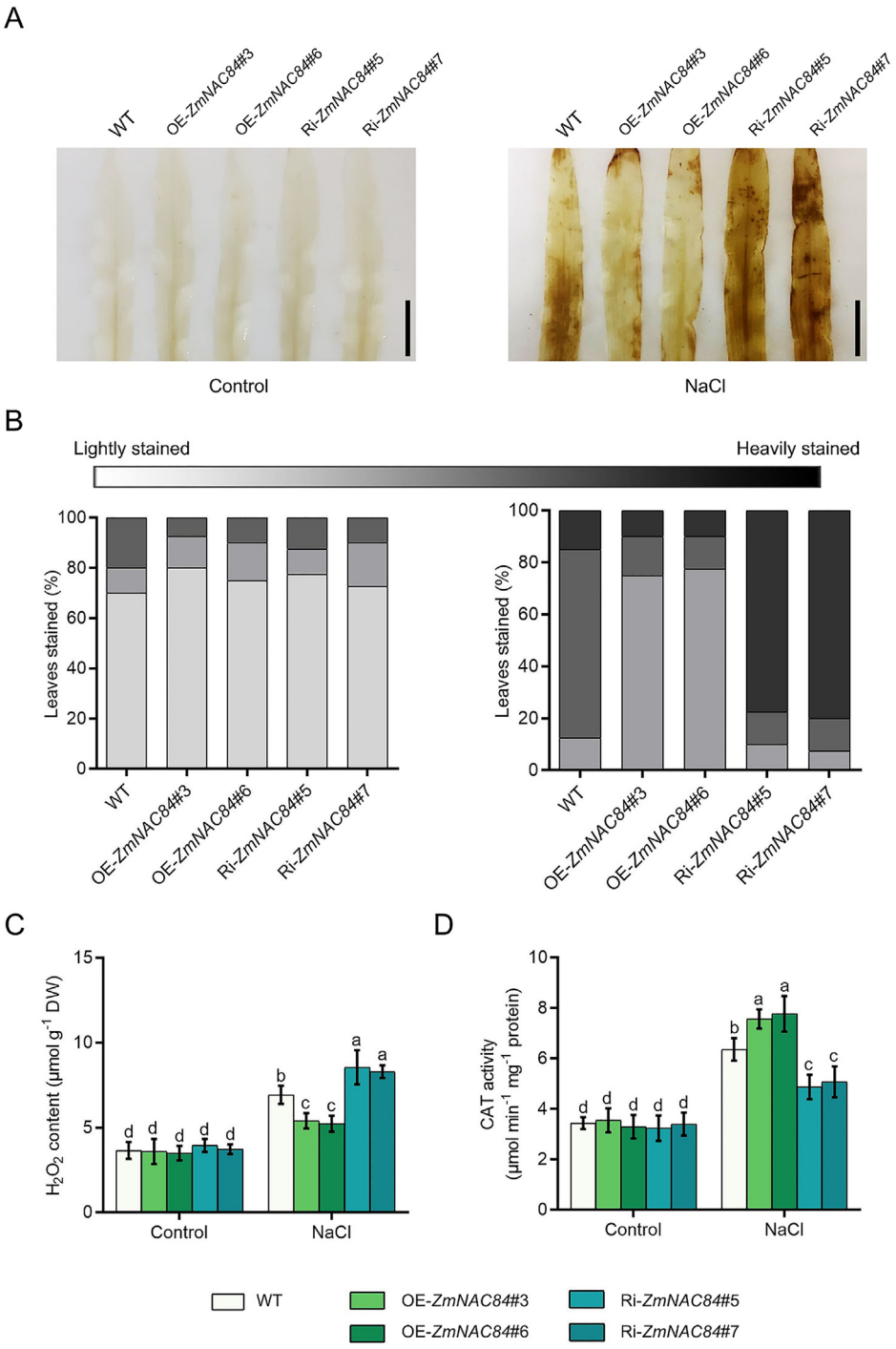

Fig. 2. Comparison of H_2O_2 accumulation and CAT activity in *ZmNAC84* transgenic plants under salt stress. (A) DAB staining intensity reflects the content of H_2O_2 in OE-*ZmNAC84*, Ri-*ZmNAC84*, and WT plants (Scale bar, 2 cm). (B) Percentage and extent of leaves stained by DAB in (A). (C) The H_2O_2 content in leaves of OE-*ZmNAC84*, Ri-*ZmNAC84*, and WT plants. (D) CAT activity in leaves of OE-*ZmNAC84*, Ri-*ZmNAC84*, and the WT plants. Values in (C) and (D) are means ± SD (n = 3), and different letters indicate differences at $P < 0.05$ by one-way ANOVA with Duncan's multiple range test.

CAT activity under salt stress. All these data indicate that ZmNAC84 can mediate H_2O_2 accumulation and CAT activity under salt stress. Consequently, it can be postulated that ZmNAC84 can affect CAT activity to modulate H_2O_2 accumulation in response to salt stress.

3.3. ZmNAC84 positively regulates ZmCAT1 expression in response to salt stress

The amino acid sequence of ZmNAC84 revealed that it contains a conserved NAC domain in its N-terminal region from 44 to 185 aa (Fig. S4A). To determine whether it has transcriptional activation activity, the full-length coding sequence (ZmNAC84), the N-terminal sequence (ZmNAC84-N), and the C-terminal sequence (ZmNAC84-C) were each fused to the GAL4 DNA-binding domain in the vector pGBKT7. Yeast cells transformed with ZmNAC84 and ZmNAC84-C grew well on SD/-Trp-His-Ade medium and appeared blue in X-α-gal assays (Fig. S4B). In contrast, cells transformed with the control vector or ZmNAC84-N did not survive on this medium. Thus, the results show that ZmNAC84 has transcriptional activation activity in yeast.

Since CAT activity was affected by ZmNAC84 in response to salt stress, we further analyzed the transcript levels of *CAT* genes (*ZmCAT1*, *ZmCAT2*, *ZmCAT3*) encoding the CAT enzyme in maize. First, we examined the expression of ZmCATs in WT exposed to NaCl treatment using RT-qPCR. *ZmCAT1* and *ZmCAT2* transcript levels were slightly induced within 1 h and peaked at 6 h (Fig. S5A, B). However, there was no change in the transcript level of *ZmCAT3* (Fig. S5C). Next, we analyzed the expression of ZmCATs in WT, *ZmNAC84*-overexpressing, and *ZmNAC84*-RNAi plants exposed to salt stress. Under NaCl treatment, only *ZmCAT1* expression was highly increased in *ZmNAC84*-overexpressing plants and decreased in *ZmNAC84*-RNAi plants compared to WT (Fig. S5D). However, there were no differences in the expression of *ZmCAT2* and *ZmCAT3* under NaCl treatment (Fig. S5E, F). These results suggest that ZmNAC84 positively regulates *ZmCAT1* expression in response to salt stress.

Six representative tissues/organs (root, stem, and young leaf of maize at the three-leaf stage; old leaf, female flower, and male flower of maize at the flowering stage) were sampled for the spatiotemporal expression analysis of *ZmCAT1*. As shown in Fig. S6, *ZmCAT1* was expressed in all tissues and organs tested, and the expression level of *ZmCAT1* was highest in the old leaf, followed by the stem and young leaf. *ZmCAT1* expression was lowest in root, which was used as a calibrator to normalize the expression level in other tissues and organs.

3.4. ZmNAC84 directly binds to the ZmCAT1 promoter in vivo and in vitro

Since ZmNAC84 positively regulates the *ZmCAT1* expression, we wondered whether ZmNAC84 directly or indirectly modulates its expression. Thus, we collected the upstream 2 kb genomic sequences relative to the translation start site of *ZmCAT1* as the putative promoter region. Next, we tested the transcriptional regulation between ZmNAC84 and *ZmCAT1* using a dual luciferase assay system. The promoter of *ZmCAT1* (2 kb) was amplified and fused them upstream of the *firefly luciferase* (LUC) gene. The CaMV35S promoter-driven *renilla luciferase* (REN) was used as an internal control. The effector plasmids contain either ZmNAC84-YFP or YFP expression cassette (Fig. 3A). Introducing equal amounts of different plasmid into *Nicotiana benthamiana* leaves (Fig. S7A). The ability of ZmNAC84 to transcriptionally activate its putative downstream genes was calculated through LUC/REN ratios. As shown in Fig. 3B, *ZmNAC84* expression activated the LUC activity driven by the *ZmCAT1* promoter. We also confirmed that *ZmNAC84* did not affect the LUC activity driven by *ZmCAT2* or *ZmCAT3* promoters.

NACs can regulate the expression of their target genes by recognizing CACG/CACGTA/CACGTG elements. To further demonstrate ZmNAC84 protein can bind to the specific binding site of the *ZmCAT1* promoter, we performed a series of 5′-deleted promoter constructs fused to the LUC reporter gene in *N. benthamiana* (namely, Δ1, Δ2, Δ3, Δ4, and Δ5, Fig. 3C) to test for interaction with ZmNAC84 using a dual luciferase assay system. The scheme of the reporter and effector vectors used in dual luciferase assay is shown in Fig. 3D. As shown in Figs. 3E and S7B, ZmNAC84 expression activated the LUC activity of full-length (FL) (−2000 to −1), Δ1 (−1597 to −1), Δ2 (−1109 to −1) and Δ3 (−557 to −1) plants. However, the deletions from position Δ4 (−207 to −1) and Δ5 (−2000 to −330 and −256 to −1) were similar to those of the control. These results indicate that ZmNAC84 may directly bind to the CACGTG sequence located within the −330 bp to −256 bp region of *ZmCAT1* promoter.

To further confirm this, an electrophoretic mobility shift assay (EMSA) was performed. We synthesized a 36-bp oligonucleotide containing the CACGTG sequence (−314 bp to −279 bp) based on the promoter sequence of *ZmCAT1* and labeled it as a probe. The unlabeled and mutated probes were used as competitors. In the EMSA, the purified His-ZmNAC84 protein was incubated with the labeled probe, and a protein-DNA complex was detected. The band was reduced when the unlabeled competitor probe with the same sequence was added. This competition was not observed when the mutant version was used (Fig. 3F). The assay showed that ZmNAC84 interacted with the CACGTG motif in the *ZmCAT1* promoter in vitro.

To determine whether ZmNAC84 directly binds to the promoter of *ZmCAT1* in vivo, a ChIP-qPCR assay using OE-*ZmNAC84*#3 plants was performed. The ChIP-qPCR assay showed that ZmNAC84 could bind to the P1, but not to the P2, which does not have a CACGTG motif (Fig. 3G). This indicates that ZmNAC84 directly binds to the specific CACGTG motif of *ZmCAT1* promoter in vivo. Together, these analyses suggest that ZmNAC84 directly binds to the CACGTG sequence within the *ZmCAT1* promoter both in vivo and in vitro.

3.5. ZmCAT1 increased salt tolerance in maize

To further investigate the function of ZmNAC84 through ZmCAT1 in maize, the transgenic maize plants *ZmCAT1*-overexpressing (OE-*ZmCAT1*#5, OE-*ZmCAT1*#8) and *ZmCAT1*-RNAi (Ri-*ZmCAT1*#1, Ri-*ZmCAT1*#2) plants were generated (Fig. S8). After NaCl treatment, *ZmCAT1*-overexpressing plants showed less severe wilting and growth inhibition than the WT plants (Fig. 4A), and the survival rate of *ZmCAT1*-overexpressing plants were higher than WT plants (Fig. 4B). Conversely, after NaCl treatment, *ZmCAT1*-RNAi plants exhibited more severe wilting and growth inhibition compared to WT plants (Fig. 4C), and the survival rate of WT plants were higher than *ZmCAT1*-RNAi plant (Fig. 4D). Taken together, these observations suggest that *ZmCAT1* enhances salt stress tolerance in maize.

Next, we examined H_2O_2 accumulation and CAT activity in *ZmCAT1* transgenic plants and WT plants under salt stress. Salt stress obviously increased H_2O_2 levels in WT, *ZmCAT1*-overexpressing, and *ZmCAT1*-RNAi plants. However, less accumulation of H_2O_2 was observed in *ZmCAT1*-overexpressing plants and more accumulation of H_2O_2 was observed in *ZmCAT1*-RNAi plants compared with WT (Fig. 5A–C). CAT activity showed the opposite result. As shown in Fig. 5D, under salt stress, the CAT activity of *ZmCAT1*-overexpressing plants was higher than WT plants, but the CAT activity of *ZmCAT1*-RNAi plants was lower than WT plants. These data suggest that *ZmCAT1* enhances salt tolerance by modulating H_2O_2 accumulation.

3.6. ZmNAC84 acts genetically upstream of ZmCAT1 in regulating salt tolerance in maize

To further confirm the relationship between ZmNAC84 and ZmCAT1 in maize, we crossed the OE-*ZmCAT1* and RNAi-*ZmNAC84* plants and generated two independent lines (OE-*ZmCAT1* × RNi-*ZmNAC84#1*, OE-*ZmCAT1* × RNi-*ZmNAC84#2*) which confirmed by RT-qPCR (Fig. S9). No growth differences were observed between them under normal conditions, but after NaCl treatment, the OE-*ZmCAT1* × RNAi-*ZmNAC84* lines showed a better degree of wilting and suffered from growth inhibition than the RNAi-*ZmNAC84* plants but worse than WT plants (Fig. 6A). After 3 d of recovery, survival rate of OE-*ZmCAT1* × RNAi-*ZmNAC84* plants was higher than RNAi-*ZmNAC84* plants but lower than WT plants (Fig. 6B). Next, we similarly analyzed the chlorophyll content, MDA content, and electrolyte leakage of these materials after salt stress (Fig. 6C–E), and the chlorophyll contents were higher in OE-*ZmCAT1*-× RNAi-*ZmNAC84* plants than in RNAi-*ZmNAC84* plants and lower than in WT plants. Content of MDA and electron leakage rate of

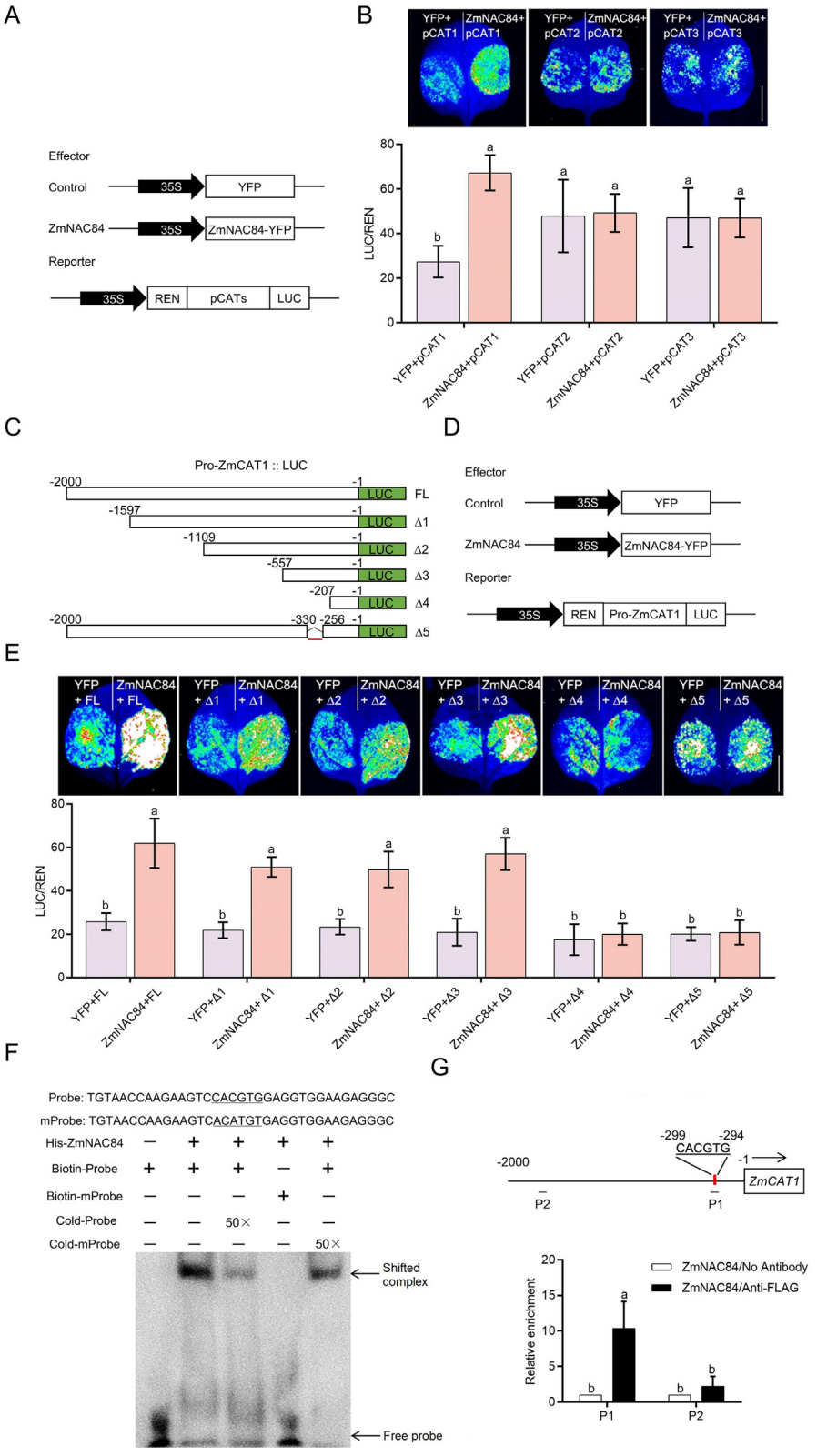

Fig. 3. ZmNAC84 up-regulates the expression of *ZmCAT1* by binding to the promoter. (A) Schematic representation of the double-reporter and effector plasmids used in the dual luciferase assay. The effector plasmid contains the ZmNAC84 or YFP. (B) Dual luciferase assay screening *ZmCATs* promoters that may bind to ZmNAC84 (Scale bar, 2 cm). (C) Schematic diagram of the construct at the 5′ terminal deletion of *ZmCAT1* promoter used for dual luciferase assay. (D) Schematic of the effector and reporter used in the dual luciferase assay. (E) Dual luciferase assay of the promoter activities co-transformed with effector and reporter constructs using 5′ terminal deletion of *ZmCAT1* promoter (Scale bar, 2 cm). (F) EMSA assay shows ZmNAC84 binding to the promoter of *ZmCAT1*. Arrows indicate protein-DNA complexes (upper arrows) or free probe (lower arrows). Cold-Probe, unlabeled probes. Cold-mProbe, unlabeled mutated probes. Biotin-Probe, labeled probes. Biotin-mProbe, labeled mutated probes. '+' indicates presence, '—' indicates absence. (G) ChIP-qPCR assay shows ZmNAC84 binding to the promoter of *ZmCAT1 in vivo*. Values in (V), (E) and (G) are means ± SD (n = 3), and different letters indicate differences at $P < 0.05$ by one-way ANOVA with Duncan's multiple range test.

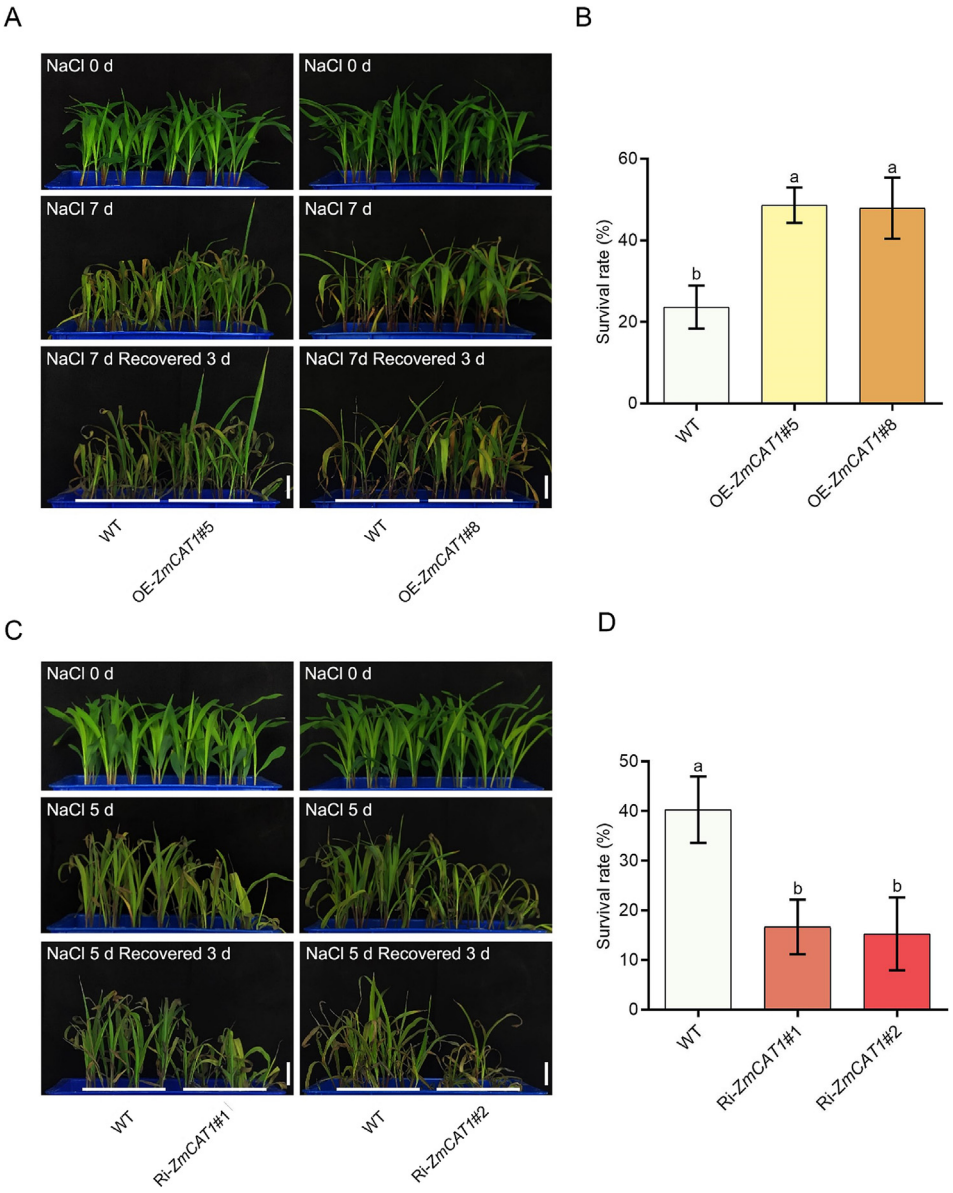

Fig. 4. Phenotype and survival rate of *ZmCAT1* transgenic plants under salt stress. (A) and (C) For OE-*ZmCAT1* transgenic plants were treated with NaCl and then recovered. Then the images were obtained (Scale bar, 5 cm). The experiments were repeated at least three times with similar results. (B) and (D) Survival rates of maize transgenic and WT plants in (A) and (C). Values in (B) and (D) are means ± SD (n = 3), and different letters indicate differences at $P < 0.05$ by one-way ANOVA with Duncan's multiple range test.

hybrid materials were higher than RNAi-*ZmNAC84* plants and lower than WT plants.

To determine the antioxidant capacity of the transgenic plants under salt stress, we detected the H_2O_2 content in the leaves by DAB staining and measured the H_2O_2 content and CAT activity in the leaves. The results showed that under salt stress, hybrid materials accumulated more H_2O_2 than RNAi-*ZmNAC84* but less than WT and OE-*ZmCAT1* plants (Fig. 7A–C), and the H_2O_2 content and CAT activity of hybrid plants were higher than RNAi-*ZmNAC84* plants and lower than WT and OE-*ZmCAT1* plants (Fig. 7D, E). The results of the study showed that the hybrid material was not as salt tolerant as the WT material, thus providing genetic evidence that *ZmNAC84* plays a role in regulating *ZmCAT1* transcript levels and influencing the material's antioxidant capacity. Combined with the fact that the survival rate of OE-*ZmNAC84* was higher than OE-*ZmCAT1* under the same salt treatment conditions. These convincing evidences proved that *ZmNAC84* is an upstream gene of *ZmCAT1* in response to salt stress.

4. Discussion

NAC proteins are proven to be involved in many regulatory and developmental processes [43,44]. Many studies have shown that NAC transcription factors can regulate many abiotic stresses [45,46] such as salt stress. For example, salt treatment of apple significantly induced the expression of *MdNAC047*, and overexpression of this gene improved salt tolerance and promoted ethylene release [47]. Overexpression *LpNAC17* in tobacco could increase antioxidant enzyme activity, reduce MDA and H_2O_2 levels, and improve the salt tolerance of tobacco [48]. *GmNAC06* control the ratio of Na^+/K^+ in hairy roots of soybean and maintain the ionic balance, and overexpression of *GmNAC06* in hairy roots could significantly improve the salt tolerance of the whole plant [49]. In this study, we found that overexpression of *ZmNAC84* could improve salt tolerance, increase CAT activity and reduce H_2O_2 accumulation in maize. However, the mechanism that NAC transcription factor regulates salt stress by regulating CAT expression is still unclear.

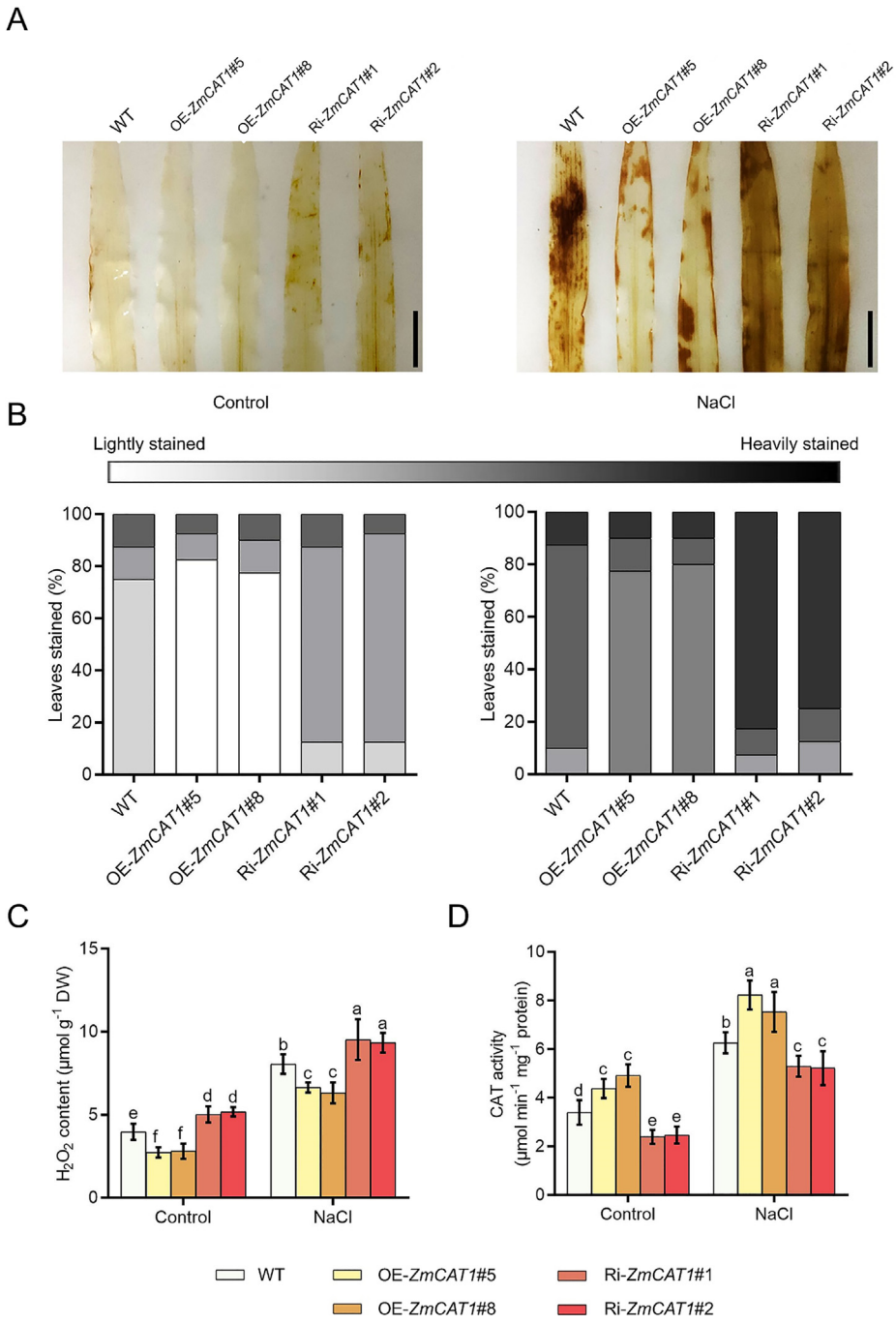

Fig. 5. Comparison of H_2O_2 accumulation and CAT activity in *ZmCAT1* transgenic plants under salt stress. (A) DAB staining intensity reflects the content of H_2O_2 in leaves of OE-*ZmCAT1*, Ri-*ZmCAT1*, and WT plants (Scale bar, 2 cm). (B) Percentage and extent of leaves stained by DAB (n = 40 individual plants). (C) The H_2O_2 content in leaves of OE-*ZmCAT1*, Ri-*ZmCAT1*, and WT plants. (D) CAT activity in leaves of OE-*ZmCAT1*, Ri-*ZmCAT1*, and WT plants. Values in (C) and (D) are means SD (n = 3), and different letters indicate differences at $P < 0.05$ by one-way ANOVA with Duncan's multiple range test.

CAT is the most abundant protein in the plant peroxisome and one of the highest catalytic rates known to biology [25]. Stronger antioxidant capacity in plants can enhance resistance to excessive ROS caused by adverse environments [50–53]. In Arabidopsis, GBF1 negatively regulates pathogen-induced *CAT2* expression and thus positively regulates the hypersensitive response [54]. ABI5 plays a role in regulating ROS homeostasis by activating *CAT1* transcription during seed germination [55]. Here, we found that salt stress increased the expression of *ZmCAT1*, and overexpression of *ZmCAT1* could reduce H_2O_2 accumulation, increase the content of MDA, and improve the survival rate of maize under salt stress. Next, we found that CACGTG motif of *ZmCAT1* can bind with ZmNAC84, thereby enhancing the transcription of *ZmCAT1*. This explains why overexpression of *ZmNAC84* enhances the ability of maize to decomposition H_2O_2, thereby increasing survival rate under salt stress, and that had never been identified in previous studies. However, ZmCATs have three members in maize, and their promoters of the other two do not bind to ZmNAC84, probably because they do not contain the CACGTG motif in their promoters, and this may explain why the expression of the two genes was not altered in the transgenic material.

NAC TFs constituting a plant-specific superfamily are characterized by the presence of a highly conserved NAC domain at the N-terminal, while the C-terminal is a variable transcriptional regula-

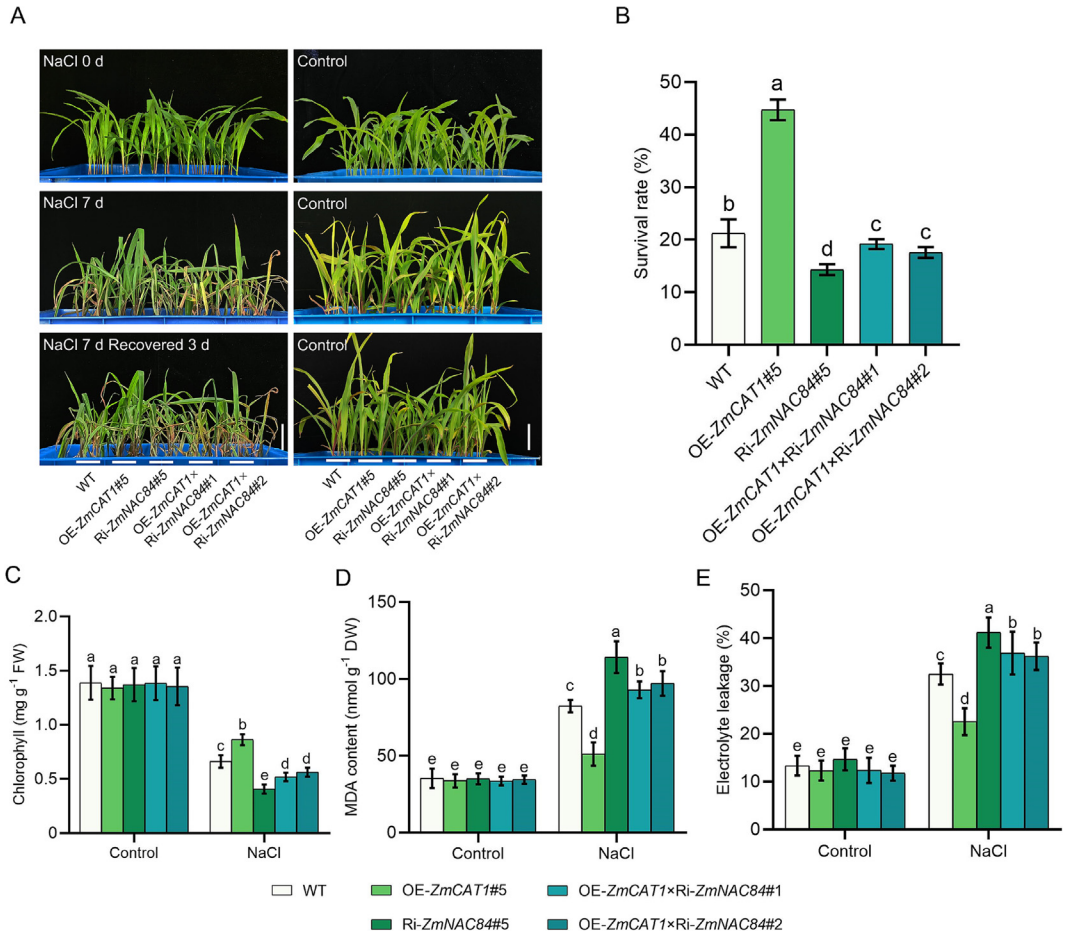

Fig. 6. Phenotype and physiological indicators of OE-*ZmCAT1* × RNi-*ZmNAC84* plants under salt stress. (A) OE-*ZmCAT1* × RNi-*ZmNAC84* transgenic plants were treated with NaCl and then recovered. Then the images were obtained (Scale bar, 5 cm). (B) Survival rates of transgenic plants and WT plants in (A). (C) Chlorophyll content in maize leaves. (D) MDA content in maize leaves. (E) Electrolyte leakage in maize leaves. Values in (B) to (E) are means SD ($n = 3$), and different letters indicate differences at $P < 0.05$ by one-way ANOVA with Duncan's multiple range test.

tory region, which might determine the diversity of their functions [4]. In the present study, the survival rate of hybrids plants under salt stress was found to be lower than WT plants but higher than Ri-*ZmNAC84* plants, demonstrating that *ZmNAC84* is an upstream gene of *ZmCAT1* in response to salt stress. It is noteworthy that salt stress also induced an increase in *ZmCAT2* expression, but there was no difference in the expression level of this gene in OE-*ZmNAC84* and Ri-*ZmNAC84* materials compared to WT, while Dual luciferase assay also indicated that ZmNAC84 did not bind to the promoter of *ZmCAT2*. These results suggest that *ZmCAT2* although responsive to salt stress, may be regulated by other transcription factors. Sequence analysis showed that the *ZmNAC84* promoter contained multiple elements that respond to hormone signals and stresses [56]. However, it fails to regulate homologous genes of *ZmCAT1*. suggesting that there are more unknowns waiting to be discovered in the regulatory network of maize under salt stress.

In summary, ZmNAC84 directly regulates *ZmCAT1* expression by binding to the CACGTG motif in the promoter region, and the activated *ZmCAT1* promotes H_2O_2 scavenging under salt stress, and in turn, protecting plants against damage.

CRediT authorship contribution statement

Yitian Pan: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Tong Han:** Validation, Data curation. **Yang Xiang:** Validation, Methodology, Formal analysis, Data curation. **Caifen Wang:** Writing – review & editing, Methodology. **Aying Zhang:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

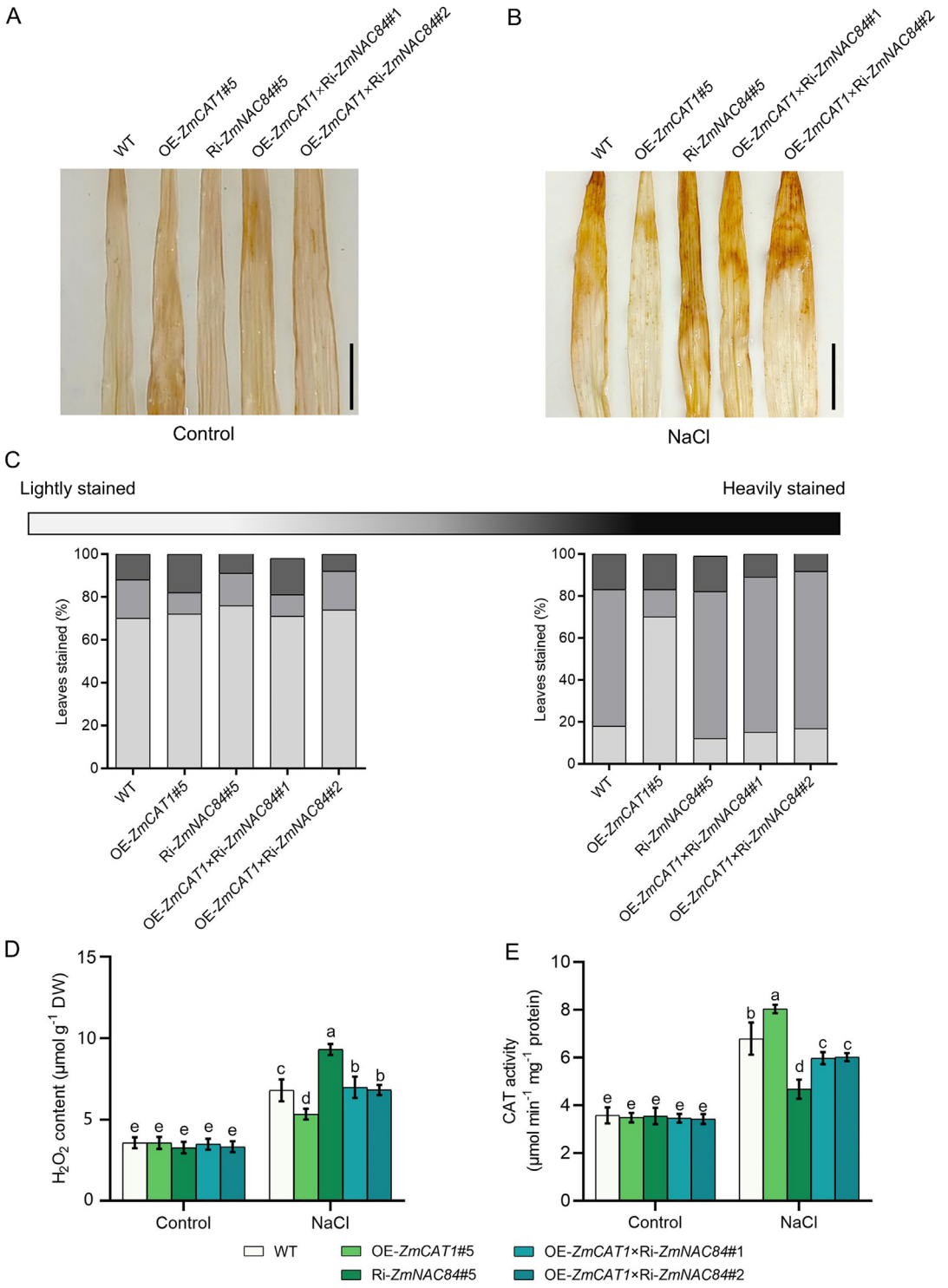

Fig. 7. Comparison of H_2O_2 accumulation and CAT activity in OE-*ZmCAT1* × RNi-*ZmNAC84* plants under salt stress. (A) and (B) DAB staining intensity reflects the content of H_2O_2 in WT, OE-*ZmCAT1*, Ri-*ZmNAC84*, and OE-*ZmCAT1* × RNi-*ZmNAC84* plants (Scale bar, 2 cm). (C) Percentage and extent of leaves stained by DAB in (A). (D) The H_2O_2 content in leaves of WT, OE-*ZmCAT1*, Ri-*ZmNAC84*, and OE-*ZmCAT1* × RNi-*ZmNAC84* plants. (E) CAT activity in leaves of WT, OE-*ZmCAT1*, Ri-*ZmNAC84*, and OE-*ZmCAT1* × RNi-*ZmNAC84* plants. Values in (D) and (E) are means SD (n = 3), and different letters indicate differences at $P < 0.05$ by one-way ANOVA with Duncan's multiple range test.

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Appendix A. Supplementary data

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2024.09.005.

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