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A *Brachypodium distachyon* calcineurin B-like protein-interacting protein kinase, BdCIPK26, enhances plant adaption to drought and high salinity stress

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Abstract: As sessile organisms, plants possess a complex system to cope with environmental changes. Ca^{2+} functions as a vital second messenger in the stress signaling of plants, and the CBL-interacting protein kinases (CIPKs) serve as essential elements in the plant Ca^{2+} signaling pathway. In this study, calcineurin B-like protein-interacting protein kinase 26 (BdCIPK26) from *Brachypodium distachyon* was characterized. Overexpression of *BdCIPK26* enhanced tolerance to drought and salt stress of transgenic plants. Further investigations revealed that BdCIPK26 participated in abscisic acid (ABA) signaling, conferred hypersensitivity to exogenous ABA in transgenic plants, and promoted endogenous ABA biosynthesis. Moreover, BdCIPK26 was found to maintain ROS homeostasis in plants under stress conditions. Therefore, this study indicates that BdCIPK26 functions as a positive regulator in drought and salt stress response.

Introduction

Global climate change tends to be extreme and causes plants to face drought or salt stress more frequently (Simpkins, 2021). As sessile organisms, plants have to passively adapt to the environment. Thus, precise control of stress signal transduction and response is crucial for plants (Zhang *et al.*, 2022). As an indispensable second messenger, Ca participates in the plant stress-response regulatory network (Zhang *et al.*, 2018b). Drought, high salt, cold, and heat trigger the alternation of cytosolic Ca^{2+} concentration, transforming direct stress signal to Ca^{2+} signal (Zhu, 2016). The calcineurin B-like proteins (CBLs) are unique Ca^{2+} sensors for plants. The CBL-interacting protein kinases (CIPKs) are responders for CBLs, and the CBLs can interact with CIPKs after binding to Ca^{2+}. The CBL-CIPK complex then acts on various substrates soon afterward (Yu *et al.*, 2014). The functional studies of CIPK involve the regulation of growth, developmental control, plant nutrition, and stress response (Ding *et al.*, 2022). For example, tonoplast-localized CBL2 and CBL3 interact with CIPK12, thus participating in modulating polarized pollen tube growth (Steinhorst *et al.*, 2015). *Brachypodium distachyon* calcineurin B-like protein-interacting protein kinases (BdCIPK31) promotes plant potassium uptake, conferring tolerance to enhanced low potassium stress (Luo *et al.*, 2022). However, until now, the research on CIPK function has focused mainly on the stress response. The salt overly sensitive (SOS) pathway plays an important role in the salt stress response of plants. The Ca^{2+} sensor SOS3 (CBL4) interacts with SOS2 (CIPK24), forming SOS3-SOS2 complex phosphorylated Na^+/H^+ antiporter SOS1 (NHX7), enhancing Na^+ extrusion under high salinity conditions (Liu and Zhu, 1998; Halfter *et al.*, 2000; Qiu *et al.*, 2002). After that, more CIPKs have been found to participate in stress response regulation. *Hordeum brevisubulatum* HbCIPK2 could enhance osmotic and salt stress resistance in *Arabidopsis* (Li *et al.*, 2012). Likewise, transient overexpression of pepper *CaCIPK3* promoted drought tolerance (Ma *et al.*, 2021b). Therefore, it is important from a research point of view to discover more stress-related CIPKs.

In addition to Ca^{2+} signaling, plants also transmit stress signals through other signaling pathways. Among these signaling pathways, abscisic acid (ABA) and reactive oxygen species (ROS) are essential elements. Abiotic stress causes

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ABA synthesis (Boominathan *et al.*, 2004), and the increased ABA content leads to stomatal closure (Kim *et al.*, 2010), inhibition of germination (Thole *et al.*, 2014), and growth repression (Sharp *et al.*, 2004). ROS production is also induced by environmental stress (Mittler and Blumwald, 2010). A moderate amount of ROS act as signal molecules that modulate plant growth and stress response (Apel and Hirt, 2004). However, ROS overaccumulation is toxic to plants, causing oxidative damage to cells (Gill and Tuteja, 2010). The CIPKs are found to be involved in the crosstalk between ABA/Ca^{2+} signaling and ROS/Ca^{2+} signaling. Under water deficiency soybean GmCBL1-GmCIPK2 localized to the plasma membrane, participated in ABA-mediated stomatal movement and induced the transcription of ABA-related genes (Xu *et al.*, 2021). Another soybean CIPK, GmCIPK21, enhanced ABA sensitivity and induced ABA-related genes under high salinity (Li *et al.*, 2022). Furthermore, overexpression of *GmCIPK21* elevated the antioxidative enzyme activity and contributed to maintaining ROS homeostasis (Li *et al.*, 2022).

B. distachyon is a model plant of the Pooideae subfamily, the largest subfamily of grasses, which includes most seasonal cereals, forage, and turf grasses (Vogel *et al.*, 2010). *B. distachyon* is more tolerant to abiotic stress than its related species, like cultivated rice (Luo *et al.*, 2011). Therefore, *B. distachyon* is a valuable model species for exploring stress tolerance genes. In our previous study, the stress-repressed gene *BdCIPK31* was found to confer tolerance to drought, high salinity, and cold stress in transgenic plants (Luo *et al.*, 2017, 2018). However, due to the contradiction between expression and phenotype, the BdCIPK31 was hypothesized as a redundant member in stress response modulation. In the present study, we characterized *BdCIPK26*, a stress-induced *CIPK* gene in *B. distachyon*. BdCIPK26 positively regulates drought and salt response of plants. Overexpressed *BdCIPK26* conferred hypersensitivity to ABA and promoted ABA biosynthesis under stress treatments. Moreover, BdCIPK26 promoted ROS-scavenging ability and accumulation of osmoprotectants and modulated the expression of stress-related transcription factors. In summary, our study demonstrated that BdCIPK26 functions positively in plant response to drought and salt stress.

Materials and Methods

Plant materials

B. distachyon inbred line Bd21 was selected to isolate and analyze *BdCIPK26*. The seeds of *B. distachyon* were sterilized by ethanol treatment. Afterward, the seeds were germinated at room temperature. The *B. distachyon* seedlings were cultured in modified Hoagland solution at 25°C, in a 16/8 h light/dark cycle. The *Nicotina tabacum* cv. Samsun was employed to generate transgenic tobaccos overexpressing *BdCIPK26*. The coding sequence (CDS) of *BdCIPK26* (Suppl. Fig. S1) was amplified by polymerase chain reaction (PCR) using gene-specific primers with *BglII* (forward) and *SpeI* (reverse) restriction sites (Suppl. Table S1), and the cDNA obtained from normally growing 10-day-old *B. distachyon* seedlings was used as PCR template. Afterward, the cloned CDS was introduced into p*Cambia1303*, downstream of the *CaMV 35S* promoter. The recombinant vector was then transformed into *Agrobacterium* strain EHA105 for *Agrobacterium*-mediated transformation (Sparkes *et al.*, 2006). The plasmid p*Cambia1303* was also introduced into tobaccos to generate the vector control (VC) lines. The T_0 transgenic seedlings were screened in half-strength Murashige and Skoog (1/2 MS) medium containing 20 mg/L hygromycin and further verified by PCR using gene-specific primers (Suppl. Table S1). The transcript level of *BdCIPK26* in transgenic tobaccos of T_2 generation was analyzed by quantitative-PCR (qPCR). Two T_2 homozygote lines of these transgenic tobaccos (OX lines) were used in this study.

For treatments of *B. distachyon* seedlings, ten-day-old seedlings were treated with 20% (w/v) PEG6000, 200 mM NaCl, 10 mM H_2O_2, or 100 μM ABA. The treated samples were collected and divided into shoots and roots for RNA preparation.

For treatments of tobacco plants, watering of one-month-old tobacco plants were stopped or watered with 300 mM NaCl solution. The leaf samples were collected 3 days after treatments. The survival rates were calculated after treatments.

Phylogenic and multiple sequence alignment assay

The BdCIPK26 homologs were searched by NCBI BlastP (https://blast.ncbi.nlm.nih.gov/Blast.cgi). BdCIPK26 functional domains and motifs were analyzed using SMART tools (http://smart.embl-heidelberg.de) (Letunic *et al.*, 2021). Multiple sequence alignment was performed by ClustalX. BdCIPKs and OsCIPKs phylogenetically analyzed by MEGA using the maximum likelihood method (Kumar *et al.*, 2018), the sequences of the OsCIPKs were obtained from Xiang *et al.* (2007).

Subcellular localization assay

The *BdCIPK26*-p*Cambia1303* was introduced into *N. tabacum* leaves by *Agrobacterium*-mediated transformation. The BdCIPK26::green fluorescent protein (GFP) fusion protein was transiently expressed in the tobacco leaf epidermis and observed by fluorescence microscopy (Olympus IX71, Japan).

Gene expression and promoter analysis

The treated samples of *B. distachyon* seedlings were subjected to RNA extraction using Total RNA Extraction Kit (Solarbio, Beijing, China). The cDNA was synthesized by FastQuant RT Kit (TIANGEN, Beijing, China). The quantitative real-time PCR was performed to detect the expression of *BdCIPK26* using 2 × SY BR Green PCR Mastermix (Solarbio, Beijing, China) on an ABI StepOnePlus Real-Time PCR System (Thermo Fisher, USA). The *Bdactin* gene was applied as endogenous control. The primers used in this assay are listed in Suppl. Table S1. The data were analyzed by the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

The promoter sequence of BdCIPK26 was obtained from Ensembl Plants database (http://plants.ensembl.org/index.html). The promoter sequence was analyzed by the

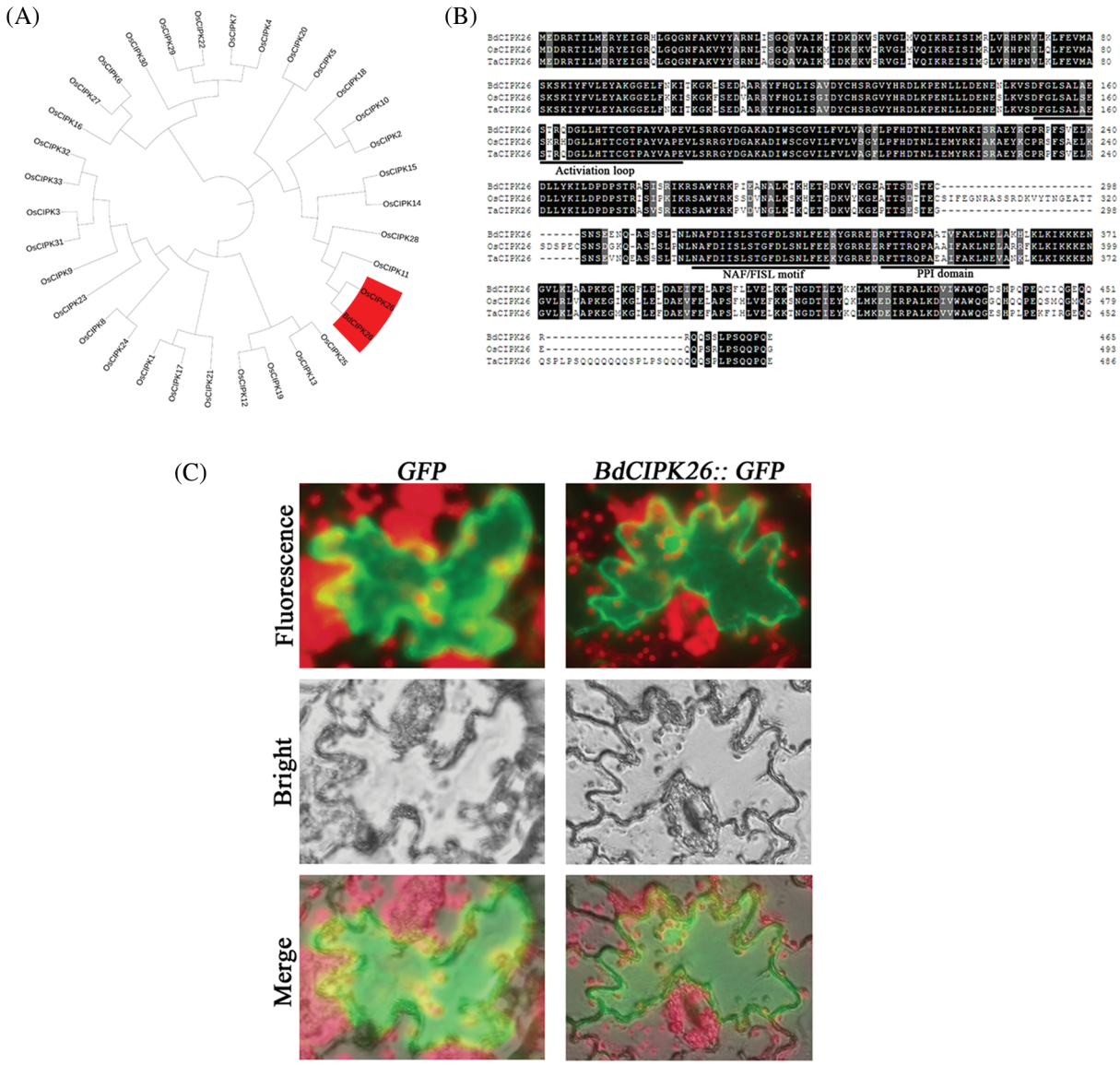

FIGURE 1. Characterization of *Brachypodium distachyon* calcineurin B-like protein-interacting protein kinases (BdCIPK26). (A) Phylogenic analysis of BdCIPKs and OsCIPKs. The red color indicates OsCIPK26 and BdCIPK26. (B) Multiple sequence alignment of BdCIPK26, OsCIPK26, and TaCIPK26 (Sun *et al.*, 2015). Black shadows indicate the same amino acid residues, and gray shadows indicate similar amino acid residues. The typical motifs of CIPKs are indicated by lines. (C) Subcellular localization of BdCIPK26. The GFP protein in the left column is applied as a positive control, and the fluorescence of the BdCIPK26::GFP fusion protein is shown in the right column. Three independent experiments exhibited the same results.

PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot *et al.*, 2002).

Seedling phenotype assays

Tobacco seeds were germinated on 1/2 MS medium with 20 mg/L hygromycin. After germination, the seeds were transferred into 1/2 MS medium containing 200 mM mannitol, 150 mM NaCl, or 2.5 μM ABA for stress treatments. The root length and fresh weight were examined after each treatment.

Estimation of abscisic acid content

Tobacco leaf samples were collected 3 days after drought or salt treatment. The samples were pretreated as previously described (Yan *et al.*, 2014), and the ABA content was determined by the ABA content detection ELISA kit (Jiancheng, Nanjing, China).

Reactive oxygen species-scavenging enzyme activity, H_2O_2 content, ion leakage, malondialdehyde (MDA) content, enzyme activity, and osmolyte content assay

Tobacco leaf samples used in these assays were collected 3 days after drought or salt treatment. The H_2O_2 content was determined by the H_2O_2 content detection kit (molybdic acid method, Jiancheng, Nanjing, China). The MDA content was determined by the MDA assay kit (TBA method, Jiancheng, Nanjing, China). The ion leakage was determined by the conductivity method as described before (Luo *et al.*, 2017). The activities of the antioxidative enzymes were determined using corresponding kits (Jiancheng, Nanjing,

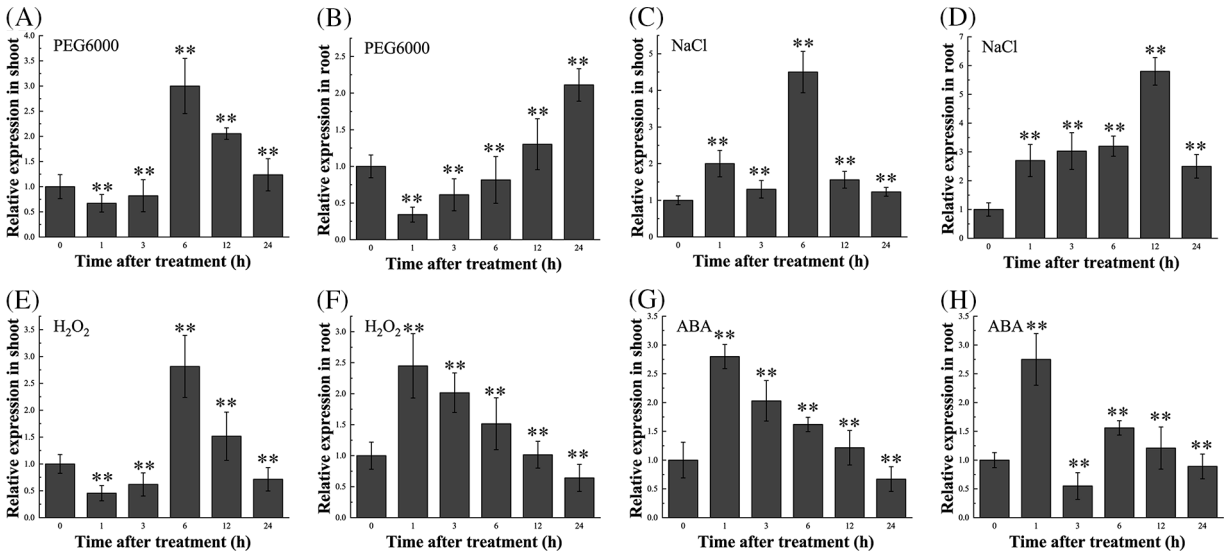

FIGURE 2. The expression profiles of *BdCIPK26*. (A and B) The expression of *BdCIPK26* in shoots or roots of *Brachypodium distachyon* seedlings under 20% PEG6000 treatment. (C and D) The expression of *BdCIPK26* in shoots or roots of *B. distachyon* seedlings under 200 mM NaCl treatment. (E and F) The expression of *BdCIPK26* in the shoots or roots of *B. distachyon* seedlings under 10 mM H_2O_2 treatment. (G and H) The expression of *BdCIPK26* in the shoots or roots of *B. distachyon* seedlings under 100 μM ABA treatment. The bars exhibit the mean ± SE of three biological replicates (n = 3). The asterisks indicate a significant difference compared to the control (*$p < 0.05$, **$p < 0.01$).

China). The contents of proline and soluble sugar were also determined using suitable kits (Jiancheng, Nanjing, China).

Assay for the expression of stress-related genes

Tobacco leaf samples used in these assays were collected 3 days after drought or salt treatment. The RNA preparation was performed as mentioned above. The primers used in this assay are listed in Suppl. Table S1.

Statistical analysis

The data was analyzed by SPSS software (Chicago, IL, USA). The significance tests were performed by student's *t*-test.

Results

Characterization of Brachypodium distachyon calcineurin B-like protein-interacting protein kinase

The BdCIPK26 had been identified previously (Wang *et al.*, 2015). Phylogenic analysis of BdCIPK26 and OsCIPKs showed the closest relationship between BdCIPK26 and OsCIPK26 (Fig. 1A). Multiple sequence alignment suggested that BdCIPK26 contains CIPK feature domains such as activation loop, the CBL interaction motif (NAF/FISL motif), and protein–phosphatase interaction domain (Fig. 1B).

The subcellular localization assays were performed by introducing *BdCIPK26::GFP* fusion plasmids into tobacco leaves, and no specific localization of BdCIPK26 was observed in the tobacco epidermis cells (Fig. 1C).

Expression patterns of Brachypodium distachyon calcineurin B-like protein-interacting protein kinases (BdCIPK26)

The qPCR analyses were performed to determine whether the transcription of *BdCIPK26* was responsive to abiotic stress and ABA treatments. The results suggested significant induction of *BdCIPK26* by PEG6000, NaCl, H_2O_2, and ABA treatments in both shoots and roots of *B. distachyon* seedlings (Fig. 2). Analysis of the promoter sequence of *BdCIPK26* revealed various stress-related *cis*-elements, including ABRE and DRE (Suppl. Table S2). These results suggested that *BdCIPK26* was induced by osmotic, salt, oxidative stress, and ABA treatment.

Brachypodium distachyon calcineurin B-like protein-interacting protein kinases conferred enhanced drought and salt adaption in transgenic tobaccos

Since the transcription level of *BdCIPK26* was induced by osmotic and salt stress treatment, we speculated the role of BdCIPK26 in plant response to corresponding stresses. Transgenic tobacco plants overexpressing the *BdCIPK26* gene were generated as experimental materials. The expression of *BdCIPK26* in six transgenic lines was detected by qPCR (Suppl. Fig. S2B). The T_2 homozygous lines OX3 and OX5 were selected for the functional characterization of BdCIPK26.

The tobacco seeds were germinated on 1/2 MS medium and then moved to treatment medium containing 200 mM mannitol or 150 mM NaCl. After two weeks of stress treatment, the OX seedlings exhibited better growth status than the WT and VC control seedlings (Fig. 3A). The results were verified by root length and fresh weight assessments. The transgenic seedlings overexpressing *BdCIPK26* displayed longer tap roots (Fig. 3B) and higher fresh weight (Fig. 3C) than the controls, suggesting that BdCIPK26 enhanced plant adaption to osmotic and high salinity stress in the seedling stage.

In the vegetative growth stage, the watering of 28-day-old tobacco plants was stopped for drought treatment. After 40 days of treatment, all the tobacco plants were shriveled, especially the control plants, while the leaves of OX plants

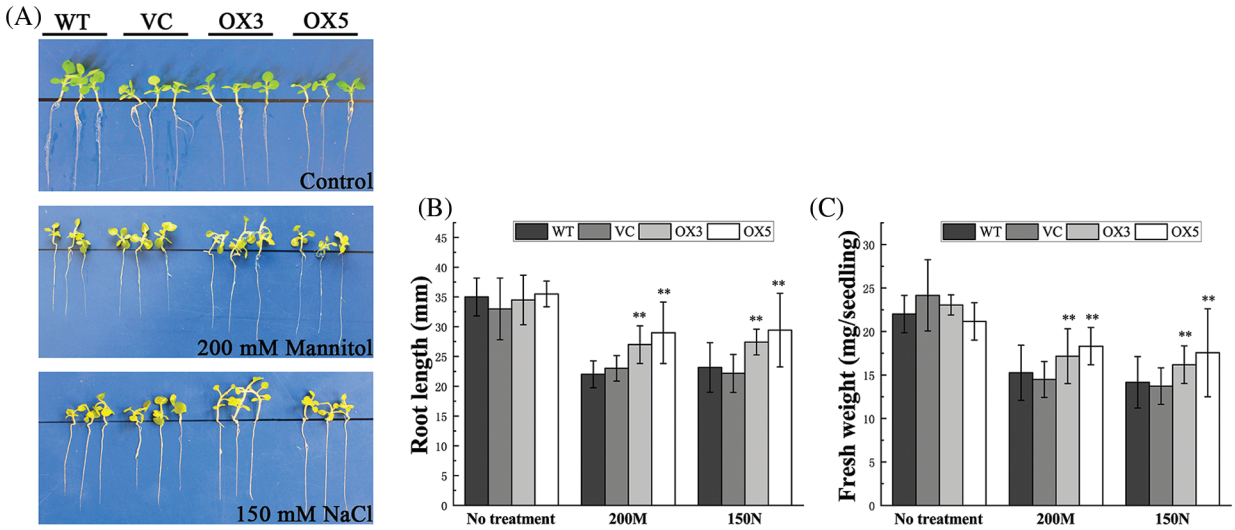

FIGURE 3. Phenotype analysis of transgenic tobacco seedlings under osmotic or high salinity stress. (A) The tobacco seedlings grown on an untreated 1/2 Murashige and Skoog (MS) medium (upper panel), 1/2 MS medium containing 200 mM mannitol (middle panel), and 1/2 MS medium containing 150 mM NaCl (lower panel). (B) The average root length of seedlings under mannitol or NaCl treatment. (C) The fresh weight analysis of seedlings under mannitol or NaCl treatment. The bars exhibit the mean ± SE of three biological replicates (n = 3). The asterisks indicate significant difference compared to the control (*$p < 0.05$, **$p < 0.01$).

were still green and unfolded (Fig. 4A). All treated plants were rewatered for 21 days, and more than 30% OX plants were recovered, whereas less than 10% control plants survived after drought treatment (Fig. 4B).

In the high salinity stress assay, tobacco plants were irrigated with 300 mM NaCl for 14 days. The treated plants exhibited phenotype representing salt damage, such as wilted and yellowing, whereas the leaves of OX plants

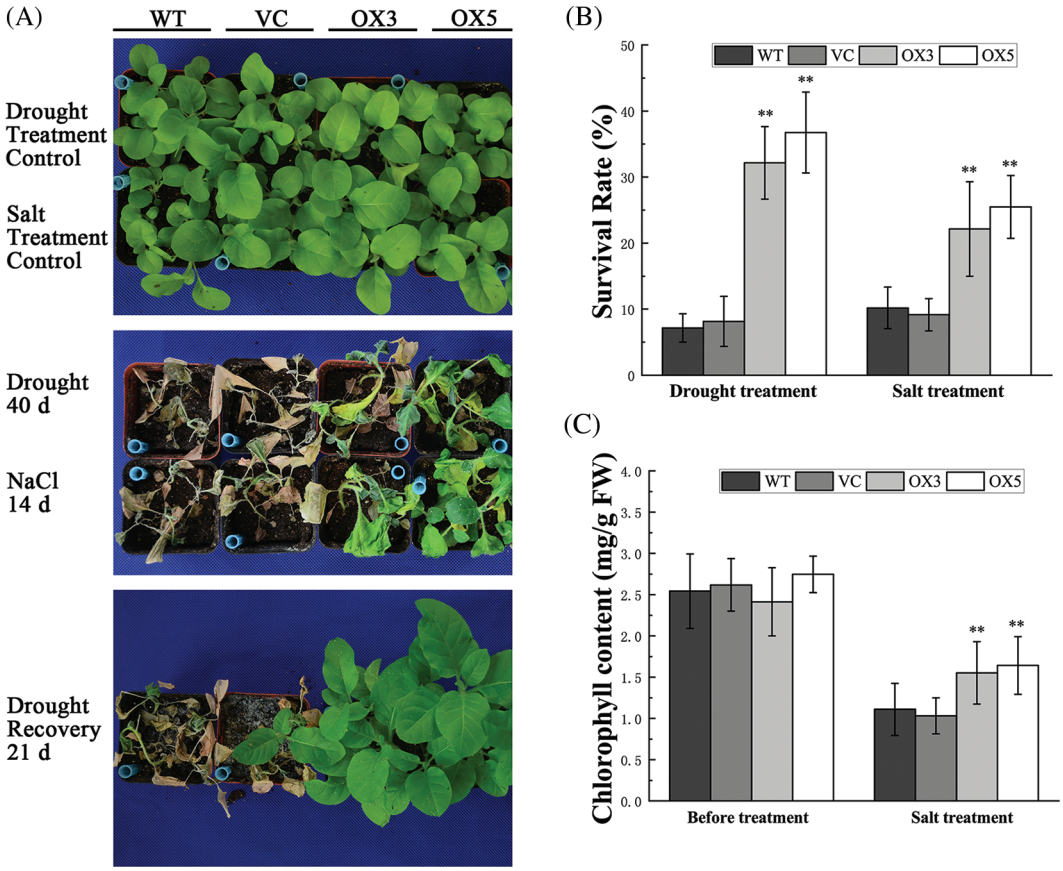

FIGURE 4. The phenotype analysis of tobacco plants under drought or salt stress. (A) The tobacco plants grown in normal conditions (upper panel), in drought or high salinity treatment (middle panel), and were rewatered (lower panel). (B) The survival rate of tobacco plants after stress treatment. (C) The chlorophyll contents in tobacco leaves under high salinity treatment. The bars in (B) and (C) exhibit the mean ± SE of three biological replicates (n = 3). The asterisks indicate a significant difference compared to the control (*$p < 0.05$, **$p < 0.01$).

remained greener than that in controls (Fig. 4A). About 22% of the OX3 line plants and 25% of the OX5 line plants survived after high salinity treatment. In contrast, only less than 10% of the control plants survived after the same treatment (Fig. 4B). In addition, the OX plants contained more chlorophyll than the controls during salt treatment (Fig. 4C), indicating that *BdCIPK26* overexpression functioned in plant tolerance to salt stress.

Brachypodium distachyon calcineurin B-like protein-interacting protein kinases enhanced abscisic acid sensitivity and biosynthesis in plants

To identify whether BdCIPK26 participates in ABA signaling, the transgenic seedlings were subjected to 2.5 μM exogenous ABA. The OX seedlings exhibited a hypersensitive phenotype in the ABA treatment (Fig. 5A), while the control seedlings showed longer tap roots than those in OX seedlings (Fig. 5B). Further analysis revealed that transgenic tobacco plants overexpressing *BdCIPK26* contained more endogenous ABA under drought or salt conditions (Fig. 5C), indicating the participation of BdCIPK26 in ABA synthesis. This result had been confirmed at the mRNA level. The expressions of *NtNCED1* and *NtNCED3* in tobacco plants were elevated by *BdCIPK26* overexpression under the same conditions (Figs. 5D and 5E).

Brachypodium distachyon calcineurin B-like protein-interacting protein kinases alleviated oxidative damage in plants under drought and high salinity

Abiotic stresses trigger ROS accumulation and cause oxidative damage to plants (Rodrigo-Moreno *et al.*, 2013). Induction of *BdCIPK26* transcripts by H_2O_2 treatment (Figs. 2E and 2F) implied that BdCIPK26 may be involved in ROS homeostasis. In the present study, H_2O_2 content was examined to estimate the ROS level. Ion leakage and MDA content were analyzed to assess oxidative damage. *BdCIPK26* overexpression reduced H_2O_2 content in transgenic tobacco plants under drought or salt treatment (Fig. 6A). Furthermore, ion leakage and MDA content level were lowered in transgenic tobacco plants (Figs. 6B and 6C), suggesting that BdCIPK26 alleviated the membrane damage caused by ROS overaccumulation.

Plant ROS-scavenging system consists of antioxidative enzymes and antioxidants. In this study, catalase (CAT), guaiacol peroxidase (POD), superoxide dismutase (SOD), glutathione s-transferase (GST), ascorbic acid, and anthocyanin were selected as the representative members of ROS-scavenging system. The enzyme activity assay revealed that the activities of CAT, POD, SOD, and GST were elevated by BdCIPK26 overexpression in the transgenic tobaccos under stress treatments (Figs. 6D–6G). Additionally, the treated transgenic tobaccos also showed higher ascorbic acid and anthocyanin content (Figs. 6H and 6I), suggesting the participation of BdCIPK26 in ROS-scavenging regulation. These effects were also verified at the transcriptional level; the transcripts of *NtCAT1*, *NtPOX2*, *NtSOD1*, *NtGST*, *NtAPX*, and *NtDFR* were all induced by *BdCIPK26* overexpression under stress conditions (Suppl. Fig. S3).

Brachypodium distachyon calcineurin B-like protein-interacting protein kinases enhanced osmolyte accumulation in plants

Osmotic pressure unbalance is caused by water and salt stress. The production and accumulation of osmoprotectants such as proline and soluble sugar can maintain the osmotic equilibrium of plant cells. To investigate whether BdCIPK26 functions in osmolytes accumulation, the proline and soluble sugar contents were examined. The results showed higher proline, and soluble sugar content in transgenic tobaccos than the control plants under drought or salt stress treatment (Figs. 7A and 7C). Furthermore, the expressions

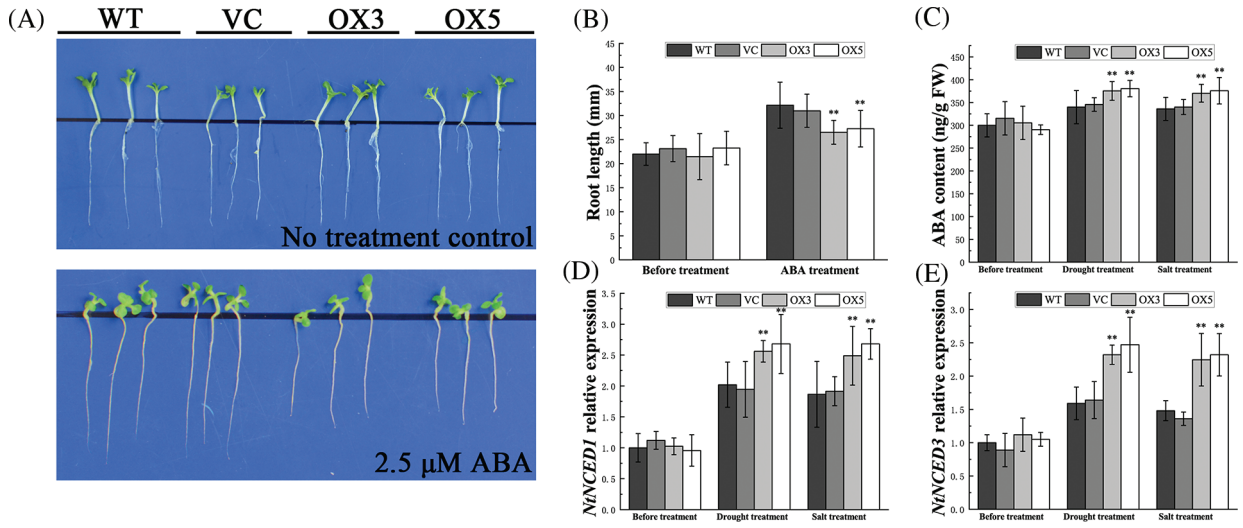

FIGURE 5. Analyses of abscisic acid (ABA) response, ABA content, and ABA synthesis-related gene expression in tobacco seedlings. (A) Tobacco seedlings under 2.5 μM ABA treatment. (B) The average root length of tobacco seedlings exposed to exogenous ABA. (C) ABA content in tobacco leaves under drought or high salinity treatment. (D) The expression of *NtNCED1* in tobacco plants under drought or high salinity treatment. (E) The expression of *NtNCED3* in tobacco plants under drought or high salinity treatment. The bars in (B–E) represent the mean ± SE of three biological replicates (n = 3). The asterisks indicate a significant difference compared to the control (*$p < 0.05$, **$p < 0.01$).

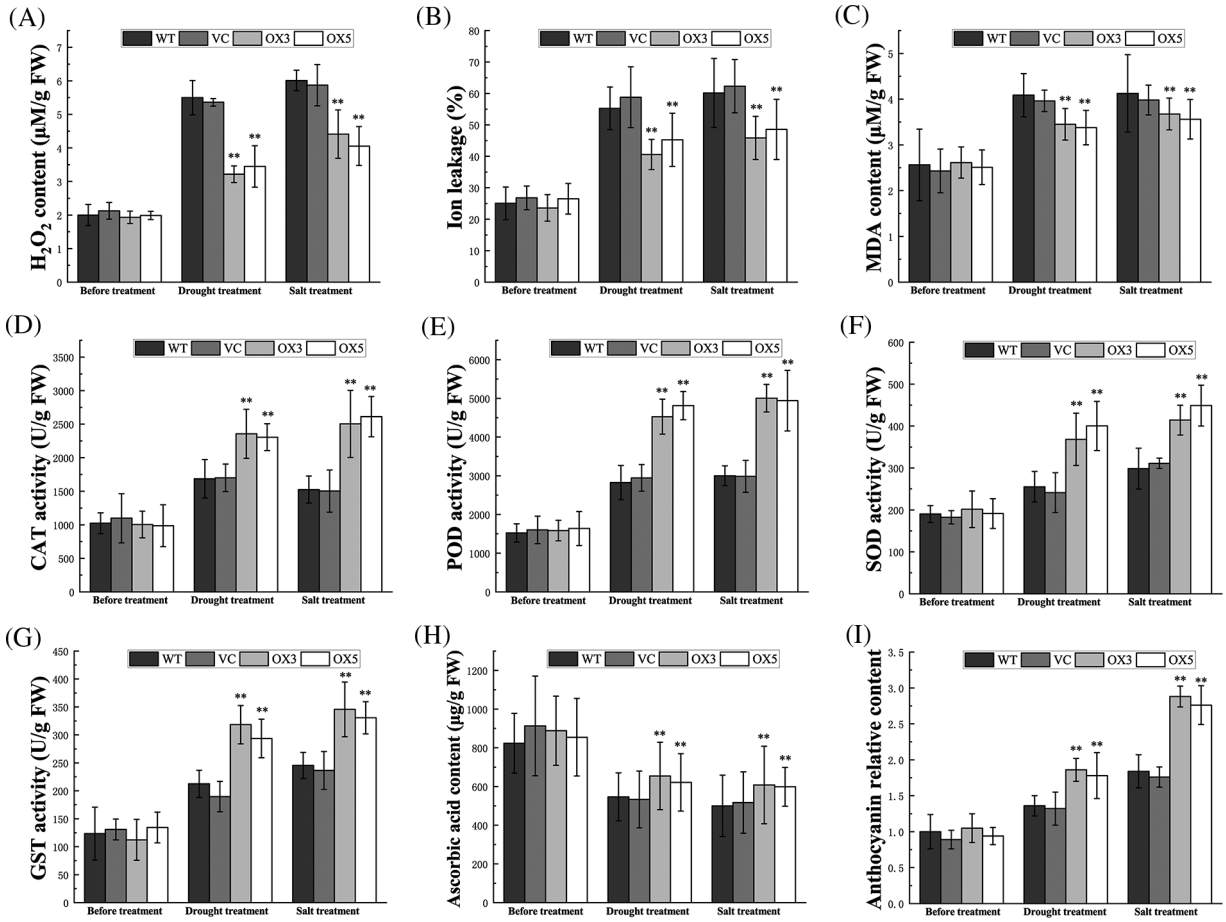

FIGURE 6. Analyses of reactive oxygen species (ROS)-related indexes. (A–C) H_2O_2 content (A), relative ion leakage (B), and *Malondialdehyde* (MDA) content (C) in tobacco plants under stress treatments. (D–I) Catalase (CAT) (D), guaiacol peroxidase (POD) (E), superoxide dismutase SOD (F), and glutathione s-transferase (GST) (G) activities in tobacco plants under stress treatments. (H–I) Ascorbic acid content (H) and relative anthocyanin content (I) in tobacco plants under stress treatments. The bars exhibit the mean ± SE of three biological replicates (n = 3). The asterisks indicate a significant difference compared to the control (*$p < 0.05$, **$p < 0.01$).

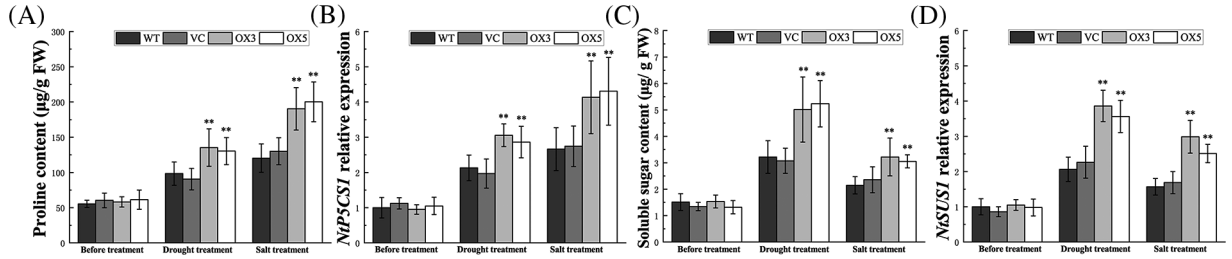

FIGURE 7. Analyses of osmolytes in tobacco plants. (A) The proline content in tobacco plants under stress treatments. (B) The level of *NtP5CS1* mRNA in tobacco plants under drought or salt stress. (C) The soluble sugar content in tobacco plants under drought or salt stress. (D) The expression of *NtSUS1* in tobacco plants under drought or salt stress. The bars exhibit the mean ± SE of three biological replicates (n = 3). The asterisks indicate a significant difference compared to the control (*$p < 0.05$, **$p < 0.01$).

of proline synthesis-related gene *NtP5CS1* and sucrose synthesis-related gene *NtSUS1* were also elevated due to *BdCIPK26* overexpression under the same conditions (Figs. 7B and 7D). These results indicated that BdCIPK26 enhanced the accumulation of osmoprotectants.

Brachypodium distachyon calcineurin B-like protein-interacting protein kinases affected the transcription of stress-related transcription factors

The transcripts of some typical transcription factors related to abiotic stress response were examined, the ABA-responsive element binding factor *NtABF2* and the dehydration-responsive element binding protein *NtDREB3* exhibited higher transcripts under stress treatments (Suppl. Fig. S4). These results indicated that BdCIPK26 can modulate the expressions of stress-related transcription factors.

Discussion

The Ca^{2+} signaling pathway is one of the most important signaling pathways which regulate the physiological and transcriptional processes of plants. As unique members in

plant Ca^{2+} signaling, CBL-CIPK complexes are extensively involved in growth, development, reproduction, and environment adaptation (Mao *et al.*, 2022). The study of CIPK function focuses on plant response to external stresses (Tang *et al.*, 2020). BdCIPK31, a stress-repressed CIPK, was characterized as a potential positive regulator in abiotic stress tolerance and plant nutrition in our previous study (Luo *et al.*, 2017, 2018, 2022). In this study, a multiple-stress-induced *CIPK* (Fig. 2), *BdCIPK26*, was discovered. Further, overexpression of *BdCIPK26* conferred enhanced adaption to drought and high salinity in plants (Figs. 3 and 4).

As the key phytohormone in stress signal transduction, ABA functions in modulating plant growth under stress conditions (Raghavendra *et al.*, 2010). The CIPKs have been reported to be involved in ABA signaling. Most *CIPK* genes in potatoes *(Solanum tuberosum* L.) are induced by ABA treatment (Ma *et al.*, 2021a). AtCIPK26 was found to interact with a series of transcription factors and keep-on-going (KEG), a RING-type E3 ligase, in ABA signaling (Lyzenga *et al.*, 2013). The AtCIPK26 was degraded by the ubiquitin-proteasome system mediated by KEG, and the AtCIPK26-mediated phosphorylation led to ABA-mediated degradation of KEG (Lyzenga *et al.*, 2017). The OsCBL8-OsCIPK17 complex negatively regulates seed germination and seedling growth by interacting with OsPP2C77, which is an ABA-mediated phosphatase (Gao *et al.*, 2022). AcCIPK5 regulates ABA signaling to mediate stress response in *Arabidopsis* (Aslam *et al.*, 2022). In the present study, *BdCIPK26* was found to be induced by ABA treatment (Figs. 2G and 2H). In addition, *BdCIPK26* overexpression conferred a hypersensitive phenotype to transgenic tobacco seedlings exposed to exogenous ABA (Figs. 5A and 5B), suggesting that BdCIPK26 functioned as a positive regulator in ABA signaling. Unlike BdCIPK31, which did not affect ABA synthesis (Luo *et al.*, 2017), further investigations revealed that BdCIPK26 enhanced endogenous ABA production under drought and salt stresses (Fig. 5C). The transcription of tobacco 9-cis-epoxycarotenoid dioxygenase genes (*NCED*), which encode rate-limiting ABA biosynthesis enzymes, were also increased in the treated transgenic plants (Figs. 5D and 5E). These results implied that BdCIPK26 might be an upstream regulator of ABA synthesis.

Besides Ca^{2+}, ROS are also essential second messenger molecules in plant stress signal transduction (Bartels and Sunkar, 2005). Environmental changes trigger the accumulation of ROS and then alter the plant responses to exogenous stimuli (Zhu, 2016). On the other hand, overproduced ROS are reactive in cells and cause oxidative damage to proteins, nucleic acids, organelles, and membranes (You and Chan, 2015). The CIPKs have been reported to affect the production of ROS. The AtCBL1/AtCBL9-AtCIPK26 complexes reduced ROS-producing activity by interacting and phosphorylating NADPH oxidase AtRBOHF (Drerup *et al.*, 2013; Kimura *et al.*, 2013; Lyzenga *et al.*, 2013). The AtCBL1-AtCIPK26 also mediated the phosphorylation of AtRBOHC, enhanced AtRBOHC activity, and affected root hair growth (Zhang *et al.*, 2018a). In addition, the plant ROS-managing system consists of enzymatic and non-enzymatic components responsible for scavenging excess ROS in cells (Gill and Tuteja, 2010). The CIPK protein kinases also take part in the regulation of ROS-managing system. Knockdown of pepper *CaCIPK13* resulted in hypersensitivity to cold stress with higher H_2O_2 content (Ma *et al.*, 2022). GmPKS4, a soybean CIPK, increased the activities of antioxidative enzymes under salt and alkali stress (Ketehouli *et al.*, 2021). The CcCBL1-CcCIPK14 complex in pigeon peas was found to positively modulate drought tolerance by enhancing flavonoid biosynthesis (Meng *et al.*, 2021). In this study, overexpression of *BdCIPK26* elevated the activities of representative ROS-scavenging enzymes, including CAT, POD, SOD, and GST, under stress conditions (Figs. 6D–6G). BdCIPK26 also affected the biosynthesis of antioxidants. During drought and salt treatments, transgenic tobacco plants exhibited higher ascorbic acid and anthocyanin levels than the control plants (Figs. 6H and 6I). Furthermore, our study revealed that BdCIPK26 modulates the ROS-scavenging system at the transcriptional level. In stress conditions, the transcripts of *NtCAT1*, *NtPOX2*, *NtSOD1*, and *NtGST* were induced by *BdCIPK26* overexpression (Suppl. Figs. S3A–S3D), together with the transcripts of tobacco ascorbate peroxidase gene *NtAPX* and dihydroflavonol 4-reductase gene *NtDFR* (Suppl. Figs. S3E and S3F). Therefore, our results reveal that BdCIPK26 contributes to maintaining ROS homeostasis in plant response to abiotic stress.

Both water shortage and salt stress induce cell dehydration, leading to osmotic stress (Zhu, 2016). Osmotic stress triggers an alternation of cytosolic Ca^{2+} concentration and transforms the stress signal into a Ca^{2+} signal (Yuan *et al.*, 2014). Natural osmolytes such as soluble amino acids and sugar play important roles in maintaining cell osmotic pressure. One previous study indicated that wheat TaCBL1-TaCIPK23 enhanced the accumulation of proline and soluble sugar in *Arabidopsis* under drought stress (Cui *et al.*, 2018). In apples, MdCIPK22 phosphorylated sucrose transporter MdSUT2.2 increased sucrose content in response to dehydration (Ma *et al.*, 2019). In the present study, *BdCIPK26* overexpression significantly improved the soluble sugar and proline contents in plants under drought and salt stress (Figs. 7A and 7C), the expression of proline synthesis-related gene *NtP5CS1* and sucrose synthesis-related gene *NtSUS1* (Figs. 7B and 7D). Therefore, our results suggested that BdCIPK26 had a positive function in adjusting osmolality in plant response to abiotic stress.

In addition to physiological regulation, CIPKs have also been identified to participate in transcriptional regulation (Tang *et al.*, 2020). The abiotic stress-related transcriptional regulatory network can be categorized into ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki, 2006). In our study, a series of transcriptional factors were analyzed at the transcriptional level, and we discovered that the *NtABF2* and *NtDREB3* transcripts were upregulated by BdCIPK26 (Suppl. Figs. S4A and S4B). The ABA-responsive element binding factor (ABF) is recognized as a key transcriptional factor in ABA-dependent pathway, while the dehydration-responsive element binding protein (DREB) is the primary transcriptional factor in the ABA-independent pathway (Yoshida *et al.*, 2014). Hence, the BdCIPK26 is demonstrated to be a regulator in plant stress-related transcriptional regulatory network.

In conclusion, BdCIPK26 is a positive regulator in plant response to drought and salt stress. The expression of *BdCIPK26* is induced by osmotic, salt, H_2O_2, and ABA treatment. Overexpression of *BdCIPK26* enhances plant tolerance to drought and salt stress and alleviates oxidative damage by promoting the activity of the ROS-scavenging system. Additionally, BdCIPK26 participates in transcriptional regulation through both ABA-dependent and ABA-independent pathways.

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Author Contributions: Q. L. conceived the study. Q. L. and J. F. performed the experiments and analyzed the data. Q. L. wrote the manuscript. X. D. performed the gene expression assays and analyzed the data.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Supplementary Materials

TABLE S1

Genes and primers in this study

Gene	Sequence (5′-3′)	Loci number	Purpose
Bdactin	F: TGCCCAGCAATGTATGTCGC R: CAGCAAGGTCCAGACGAAGG	LOC100834406	Endogenous control of qPCR
BdCIPK26	F: GCAGATCTGATGGAGGATAGGAGGACGAT R: GCACTAGTCTCCTGTGGCTGCTGTGATG	LOC100830445	*BdCIPK26*-p*Cambia1303* construction
qBdCIPK26	F: AGTTCCACTTCCTACCATT R: GCACACGCATATTCTCAA	LOC100830445	Expression of *BdCIPK26*
NtUbiquitin	F: TTGTGTCGTGTTGATTGA R AAACAGCAAACAGAAATAGC	LOC104090883	Endogenous control of qPCR
NtNCED1	F: AAGAATGGCTCCGCAAGTTA R: GCCTAGCAATTCCAGAGTGG	LOC107827644	*NtNCED1* gene expression analysis
NtNCED3	F: AATTGTGGTGATTGGTTC R: ATTGCTCTTCTTGTTGATT	LOC107799511	*NtNCED3* gene expression analysis
NtCAT1	F: AGGTACCGCTCATTCACACC R: AAGCAAGCTTTTGACCCAGA	LOC107799583	*NtCAT* gene expression analysis
NtPOX2	F: CTTGGAACACGACGTTCCTT R: TCGCTATCGCCATTCTTTCT	LOC107783527	*NtPOX2* gene expression analysis
NtSOD	F: CTCCTACCGTCGCCAAAT R: GCCCAACCAAGAGAACCC	LOC107830263	*NtSOD* gene expression analysis
NtGST	F: AGCACCCTTACCTTTCCCTCA R:GACATACTGGGCATCTTCTTTGG	LOC107795177	*NtGST* gene expression analysis
NtDFR2	F: TATATGCTAAGAAGATGAC R: GTATGATGCTAATGAAATC	LOC107803097	*NtDFR2* gene expression analysis
NtAPX1	F: GACATTGCTATCAGACTC R: CTCCAGTAACTTCAACAG	LOC107759703	*NtAPX1* gene expression analysis
NtP5CS1	F: ATCTTCTAGTTCTGTTGA R: CTCTCCTTAATGTATGTG	LOC107819560	*NtP5CS1* gene expression analysis

(Continued)

Table S1 (continued)

Gene	Sequence (5′-3′)	Loci number	Purpose
NtSUS1	F: GTGGGGAAACACCGCTGAA R: CAACAAGGATGCGAGGGATGA	LOC107804066	*NtSUS1* gene expression analysis
NtABF2	F: GCAGCCATCTATCTATTC R: GCAACTCATCCATATTCA	LOC107824387	*NtABF2* gene expression analysis
NtDREB3	F: GCCGGAATACACAGGAGAAG R: CCAATTTGGGAACACTGAGG	LOC107807923	*NtDREB3* gene expression analysis

TABLE S2

The *cis*-elements predicted in *BdCIPK26* promoter region

Cis-elements	Position	Description
ABRE	791	AREB/ABF binding sites, involved in the abscisic acid responsiveness.
CGTCA motif	128 727	Cis-acting regulatory element involved in the MeJA-responsiveness
DRE core	297 566 485	DREB binding sites, involved in abiotic stress response
GARE	645 1448 1565	Gibberellin-responsive element
G box	790 1425	Cis-acting regulatory element involved in light responsiveness
LTR	154	*Cis*-acting element involved in low-temperature responsiveness
MRE	647	MYB binding site involved in light responsiveness
MYB-binding site	718 1442 1317	MYB binding site.
TCCC-motif	685	Part of a light responsive element
TCT-motif	928	Part of a light responsive element
TGACG-motif	128 727	Cis-acting regulatory element involved in the MeJA-responsiveness
TATA box	854 1132 1347 1432 1486 1496	Core promoter element around-30 of transcription start

1 ATGGAGGATA GGAGGACGAT TTGATGGAA CGTTATGAAA TTGGGAGACA CTTAGGGCAA GGGAACTTTG
71 CCAAGGTATA TTATGCTCGG AATCTTATCA GTGGACAAGG TGTTGCAATA AAGATAATTG ATAAGGACAA
141 GGTTTCAAGG GTTGGGCTAA TGGTGCAGAT AAAGAGGGAG ATTTCTATAA TGAGATTGGT CAGGCATCCA
211 AACGTCCTAA AACTTTTTGA GGTAATGGCT AGCAAGAGCA AGATTTACTT TGTTTTGGAG TATGCTAAAG
281 GTGGCGAGCT TTTCAACAAA ATAACCAAGG GAAAGTTAAG TGAGGATGCT GCAAGGAAGT ACTTCCATCA
351 ATTGATCAGT GCTGTGGACT ACTGCCATAG CAGAGGTGTT TATCATCGCG ACTTGAAGCC GGAAAACCTA
421 CTCCTGGATG AGAATGAGAA CCTAAAAGTC TCTGATTTTG GTTTAAGCGC TCTAGCCGAG TCCACGAGAC
491 AAGATGGCCT CCTCCATACC ACATGTGGAA CTCCAGCTTA TGTTGCTCCA GAAGTGCTTA GCAGGAGAGG
561 CTATGACGGT GCGAAGGCTG ACATATGGTC TTGTGGAGTA ATTCTATTTG TGCTGGTGGC TGGTTTCCTT
631 CCTTTCCATG ATACAAATCT TATAGAGATG TATAGGAAGA TTTCCAGAGC TGAATATAGA TGCCCTCGTC
701 CTTTTTCTGT TGAGTTGAAG GATCTACTAT ATAAGATTCT TGATCCAGAT CCAAGTACTA GAGCTTCTAT
771 CTCAAGGATA AAGAGAAGTG CTTGGTACCG AAAACCCATC GAGGCAAATG CACTGAAGAT CAAACATGAA
841 ACAAGAGACA AGGTATACAA AGGTGAAGCC ACAACCTCTG ACTCGACAGA ATGCAGTAAT TCAGAGGAAA
911 ATCAAGCGTC CTCAAGCCTC ACAAACTTGA ATGCATTTGA CATCATCTCT CTCTCAACAG GATTTGACCT
981 ATCCAATTTA TTTGAAGAGA AGTATGGTCG GAGGGAGGAC AGATTTACCA CTAGGCAGCC AGCAGCGACT
1051 GTATTTGCTA AGTTAAATGA ACTGGCCAAG CATTTGAAGC TCAAAATTAA GAAGAAAGAA AATGGTGTTT
1121 TGAAATTGGC AGCACCAAAG GAAGGGATAA AGGGTTTTCT TGAGCTTGAC GCGGAGATTT TTGAGCTCGC
1191 GCCTTCTTTT CTGTTAGTTG AATTAAAAAA GACTAATGGA GATACTCTAG AGTATAAAAA ACTCATGAAA
1261 GATGAAATAA GGCCCGCACT CAAGGATGTG ATTTGGGCGT GGCAAGGTGA CTCACACCCG CAGCCTGAGC
1331 AATGTATCCA AGGAGAGCAG CAGCGACGGC AGCAGTCGTC TTTGCCATCA CAGCAGCCAC AGGAGTAA

FIGURE S1. The coding sequence of *BdCIPK26* gene (LOC100830445).

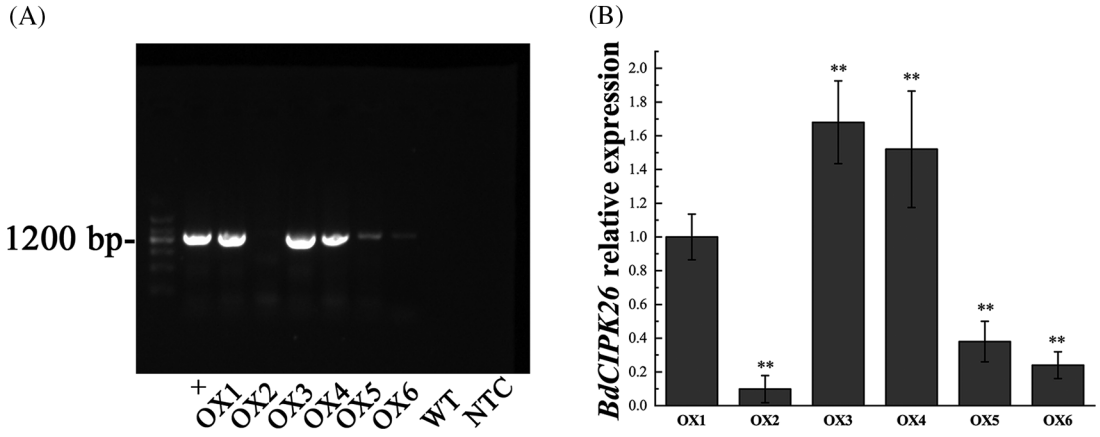

FIGURE S2. Generation of transgenic tobacco plants overexpressing *BdCIPK26*. (A) PCR screening of T_0 transgenic tobacco plants. + indicates the positive control, WT indicates the wildtype control using genome DNA extracted from WT tobaccos as PCR template. NTC indicates non-template control using ddH_2O as PCR template. (B) The expression of *BdCIPK26* in different transgenic tobacco lines. The bars exhibit the mean ± SE of three biological replicates (n = 3). The asterisks indicate significant difference compares to the control (*$p < 0.05$, **$p < 0.01$).

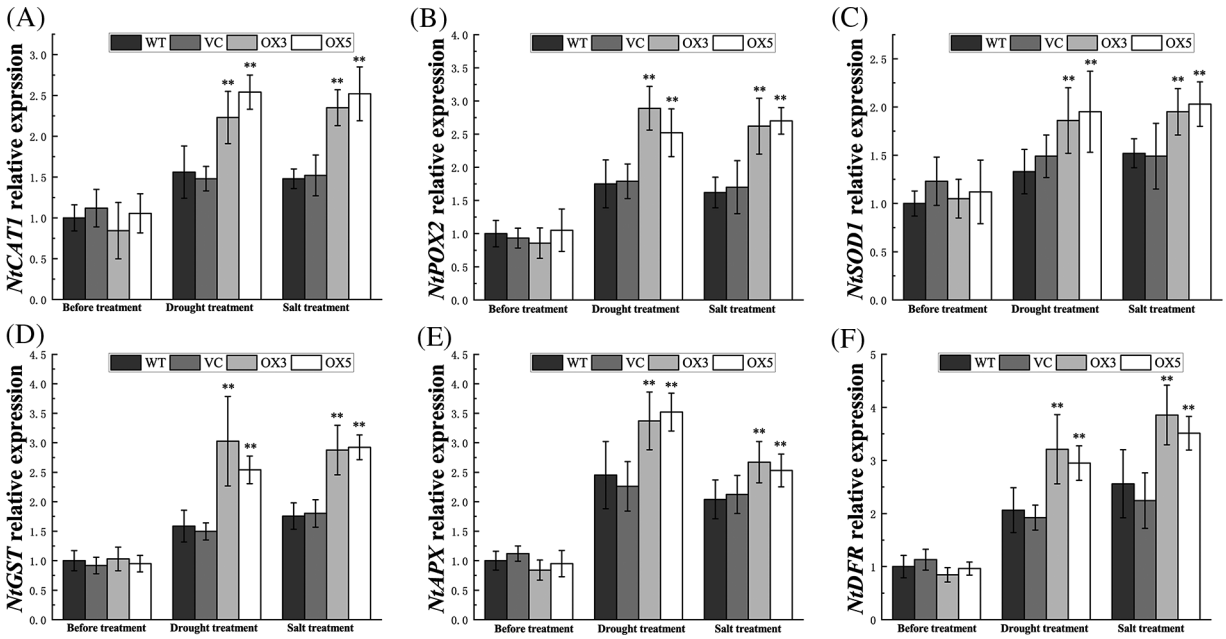

FIGURE S3. Expression of ROS-related genes. Expression of (A) *NtCAT1*, (B) *NtPOX2*, (C) *NtSOD1*, (D) *NtGST*, (E) *NtAPX*, and (F) *NtDFR* in transgenic tobacco plants under drought or salt treatment. The bars exhibit the mean ± SE of three biological replicates (n = 3). The asterisks indicate significant difference compares to the control (*$p < 0.05$, **$p < 0.01$).

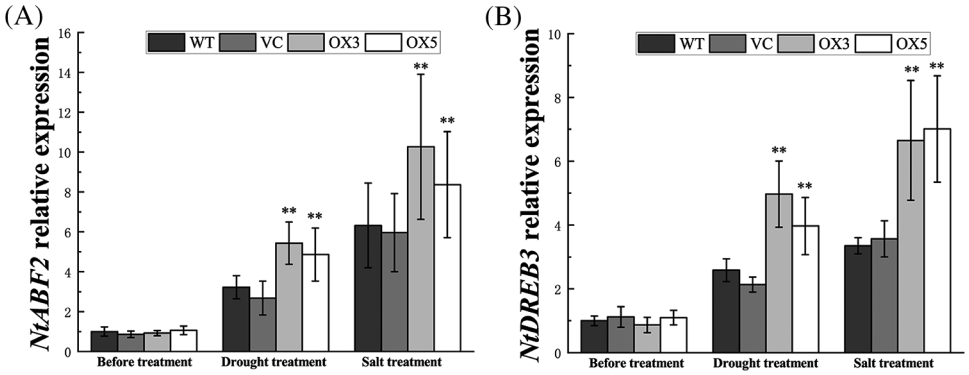

FIGURE S4. Expression of stress-related transcription factors. Expression of (A) *NtABF2* and (B) *NtDREB3* in transgenic tobacco plants under drought or salt treatment. The bars exhibit the mean ± SE of three biological replicates (n = 3). The asterisks indicate significant difference compares to the control (*$p < 0.05$, **$p < 0.01$).