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CaWRKY6-CaERF3 regulate ROS homeostasis to positively modulate salt stress in pepper (*Capsicum annuum* L.)

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Key message

CaWRKY6 functions together with *CaERF3* to regulate ROS production, thus positively modulating pepper salt stress response.

Abstract

Salt stress significantly inhibits the growth and productivity of plants. Pepper (*Capsicum annuum* L.), a widely cultivated economic horticultural crop, exhibits high sensitivity to salinity. This study investigates the role of the transcription factor *CaWRKY6* in modulating pepper's responses to salt stress. Our findings indicate that CaWRKY6 predominantly localizes at the cell nucleus, and its expression is significantly induced upon salt treatment. The *CaWRKY6*-silenced pepper plants exhibit heightened sensitivity to salt stress, as evidenced by increased malondialdehyde (MDA) levels and decreased proline content. Additionally, *CaWRKY6*-silenced pepper plants show elevated levels of reactive oxygen species (ROS). Through a yeast two-hybrid screen, *CaERF3* is identified as an interactor of *CaWRKY6*. Silencing *CaERF3* expression induces a similar sensitive salt response and elevated ROS levels as observed in *CaWRKY6*-silenced plants. Collectively, our results demonstrate that the CaWRKY6-CaERF3 module positively regulates salt stress responses in pepper by modulating ROS homeostasis.

Keywords: *Capsicum annuum* L., salt stress, *CaWRKY6*, *CaERF3*, ROS

1. Introduction

Soil salinization represents a critical environmental issue that suppresses plant growth and development, consequently impairing global agricultural productivity and food supplement (Tibesigwa et al., 2025; Zhang et al., 2025). Pepper (*Capsicum annuum* L.) exhibits high sensitivity to salinity, and elevated salt concentrations can markedly inhibit germination rates, delay fruit ripening, decrease the number of fruits reduce fruit size, and reduce fruit size, weight, and nutrient content (Taffouo et al., 2017; Kpinkoun et al., 2019; Zhang et al., 2024). Extensive research has been undertaken to identify the key factors involved in the salt stress response in pepper (Zhang et al., 2024). Nevertheless, the precise mechanisms remain inadequately understood.

Transcription factors (TFs) are proteins that modulate transcriptional activity by binding to specific DNA sequences within the DNA-binding domain or interacting with the regulatory elements of target genes, thereby either enhancing or repressing transcription (Weidemüller et al., 2021; Strader et al., 2022). The WRKY family of transcription factors is characterized by a conserved WRKYGQK motif at the N-terminus, and they play essential roles in salinity responses (Li et al., 2020; Ma and Hu, 2024; Yang et al., 2025). In the pepper genome, 61 *CaWRKY* genes have been identified (Cheng et al., 2016a; Zheng et al., 2019), and their expression levels are influenced by abiotic stress factors such as high temperature, salinity, and drought (Cheng et al., 2016b), indicating the involvement of *CaWRKY*s in stress response pathways. Among these *CaWRKY*s, *CaWRKY27* has been shown to regulate the expression level of genes associated with ROS, ABA biosynthesis, and stress responses, thereby fine-tuning the salt stress response in peppers (Lin et al., 2019). Nonetheless, the biological functions of *CaWRKY*s in mediating salt stress responses in pepper remain largely unexplored.

The APETALA2/ETHYLENE-RESPONSIVE FACTORS (AP2/ERF) superfamily represents one of the most expansive TF families that is unique to the plant kingdom. This family is crucial in regulating a myriad of biological processes, including growth, development, secondary metabolism, and importantly, the response to both biotic and abiotic stresses (Wu et al., 2022; Tang et al., 2024). The pepper genome harbors over 100 *CaERF* genes (Jin et al., 2018; Song et al., 2020). Under salinity stress, *CaERF2*-silenced pepper plants exhibited reduced activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), alongside

decreased transcriptional levels of genes associated with reactive oxygen species (ROS) scavenging, resulting in elevated H_2O_2 accumulation and heightened salt sensitivity (Zhao et al., 2025). However, the precise mechanisms of salt stress responses mediated by *CaERF*s remain largely unknown.

In this study, we aim to elucidate the role of the CaWRKY6-CaERF3 regulatory module in the response of pepper to salt stress. Our qRT-PCR analyses reveal a significant induction of *CaWRKY6* and *CaERF3* transcription levels following salt exposure. Silencing of either *CaWRKY6* or *CaERF3* in pepper plants results in heightened salt sensitivity compared to control plants, as indicated by increased malondialdehyde (MDA) content and decreased proline levels. Additionally, we demonstrate that *CaERF3* physically interacts with and acts downstream of *CaWRKY6*. Moreover, both *CaERF3* and *CaWRKY6* modulate the activities of SOD and CAT enzymes, leading to increased ROS levels. Collectively, our findings elucidate the physiological role of the CaWRKY6-CaERF3 module in mediating pepper's response to salt stress, offering novel insights for genetic breeding strategies aimed at developing salt-tolerant pepper cultivars.

2. Materials and methods

2.1 Plant materials and growth conditions

Zunla-1 seeds were sterilized with 0.1% potassium permanganate solution for 15-20 minutes, then the seeds were washed with ddH_2O for 3-4 times. The sterilized seeds were incubated at 30℃ to induce germination, then the seedlings were transplanted to soil ad were cultured in the growth room under long-day conditions (16 h light/8 h dark), the light intensity was 110 μmol photons m^{-2} s^{-1} white light.

2.2 Bioinformatics analysis

The multiple sequence alignment of CaWRKY6 and CaERF3 proteins alongside various ERF and WRKY proteins was executed utilizing the MEGA and GENEDoc software. Phylogenetic trees were conducted via MEGA software (version 12), employing the neighbor-joining (NJ) method with 1000 bootstrap iterations (Kumar et al., 2024). A 2000 bp promoter sequence was used to identify *cis*-regulatory elements using the PlantCARE online resource (Lescot et al., 2002). The structural analysis of the CaWRKY6 protein was performed using the SWISS-MODEL tool (SWI https://swissmodel.expasy.org/ SS-MODEL).

2.3 Subcellular localization of CaWRKY6

The full-length *CaWRKY6* cDNA lacking the stop codon was amplified via PCR utilizing specific primers (Table S1) and subsequently cloned into the pC2300S vector. Both pC2300S:CaWRKY6-GFP and the empty pC2300S-GFP vector were co-transfected with a nuclear localization marker into *Nicotiana tabacum* leaves, followed by an 8-hour incubation under low-light conditions. GFP fluorescence was then visualized using laser scanning confocal microscopy (OLYMPUS, Japan).

2.4 Virus-induced gene silencing (VIGS) and plant transformation

The generation of VIGS vectors and subsequent plant transformation was followed to established method (Sun et al., 2025). Specifically, a targeted 300 bp fragment of either *CaWRKY6* or *CaERF3* was cloned into the TRV2 vector, yielding TRV2:CaWRKY6 and TRV2:CaERF3 constructs. Pepper cotyledons were infiltrated with Agrobacterium tumefaciens strain GV3101 harboring TRV1, TRV2:00, TRV2:PDS, TRV2:CaWRKY6, or TRV2:CaERF3. Around 30 days post-inoculation, photobleaching was evident in the TRV2:PDS-inoculated pepper plants. Real-time quantitative PCR (qRT-PCR) analysis was carried out to verify the expression levels of *CaWRKY6* and *CaERF3* in the TRV2:*CaWRKY6* and TRV2:*CaERF3* transgenic pepper plants, respectively, with gene silencing efficiency surpassing 50%. Primer sequences were listed in Table S1.

2.5 RNA extraction and qRT-PCR assay

Pepper samples were collected and immediately ground in liquid nitrogen. Total RNA was isolated by the TRIzol reagent kit (GenSter, Beijing, China) following the manufacturer's protocol. Subsequently, 1 μg of RNA was utilized for cDNA synthesis via the 5 × HiScript III qRT SuperMix kit (GenSter, Beijing, China). qRT-PCR assays were conducted with the 2 × ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) on the ABI 7500 Fast Real-Time PCR System (Thermo Fisher). The *CaACTIN* gene was used an internal reference, and relative transcript levels were quantified using the $2^{-\Delta\Delta CT}$ method. For each biological replicate, the relative transcription level was determined as the average of three technical replicates. All qPCR primers used in this study were listed in Table S1.

2.6 DAB and NBT staining

The DAB (Coolaber, Beijing, China) and NBT (Coolaber, Beijing, China) kits were purchased from Macklin company and the staining was performed following the

manufacturer's instructions. Briefly, 28 days old pepper plants were subjected to 300 mM NaCl for 8 hours, then the leaves with a similar size were collected for DAB and NBT staining. The images were captured with *Nikon* camera.

2.7 MDA and Proline measurement

The MDA quantification kit (COMIN, Suzhou, China) and Proline assay kit (COMIN, Suzhou, China) were used to determine MDA and Proline levels, respectively. In brief, 28-day-old pepper plants were exposed to a 300 mM NaCl solution for 8 hours. Subsequently, the leaves were harvested and subjected to the MDA and Proline assay following the manufacturer's instructions.

2.8 Transcriptional activity assay

The full-length cDNA sequence of *CaWRKY6* was subcloned into the pGBKT7 vector and subsequently introduced into the Y2H Gold yeast strain. A single colony was selected and cultured on SD/-Trp and SD/-Trp/X-α-Gal medium at 30°C for 2-3 days. Yeast growth was then captured using a *Nikon* camera.

2.9 SOD and CAT enzyme activity assay

The SOD quantification kit (COMIN, Suzhou, China) and CAT content assay kit (COMIN, Suzhou, China) were used to determine SOD and CAT levels, respectively. In brief, 30-day-old pepper plants were exposed to a 300 mM NaCl solution for 8 hours. Subsequently, the leaves were harvested and subjected to the SOD and CAT assay following the manufacturer's instructions.

2.10 Plant transformation, phenotyping

The full cDNA length of *CaWRKY6* was amplified without a stop codon and inserted into the p3301 vector to produce 35S::CaWRKY6 construct. The 35S::CaWRKY6 construct was transformed into *Agrobacterium tumefaciens* strain GV3101 and then used to transform *Arabidopsis thaliana* (Col-0) by the floral-dip method. The homozygous lines were screened on 1/2 MS medium with 50 mg/L kanamycin. The expression level of *CaWRKY6* was confirmed by qRT-PCR. *2.11 Bimolecular fluorescence complementation (BiFC) assay*

The BiFC assay was conducted to examine *in vivo* interaction between CaWRKY6 and CaERF3 proteins. The full-length cDNA of *CaWRK6* and *CaERF3* were fused p1300S-

YNE and p2300S-YCE vector, generating the base constructs CaWRKY6-nYFP and CaERF3-cYFP constructs, respectively. All constructs were confirmed by sequencing before transformation into *Agrobacterium tumefaciens* GV3101. For transient expression, bacterial suspensions were co-infiltrated into *Nicotiana benthamiana* leaves using. Infiltrated leaves were incubated in darkness at 22 °C for 48 h. Fluorescence signals were imaged using a STELLARIS 8, Leica confocal laser scanning microscope. YFP excitation was performed at 587 nm, and emission was collected between 590 and 610 nm.

2.12 Statistical analysis

All statistical analysis was performed using One-way ANOVA test via GraphPad Prism 10 with a significant difference (* $P < 0.05$, ** $P < 0.01$).

3. Results

3.1 Expression pattern and subcellular localization of CaWRKY6

The cDNA sequence of *CaWRKY6* was 648 bp and it encoded a protein comprising 215 amino acids. Protein sequence alignment indicated that *CaWRKY6* harbors a distinctive WRKY DNA-binding domain and shares the highest sequence homology with *Solanum dulcamara* WRKY70 (SdWRKY70) (Figure S1). The CaWRKY6 protein was featured a hydrophobic region, but without a signal peptide. Secondary structure analysis of CaWRKY6 protein consisted of β-sheet region (2.79%), α-helix region (24.65%), random coil region (60.93%). The tertiary structure of CaWRKY6 was determined with a precision of 90%. (Figure S2). Moreover, *cis*-regulatory elements responsive to Abscisic acid (ABA), jasmonic acid (JA), auxin were identified within the 3000 bp upstream region of the *CaWRKY6* promoter (Figure S3), indicating its potential involvement in plant hormone signaling and stress responses.

Subsequently, we analyzed the expression pattern of *CaWRKY6*. The expression levels of *CaWRKY6* were relatively higher in flowers, fruits, and stems, while lower levels were observed in roots and leaves (Figure 1A). Additionally, the transcriptional levels of *CaWRKY6* were significantly elevated at 2h, 4h, and 8h following salt treatment, but were downregulated after 12h and 24h of treatment (Figure 1B). These results collectively suggest that *CaWRKY6* is involved in the plant's response to salinity stress.

We also investigated the transcriptional activity of *CaWRKY6*. Our findings

demonstrated that yeast cells transformed with *CaWRKY6* were able to grow in SD/-Trp medium. Moreover, these *CaWRKY6* transformed yeast cells exhibited a blue coloration in SD/-Trp/X-α-Gal medium, indicating that *CaWRKY6* possesses transcriptional activity (Figure 1C). We then cloned the full-length cDNA of *CaWRKY6* into the pC2300S vector to assess its subcellular localization. Confocal microscopic observations showed that tobacco leaf infiltrated with pC2300S:CaERF2-GFP fusion proteins were localized to the nucleus and cytoplasm (Figure 1D).

3.2 CaWRKY6-silenced pepper plants are sensitive to salt stress

To further elucidate the biological function of *CaWRKY6* in the response to salt stress, we generated *CaWRKY6*-silenced (TRV2:*CaWRKY6*) pepper plants using the virus-induced gene silencing (VIGS) technique. A 300 bp fragment of *CaWRKY6* was designed to suppress *CaWRKY6* expression (Figure S4). The mRNA levels of *CaWRKY6* in the silenced plants were markedly reduced compared to the control plants (Figure S5). Under normal growth conditions, there were no observable differences in growth between the control (TRV2:*00*) and TRV2:*CaWRKY6* pepper plants (Figure S6). However, when subjected to salt stress for 8 hours, the leaves of TRV2:*CaWRKY6* pepper plants exhibited more wilting compared to the control plants (Figure 2A). Plants with increased sensitivity to salt stress typically show elevated levels of MDA and reduced proline content (Lin et al., 2019; Yuce et al., 2025). Consistently, we detected higher MDA levels and lower proline content in the leaves of salt-treated TRV2:*CaWRKY6* plants (Figure 2B, C). To further examine the role of *CaWRKY6* in salt stress response, we then cloned the full-length cDNA of *CaWRKY6* into pYES2 plasmid, and transformed into yeast. As shown, yeast did not growth under salt stress conditions, while *CaWRKY6*-transfomed yeast can still grow (Figure S7). Therefore, *CaWRKY6* functions as a positive regulator in the pepper plant's response to salt stress.

To further substantiate the role of *CaWRKY*6 under saline stress, we overexpressed of *CaWRKY6* in Arabidopsis. Two independent transgenic lines with elevated expression level of *CaWRKY6* were chosen for subsequent analysis (Figure S8). Previous study has demonstrated that salt stress significantly hampers seed germination (Liang and Jiang, 2024). When the seeds of WT and *35S::CaWRKY6* subjected to 150 mM NaCl for 7 days, *35S::CaWRKY6* seeds exhibited a markedly higher survival rate (Figure 3A, B). We next transferred 3-day-old WT and *35S::CaWRKY6* seedlings to new plates containing 150 mM NaCl for 5 days. The growth of the WT primary root

was severely inhibited by salinity, while the *CaWRKY6* overexpression lines displayed notable tolerance to salt stress (Figure 3C, D). Collectively, these results indicate that *CaWRKY6* acts as a positive regulator in the pepper response to salt stress.

3.3 CaWRKY6 regulates ROS homeostasis

ROS are crucial mediators in the plant response to salt stress (Ahmad et al., 2024; Jiang et al., 2025). Consequently, we measured ROS levels in TRV2:*00* and TRV2:*CaWRKY6* pepper plants. In the absence of salt stress, there was no obvious difference in the ROS level between TRV2:*00* and TRV2:*CaWRKY6* pepper plants, as demonstrated by DAB and NBT staining assays. However, after 8 hours of salt exposure, ROS levels in TRV2:*CaWRKY6* leaves were significantly higher than the TRV2:*00* pepper plants (Figure 4A). Superoxide dismutase (SOD) and catalase (CAT) are pivotal enzymes involved in the production and decomposition of ROS, respectively (Bose et al., 2014; Sofo et al., 2015; Hasanuzzaman et al., 2020; Mittler et al., 2022). Therefore, we quantified the activities of SOD and CAT enzymes. Under saline conditions, TRV2:*CaWRKY6* pepper plants displayed increased SOD activity and decreased CAT activity, resulting in elevated ROS accumulation (Figure 4B, C). These findings suggest that *CaWRKY6* modulates ROS homeostasis in response to salinity.

3.4 CaERF3 interacts with CaWRKY6

We next performed yeast two-hybrid screen to identify the potential interactors of CaWRKY6. We cloned the full length of CaWRKY6 cDNA into the pGBKT7 vector and screened the pepper cDNA library (Figure 5A). To this end, we identified 9 top potential interactors (Table 1). We further confirmed the interaction between CaWRKY6 and CaERF3 by yeast two-hybrid and BiFC assay (Figure 5B - C). As *CaERF2* has been shown to regulate salt stress (Zhao et al., 2025), we hence focused on *CaERF3*. *CaERF3* cDNA length was 708 bp and encoded a protein with 235 amino acid residues. Protein alignment showed that CaERF3 contained a typical AP2/ERF domain (Figure S9).

3.5 CaERF3 is involved in salt stress response

To elucidate the physiological function of *CaERF3* in the salt stress response of pepper, we initially assessed the expression profile of *CaERF3*. Our qRT-PCR results demonstrated the differential expression levels of *CaERF3* in the leaves, stem, roots, and fruit, with the highest expression in leaves (Figure 6A). Moreover, *CaERF3*

expression was significantly upregulated upon 8h, 12h, and 24h of salt stress treatment (Figure 6B), indicating the involvement of *CaERF3* in the salt stress response.

Subsequently, we generated *CaERF3*-silenced pepper plants (TRV2:*CaERF3*) to analyze their response to salt stress. The mRNA levels of *CaERF3* in these plants were lower than in control plants (Figure S10), and no noticeable growth defects were observed when comparing TRV2:*00* and TRV2:*CaERF3* pepper plants (Figure S11). After 8 hours of salt treatment, the leaves of TRV2:*CaERF3*-silenced plants showed increased wilting compared to control pepper plants (Figure 6C). Correspondingly, higher MDA content and lower proline levels were detected in leaves of TRV2:*CaERF3* pepper plants under salinity conditions (Figure 6D, E).

We also examined the role of *CaERF3* in yeast under salt conditions. As shown, yeast did not growth under salt stress conditions, while *CaERF3*-transfomed yeast can still grow (Figure S12). Additionally, the transcriptional level of *CaERF3* was downregulated in the TRV2:*CaWRKY6* pepper plants(Figure S13). These data collectively suggest that CaERF3 interacts with CaWRKY6 and act down stream of *CaWRKY6* to regulate salt stress response.

As *CaERF3* functioned downstream of *CaWRKY6* (Figure S13), and *CaWRKY6* modulated ROS production (Figure 4). We hence investigated whether *CaERF3* also influenced ROS signaling under salt stress conditions. Under salinity conditions, DAB and NBT staining revealed higher ROS accumulation in the leaves of TRV2:*CaERF3* pepper plants compared to control pepper plants (Figure 7A). Furthermore, increased SOD activity and decreased CAT activity were detected in the leaves of TRV2:*CaERF3* plants under saline conditions (Figure 7B, C). Collectively, our findings indicate that *CaERF3* is involved in the salt stress response by modulating ROS production.

4. Discussion

In this study, we uncover the physiological roles of *CaWRKY6* and its interactor, *CaERF3*, in pepper salt stress responses. Our findings illustrate the transcriptional level of *CaWRKY6* and *CaERF3* is induced by salinity. Furthermore, *CaWRKY6* and *CaERF3* regulate ROS homeostasis to positively modulate salt stress responses.

The acquisition of Na^+ is mediated by an array of channels and transporters, encompassing nonselective cation channels (NSCCs) such as cyclic nucleotide-gated channels (Balasubramaniam et al., 2023), high-affinity K^+ transporters (HKT) (Riedelsberger et al., 2021), Na^+/H^+ exchangers (NHX) (Shabala et al., 2025), voltage-

gated channels (e.g., inward rectifying K+ channels and outward rectifying channels), and voltage-independent channels (Yang and Guo, 2018). The SALT OVERLY SENSITIVE 1 (SOS1) protein functions as a plasma membrane Na^+/H^+ antiporter that exports excess Na^+ from plant cells to maintain ionic balance and prevent Na^+ toxicity (Ma et al., 2022; Tibesigwa et al., 2025). Additionally, plants have developed sophisticated salt stress-responsive signaling pathways such as Ca^{2+} signaling, MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) signaling, epigenetic modifications, and posttranslational adjustments to cope with salt stress (Wu, 2018; Isayenkov et al., 2019; Wang et al., 2022; Tibesigwa et al., 2025). Our research elucidates the involvement of the CaWRKY6-CaERF3 module in the salt stress response (Figure 2; Figure 6). However, the precise mechanisms by which the CaWRKY6-CaERF3 module regulates Na^+ uptake, transport, export and salt stress-responsive via these well-known signaling pathways remain to be explored.

The WRKY domain (WD) encompasses a highly conserved WRKYGQK heptad motif, followed by various zinc finger structures that impart potential DNA-binding capabilities. WRKY TFs typically associate with W-box *cis*-elements (TTGACY, with TGAC as the core sequence) (Chen et al., 2025). Our binding assay demonstrates that CaWRKY6 interacts with CaERF3 and modulates *CaERF3* expression (Figure 5, Figure S13). The unresolved question is whether CaWRKY6 binds to the W-box in the promoter region of *CaERF3*, thereby influencing the transcriptional activity of *CaERF3*.

ROS are pivotal mediators in plant responses to salt stress (Ahmad et al., 2024; Jiang et al., 2025). Key enzymes such as PEROXIDASEs (PRXs), RESPIRATORY BURST OXIDASE HOMOLOG (RBOH), and ASCORBATE PEROXIDASEs (APXs) are integral to ROS production (Mittler et al., 2022; Lopez et al., 2024). The sensor HYDROGEN PEROXIDE-INDUCED Ca^{2+} INCREASES 1 (HPCA1) specifically detects H_2O_2 in plants (Wu et al., 2020). Various ROS and redox-regulating proteins, including Glutaredoxins (GRXs), Thioredoxins (TRXs), Peroxiredoxins (PrxRs), and Glutathione Peroxidases (GPXs), along with Glutathione (GSH), modulate ROS signals in the nucleus (Mittler et al., 2022). Additionally, ROS transport across cellular compartments is facilitated by Aquaporins (AQPs) (Rodrigues et al., 2017). Our finding indicates that the CaWRKY6-CaERF3 module orchestrates ROS production by modulating SOD activity and CAT activity (Figure 4 and Figure 7). Further studies are necessary to elucidate how the CaWRKY6-CaERF3 module regulates ROS production, sensing, transport, and signaling via these well-known ROS regulators under salt stress

in pepper.

Phytohormones are pivotal in modulating salt stress responses (Waadt et al., 2022; Aizaz et al., 2024; Joshi et al., 2025). Exogenous application of abscisic acid (ABA) facilitates the transcriptional activation of *CaWRKY27*, while *CaWRKY27* overexpression markedly induces the expression of ABA biosynthesis genes, such as *NCED1* and *DREB3*. This indicates that *CaWRKY27* plays a role in the ABA-mediated salt stress response in pepper (Lin et al., 2019). It is plausible that *CaWRKY6* might also participate in ABA signaling as ABRE (abscisic acid responsiveness) *cis*-regulatory was identified in the promoter region of *CaWRKY6* (Figure S3). Additionally, *cis*-regulatory elements responsive JA, and auxin are also identified, implying that *CaWRKY6* could integrate multiple phytohormonal pathways to modulate pepper's salt stress response (Waadt et al., 2022). Nevertheless, the precise mechanism by which *CaWRKY6* orchestrates these hormonal pathways under salt stress conditions necessitates further investigation.

5. Conclusion

In this study, we elucidate the involvement of *CaWRKY6* and its interactor, *CaERF3*, in the salt stress response of pepper plants. Salt treatment resulted in the induction of *CaWRKY6* and *CaERF3* expression. *CaWRKY6*- and *CaERF3*-silenced pepper plants exhibit heightened sensitivity to salinity, as demonstrated by increased levels of MDA and reduced proline content. Moreover, we demonstrate that *CaWRKY6* and *CaERF3* play a crucial role in regulating ROS homeostasis. Consequently, our findings provide valuable insights into the transcription factor-mediated mechanisms underlying salt stress response in pepper plants.

CRediT author statement

Zhimin Li: Funding acquisition, Writing- Reviewing and Editing. **Huibin Han**: Formal analysis, Writing-Reviewing and Editing. **Zhen An, Hui Li, Liping Qiu**: Methodology, Investigation, Formal analysis. **Qinghong Zhou**, **Jianping Liu**: Funding acquisition, Supervision, Conceptualization, Formal analysis, Writing-Reviewing and Editing.

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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.

References

Ahmad, B., Mukarram, M., Choudhary, S., Petrík, P., Dar, T.A., Khan, M.M.A., 2024. Adaptive responses of nitric oxide (NO) and its intricate dialogue with phytohormones during salinity stress. Plant Physiol. Biochem. 208, 108504.

Aizaz, M., Lubna., Jan, R., Asaf, S., Bilal, S., Kim, K.M., Al-Harrasi, A., 2024. Regulatory dynamics of plant hormones and transcription factors under salt stress. Biology 13, 673.

Bose, J., Rodrigo-Moreno, A., Shabala, S., 2014. ROS homeostasis in halophytes in the context of salinity stress tolerance. J. Exp. Bot. 65, 1241–1257.

Balasubramaniam, T., Shen, G., Esmaeili, N., Zhang, H., 2023. Plants' response mechanisms to salinity stress. Plants 12: 2253

Cheng, Y., Yao, Z.P., Ruan, M.Y., Ye, Q.J., Wang, R.Q., Zhou, G.Z., Luo, J., 2016a. In silico identification and characterization of the WRKY gene superfamily in pepper (*Capsicum annuum* L.). Genet. Mol. Res. 15, 3.

Cheng, Y., Ahammed, G.J., Yu, J., Yao, Z., Ruan, M., Ye, Q., Li, Z., Wang, R., Feng, K., Zhou, G., Yang, Y., Diao, W., Wan, H., 2016b. Putative WRKYs associated with regulation of fruit ripening revealed by detailed expression analysis of the *WRKY* gene family in pepper. Sci. Rep. 6, 39000.

Chen, X., Zhang, T., Wang, H., Zhao, W., Guo, Z., 2025. Transcription factor WRKY complexes in plant signaling pathways. *Phytopathol. Res.* 7, 54.

Hasanuzzaman, M., Bhuyan, M.H.M.B., Parvin, K., Bhuiyan, T.F., Anee, T.I., Nahar, K., Hossen, M.S., Zulfiqar, F., Alam, M.M., Fujita, M., 2020. Regulation of ROS metabolism in plants under environmental stress: A review of recent experimental evidence. Int. J. Mol. Sci. 21, 8695.

Isayenkov, S.V., Maathuis, F.J.M., 2019. Plant salinity stress: many unanswered questions remain. Front. Plant Sci. 10, 80.

Jin, J.H., Wang, M., Zhang, H.X., Khan, A., Wei, A.M., Luo, D.X., Gong, Z.H., 2018.

Genome-wide identification of the AP2/ERF transcription factor family in pepper (*Capsicum annuum* L.). Genome 61, 663-674.

Jiang, L., Xiao, M., Huang, R., Wang, J., 2025. The regulation of ROS and phytohormones in balancing crop yield and salt tolerance. Antioxidants 14, 63.

Joshi, P.S., Singla Pareek, S.L., Pareek, A., 2025. Shaping resilience: The critical role of plant response regulators in salinity stress. Biochim. Biophys. Acta. Gen. Subj. 1869, 130749.

Kpinkoun, J.K., Amoussa, A.M., Mensah, A.C.G., Komlan, F.A., Kinsou, E., Lagnika, L., Gandonou, C.B., 2019. Effect of salt stress on flowering, fructification and fruit nutrients concentration in a local cultivar of chili pepper (*Capsicum frutescens* L.). Int. J. Plant Physiol. Biochem. 11, 1–7.

Kumar, S., Stecher, G., Suleski, M., Sanderford, M., Sharma S, Tamura, K., 2024. MEGA12: molecular evolutionary genetic analysis version 12 for adaptive and green computing. Mol. Biol. Evol. 41, msae263.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P., Rombauts, S., 2002. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res. 30, 325-327.

Lin, J., Dang, F., Chen, Y., Guan, D., He, S., 2019. CaWRKY27 negatively regulates salt and osmotic stress responses in pepper. Plant Physiol. Biochem. 145, 43-51.

Li, W., Pang, S., Lu, Z., Jin, B., 2020. Function and mechanism of WRKY transcription factors in abiotic stress responses of plants. Plants 9, 1515.

Lopez, L.E., Ibeas, M.A., Diaz Dominguez, G., Estevez, J.M., 2024. Exploring the puzzle of reactive oxygen species acting on root hair cells. J. Exp. Bot. 75, 4589-4598.

Mittler, R., Zandalinas, S.I., Fichman, Y., Van Breusegem, F., 2022. Reactive oxygen species signalling in plant stress responses. Nat. Rev. Mol. Cell Biol. 23, 663-679.

Liang, X., Jiang, C. 2024. Coping with salt stress. Elife 13, e101732.

Ma, L., Liu, X., Lv, W., Yang, Y., 2022. Molecular Mechanisms of Plant Responses to Salt Stress. Front. Plant Sci. 13, 934877.

Ma, Z., Hu, L., 2024. WRKY transcription factor responses and tolerance to abiotic stresses in plants. Int. J. Mol. Sci. 25, 6845.

Rodrigues, O., Reshetnyak, G., Grondin, A., Saijo, Y., Leonhardt, N., Maurel, C., Verdoucq, L., 2017. Aquaporins facilitate hydrogen peroxide entry into guard cells to mediate ABA- and pathogen-triggered stomatal closure. Proc. Natl. Acad. Sci. USA 114,

9200-9205.
Riedelsberger, J., Miller, J.K., Valdebenito-Maturana, B., Piñeros, M.A., González, W., Dreyer, I., 2021. Plant HKT Channels: An Updated View on Structure, Function and Gene Regulation. Int. J. Mol. Sci. 22, 1892.
Sofo, A., Scopa, A., Nuzzaci, M., Vitti, A., 2015. Ascorbate peroxidase and catalase activities and their genetic regulation in plants subjected to drought and salinity stresses. Int. J. Mol. Sci. 16, 13561–13578.
Song, J., Chen, C., Zhang, S., Wang, J., Huang, Z., Chen, M., Cao, B., Zhu, Z., Lei, J., 2020. Systematic analysis of the Capsicum ERF transcription factor family: identification of regulatory factors involved in the regulation of species-specific metabolites. BMC Genomics 21, 573.
Strader, L., Weijers, D., Wagner, D., 2022. Plant transcription factors - being in the right place with the right company. Curr. Opin. Plant Biol. 65, 102136.
Sun, F., Chen, Y., Luo, Y., Yang, F., Yu, T., Han, H., Yang, Y., Zhou, Y., 2025. Cryptochromes (CRYs) in pepper: Genome-wide identification, evolution and functional analysis of the negative role of CaCRY1 under *Phytophthora capsici* infection. Plant Sci. 355, 112460.
Shabala, S., Chen, X., Yun, P., Zhou, M., 2025. Salinity tolerance in wheat: rethinking the targets. J. Exp. Bot. eraf152. doi: 10.1093/jxb/eraf152.
Wu, H., 2018. Plant salt tolerance and Na^+ sensing and transport. The Crop Journal 6, 3, 215-225.
Wu, F., Chi, Y., Jiang, Z., Xu, Y., Xie, L., Huang, F., Wan, D., Ni, J., Yuan, F., Wu, X., et al., 2020. Hydrogen peroxide sensor HPCA1 is an LRR receptor kinase in Arabidopsis. Nature 578, 577–581.
Weidemüller, P., Kholmatov, M., Petsalaki, E., Zaugg, J.B., 2021. Transcription factors: Bridge between cell signaling and gene regulation. Proteomics 21, e2000034.
Waadt, R., Seller, C.A., Hsu, P.K., Takahashi, Y., Munemasa, S., Schroeder, J.I., 2022. Plant hormone regulation of abiotic stress responses. Nat. Rev. Mol. Cell Biol. 23, 680-694.
Taffouo, V.D., Nouck, A.E., Nyemene, K.P., Tonfack, B., Meguekam, T.L., Youmbi, E., 2017. Effects of salt stress on plant growth, nutrient partitioning, chlorophyll content, leaf relative water content, accumulation of osmolytes and antioxidant compounds in pepper (*Capsicum annuum* L.) cultivars. Not. Bot. Horti Agrobot. Cluj-Napoca 45, 481–490.

Tang, Q., Wei, S., Zheng, X., Tu, P., Tao, F., 2024. APETALA2/ethylene-responsive factors in higher plant and their roles in regulation of plant stress response. Crit. Rev. Biotechnol. 44, 1533-1551.

Tibesigwa, D.G., Zhuang, W., Matola, S.H., Zhao, H., Li, W., Yang, L., Ren, J., Liu, Q., Yang, J., 2025. Molecular insights into salt stress adaptation in plants. Plant Cell Environ. doi: 10.1111/pce.15544.

Wu, Y., Li, X., Zhang, J., Zhao, H., Tan, S., Xu, W., Pan, J., Yang, F., Pi, E., 2022. ERF subfamily transcription factors and their function in plant responses to abiotic stresses. Front. Plant Sci. 13, 1042084.

Wang, C.F., Han, G.L., Yang, Z.R., Li, Y.X., Wang, B.S., 2022. Plant Salinity Sensors: Current Understanding and Future Directions. Front. Plant Sci. 13, 859224.

Yang, Y., Guo, Y., 2018. Elucidating the molecular mechanisms mediating plant salt-stress responses. New Phytol. 217, 523-539.

Yuce, M., Aydin, M., Turan, M., Ilhan, E., Ekinci, M., Agar, G., Yildirim, E., 2025. Ameliorative effects of SL on tolerance to salt stress on pepper (*Capsicum annuum* L.) plants. Plant Physiol. Biochem. 223, 109798.

Yang, L., Fang, S., Liu, L., Zhao, L., Chen, W., Li, X., Xu, Z., Chen, S., Wang, H., Yu, D., 2025. WRKY transcription factors: Hubs for regulating plant growth and stress responses. J. Integr. Plant Biol. 67, 488-509.

Zheng, J., Liu, F., Zhu, C., Li, X., Dai, X., Yang, B., Zou, X., Ma, Y., 2019. Identification, expression, alternative splicing and functional analysis of pepper WRKY gene family in response to biotic and abiotic stresses. PLoS One 14, e0219775.

Zhang, L., Wu, D., Zhang, W., Shu, H., Sun, P., Huang, C., Deng, Q., Wang Z, Cheng, S., 2023. Genome-wide identification of WRKY gene family and functional characterization of *CcWRKY25* in *Capsicum chinense*. Int. J. Mol. Sci. 24, 11389.

Zhang, X., Ma, X., Wang, S., Liu, S., Shi, S., 2024. Physiological and genetic aspects of resistance to abiotic stresses in *Capsicum* Species. Plants 13, 3013.

Zhao, J., Huang, M., Liu, J., Cai, J., He, Y., Zhao, W., Liu, C., Wu, Y., 2025. Pepper (*Capsicum annuum* L.) AP2/ERF transcription factor, *CaERF2* enhances salt stress tolerance through ROS scavenging. Theor. Appl. Genet. 138, 44.

Zhang, H., Yu, C., Zhang, Q., Qiu, Z., Zhang, X., Hou, Y., Zang, J., 2025. Salinity survival: molecular mechanisms and adaptive strategies in plants. Front. Plant Sci. 16, 1527952.

491

492 **Figure legends**

493 **Fig. 1. *CaWTKY6* expression pattern and subcellular localization.** (A) Expression
494 level of *CaWRKY6* in pepper stem, root, leaf, flower and fruit. (B) Expression level of
495 *CaWRKY6* in leaves under salt stress. (C) Transcriptional activity of CaWRKY6 in
496 yeast. (D) Subcellular localization of CaWRKY6. Scale bar = 50 μm. Data were mean
497 ± SD, and the statistically significant differences were determined by one-way ANOVA,
498 ** $P < 0.01$, *** $P < 0.001$.

499

500 **Fig. 2. *CaWRKY6*-silenced pepper plants are hypersensitive to salt stress.** (A)
501 Phenotype of TRV2:*00* and TRV2:*CaWRKY6* pepper under salt stress treatment for 8
502 hours. Scale bar = 1 cm. (B, C) Quantification of MDA (B) and proline content (C) in
503 the leaf of TRV2:*00* and TRV2:*CaWRKY6* pepper plants under salt stress treatment for
504 8 hours. Data were mean ± SD, and the statistically significant differences were
505 determined by one-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

506

507 **Fig. 3. *35S::CaWRKY6* transgenic Arabidopsis seedings are tolerate to salt stress.**
508 (A, C) Phenotype of WT and *35S::CaWRKY6* Arabidopsis seedlings under salt stress
509 treatment. Scale bar = 1 cm. (B, D) Quantification of survival rate (B) and primary root
510 length (D) under salt stress treatment. Data were mean ± SD, and the statistically
511 significant differences were determined by one-way ANOVA, * $P < 0.05$, *** $P < 0.001$.

512

513 **Fig. 4. CaWRKY6 regulates ROS production.** (A) Images showing the DAB and
514 NBT staining in TRV2:*00* and TRV2:*CaWRKY6* pepper under salt stress treatment for
515 8 hours. Scale bar = 100 μm. (B, C) Quantification of SOD (B) and CAT (C) enzyme
516 activity in in TRV2:*00* and TRV2:*CaWRKY6* pepper under salt stress treatment for 8
517 hours. Data were mean ± SD, and the statistically significant differences were
518 determined by one-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

519

520 **Fig. 5. CaWRKY6 interacts with CAERF3.** (A) Y2H assay between CaWRKY6 and
521 CaERF3. (B) BiFC assay between CaWRKY6 and CaERF3. Scale bar = 100 μm.

522

523 **Fig. 6. *CaERF3*-silenced pepper plants are hypersensitive to salt stress.** (A)
524 Expression level of *CaERF3* in stem, flower, leaf, root and fruit of pepper plants. (B)

Expression level of *CaERF3* in response to salt stress. (C) Phenotype of TRV2:*00* and TRV2:*CaERF3* pepper under salt stress treatment for 8 hours. Scale bar = 1 cm. (D, E) Quantification of MDA (D) and proline content (E) in the leaf of TRV2:*00* and TRV2:*CaERF3* pepper plants under salt stress treatment for 8 hours. Data were mean ± SD, and the statistically significant differences were determined by one-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 7. *CaERF3* regulates ROS production. (A) Images showing the DAB and NBT staining in TRV2:*00* and TRV2:*CaERF3* pepper under salt stress treatment for 8 hours. Scale bar = 100 μm. (B, C) Quantification of SOD (B) and CAT (C) enzyme activity in in TRV2:*00* and TRV2:*CaERF3* pepper under salt stress treatment for 8 hours. Data were mean ± SD, and the statistically significant differences were determined by one-way ANOVA, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Supplementary Files

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- CaWRKY6Supplementaryfigures20250929.pdf