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Effect of Silencing CaLEA-Dc3 Gene on Salt Tolerance in Capsicum annuum L.

S. L. Tian
J.Y. Li
Y. Wang
A. M. Shen
J. F. Li
L. Li
lili-8686860163.com

Huanghuai University

Research Article

Keywords: Transcriptome sequencing, salt stress, gene silencing, NBT staining, DAB staining

Posted Date: March 2nd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8902440/v1

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Abstract

Aims

Pepper (*Capsicum annuum* L.) is a widely cultivated vegetable crop. However, soil salinization and alkalization have increasingly threatened its healthy growth. Exploring key genes and molecular mechanisms underlying salt tolerance in pepper is crucial for the sustainable development of pepper industry. In this study, transcriptome sequencing analysis of pepper plants under salt stress revealed that *CaLEA-Dc3* (CA03g31580) gene was highly upregulated, suggesting its potential role in salt stress response.

Methods

Using virus-induced gene silencing (VIGS) technology, *CaLEA-Dc3* gene was silenced and the related physiological indicators were measured. Compared with control plants, the expression of *CaLEA-Dc3* gene was disappeared in silenced plants, accompanied by decreased activities of superoxide dismutase (SOD) and peroxidase (POD), and increased catalase (CAT) activity and malondialdehyde (MDA) content. Additionally, proline content decreased, root activity declined, chlorophyll content decreased, and root system shrank. After NBT and DAB staining, reactive oxygen species (ROS) damage in the leaves of silenced plants intensified.

Results

These results indicated that *CaLEA-Dc3* gene was involved in salt stress response. Silencing *CaLEA-Dc3* gene reduced salt tolerance of pepper plants.

Conclusions

Our findings provided a theoretical basis for breeding salt-tolerant pepper varieties.

Introduction

In recent years, rising groundwater levels, excessive chemical fertilizer application, irrational irrigation practices, and the rapid expansion of facility agriculture have exacerbated soil degradation issues. Problems such as soil acidification, secondary salinization, microecological imbalance, and heavy metal pollution have increasingly constrained the productivity improvement and sustainable development of facility vegetable cultivation. Notably, soil salinization has become a major limiting factor for modern agriculture in China (Chen et al. 2025). Therefore, addressing soil salinization in facility vegetable production is critically urgent.

Capsicum annuum L., a member of the *Capsicum* genus in *Solanaceae* family, produces fruits rich in nutrients such as vitamin C, capsaicin, and carotenoids, making it a consumer-favorite vegetable. Throughout its production cycle-from sowing to harvesting-pepper plants face persistent abiotic stresses like salinization. Such stress may moderately hinder growth and development or, in severe cases, cause whole-plant mortality. Therefore, identifying salt-tolerant genes has become a key challenge for breeders.

Studies have identified several key genes involved in pepper (*Capsicum spp.*) stress tolerance. Liu et al. (2025) transferred the overexpression vector pRI101-ANCaNAC014-EGFP and VIGS vector pTRV2-*CaNAC014* into leaves of 'FJNY-1' and 'FJYM-1' by an Agrobacterium-mediated method, the results showed that overexpression of *CaNAC014* increased salt tolerance in pepper, promoted the plant growth and root development, and maintained significantly higher chlorophyll levels and photosynthetic performance under salt stress. zhao et al. (2025) found that *CaERF2* expression was dramatically increased in pepper plants following salt stress treatment, *CaERF2* effectively enhances the salt tolerance in pepper by adjusting ROS homeostasis, *CaERF2* played a significant role in the salt tolerance of *Capsicum*. Sun et al. (2025) demonstrated that *CaDREB32* increased the resistance of transgenic plants to salt and osmotic stresses, increased superoxide dismutase and peroxidase activities, and proline, total soluble sugar and chlorophyll accompanied with up-regulated expression of stress-related genes. Ou et al. (2024) found that *CaROS1* positively regulates salt tolerance ability, and CaROS1-silenced pepper were more sensitive to salt stress. Zhou et al. (2023) found that *CaCP15* was upregulated under salt stress, and *CaCP15* silencing in pepper enhanced salt resistance, and transient overexpression of *CaCP15* increased the sensitivity to salt by reducing the antioxidant enzyme activities and negatively regulating the stress-related genes. Similarly, Xiao et al. (2021) identified *CaFtsH06* as a novel filamentous thermosensitive protease gene that contributes to heat, salt, and drought tolerance. Wu et al. (2021) further investigated *CaAnn9* via VIGS and observed increased salt sensitivity in silenced plants, characterized by chlorophyll degradation, reactive oxygen species (ROS) accumulation, and weakened antioxidant defenses, underscoring its role in pepper growth and stress adaptation. Additionally, Hyun et al. (2008) proposed that *CaMRP1*, a membrane-localized receptor-like protein, modulates disease susceptibility, methyl jasmonate (MeJA) insensitivity, and salt tolerance, suggesting its membership in a novel subfamily of stress-related proteins.

LEA proteins, widely distributed in vascular and non-vascular plants, constitute a large protein family implicated in stress defense mechanisms (Hincha & Thalhammer 2012). Functionally, they contribute to plant resilience by mitigating biotic and abiotic stress through physiological adaptations, playing a crucial role in enhancing stress tolerance (Hand et al. 2011). *CaLEA-Dc3* (CA03g31580) is classified as a member of the late embryogenesis abundant (LEA) protein family. Its potential role in pepper salt stress responses has not been previously investigated. In current study, three pepper varieties (*Capsicum annuum* cv. pod pepper, sweet pepper, and screw pepper) were subjected to salt stress treatment and subsequently analyzed through transcriptome sequencing. Through comparative transcriptomic analysis, 2,986 differentially expressed genes were identified as being commonly regulated across all three varieties. Among these, *CaLEA-Dc3* was found to exhibit the most significant upregulation.

To elucidate the functional role of *CaLEA-Dc3*, virus-induced gene silencing (VIGS) was employed. Following gene silencing, several physiological changes were observed: (1) antioxidant enzyme activities were altered, (2) reactive oxygen species accumulation was increased, (3) chlorophyll content was reduced, and (4) expression patterns of stress-responsive genes were modified. Based on these observations. These results will contribute to a more comprehensive understanding of the role of the *CaLEA-Dc3* gene in response to salt-induced stress. Furthermore, the findings provide experimental evidence supporting the involvement of *CaLEA-Dc3* in salt tolerance in pepper, offering potential applications in molecular breeding programs aimed at developing salt-resistant pepper cultivars.

Materials and Methods

Ethical approval

for this study was granted by the Huanghuai University of the Ethics Committee. Written informed consent was obtained from all participants prior to their inclusion in the study, and the study did not involve animal experiments.

Materials

This study selected three pepper varieties provided by the Horticulture Research Institute of Zhumadian Academy of Agricultural Sciences: Screw Pepper (Sc-1), Sweet Pepper (Sw-1), and Pod Pepper (Po-1), which were used as experimental materials. All materials were uniformly cultivated in an intelligent solar greenhouse located in Huanghuai University. Field management was conducted in accordance with the natural growth requirements of peppers, including controlled light exposure (12 h / d), humidity levels (50%–58%), and temperature conditions (22–25°C).

Methods

Salt stress treatment

Pepper seedlings from three different varieties: Screw pepper (Sc-1), Sweet pepper (Sw-1), and Pod pepper (Po-1) were selected, each with a seedling age of 7 to 8 leaves. The seedlings were carefully excavated from the soil, and the roots were thoroughly rinsed to remove all soil particles until completely clean. Subsequently, they were placed in separate 250 mL conical flasks (Fig. 1). For each variety, three samples were subjected to treatment while three served as controls. The treatment group was exposed to a NaCl solution at a concentration of 150 $mmol.L^{-1}$, whereas the control group received distilled water under salt stress conditions for a duration of 24 h. Following this period, all samples were ground to extract RNA for transcriptome sequencing analysis.

Transcriptome sequencing

Total RNA was extracted from pepper plants in both salt stress group and control group using RNA extraction kits provided by Shanghai Shenggong Bioengineering Co., Ltd. Following successful agarose

gel electrophoresis and purity concentration assessments, the samples were sent to Suzhou Nuomi Metabolism Co., Ltd. The integrity and total quantity of RNA were then accurately evaluated using an Agilent 2100 bioanalyzer. Upon passing these evaluations, RNA library construction was performed, followed by Illumina transcriptome sequencing. Gene expression levels were quantified using FPKM (Fragments per kilobase of transcript per million fragments mapped) values. Genetic data utilized in this study is sourced from the Korean *Capsicum* Genome Database (http://passport.pepper.snu.ac.kr/?t=PGENOME).

The differential expression analysis among samples was conducted using DESeq2 software (version 1.20.0), with an adjusted P-value threshold of padj < 0.05. GO enrichment and KEGG pathway enrichment analyses for the differentially expressed genes were performed utilizing clusterProfiler software (version 3.4.4). Significantly enriched GO categories and KEGG metabolic pathways, with a padj value of less than 0.05, were selected for further examination. The interactions between proteins corresponding to the differentially expressed genes were analyzed using String version 11.0 (https://string-db.org/cgi/input?sessionId=b5cXpYokkgLb&input_page_show_search=on).

Gene fragment amplification and VIGS vector construction

RNA extraction from pepper leaves was performed using the Trizol method. Following RNA extraction, reverse transcription into cDNA was carried out according to the operational instructions provided by the reverse transcription kit from Shanghai Shengong Bioengineering Co., Ltd. The gene sequence for *CaLEA-Dc3* (CA03g31580) was retrieved from the South Korean *Capsicum* genome database (http://passport.pepper.snu.ac.kr/?t=PGENOME). Based on primer specificity design principles, primers required for PCR amplification of *CaLEA-Dc3* gene were designed using Prime 5.0 software, as detailed in Table 1.

TRV2 plasmid was selected as the target gene vector. Following the extraction of TRV2 plasmid from bacterial culture, two restriction enzymes, EcoRI and BamHI, were employed to perform double digestion on both TRV2 plasmid and *CaLEA-Dc3* gene after PCR amplification. Post-digestion, TRV2 plasmid was ligated with *CaLEA-Dc3* gene using T4 DNA ligase.

Ecoli. containing TRV2-*CaLEA-Dc3* gene vector was then spread onto solid LB agar plates supplemented with 50 $\mu g \cdot mL^{-1}$ kanamycin via a plating method. The solid LB agar plates were incubated in a constant temperature chamber under dark conditions for approximately 12 h. Once dense colonies emerged on the agar surface, individual colonies were carefully picked using sterilized pipette tips and transferred into 3 mL centrifuge tubes containing liquid LB medium. These tubes were sealed and placed on a floating platform before being incubated in a shaking water bath at 37°C with agitation at 220 rpm for 12 h. Subsequently, 1.5 mL of cultured Ecoli liquid was sent to Shenggong (Shanghai) Bioengineering Co., Ltd. for sequencing analysis.

Preparation of infectious bacterial liquid

A total of 10 μL of TRV2-*CaLEA-Dc3* plasmid was introduced into Agrobacterium in a competent state. Subsequently, the Agrobacterium suspension containing *CaLEA-Dc3* gene was spread onto solid LB culture plates supplemented with antibiotics. These solid LB culture plates were sealed and incubated at a constant temperature of 28°C for 36 h to allow for the growth of single colonies, which were then harvested.

Single colonies were selected and transferred into 3 mL of liquid LB medium containing antibiotics (50 $\mu g \cdot mL^{-1}$). The cultures were propagated in a constant-temperature shaker set at 220 rpm and maintained at 30°C for a duration of 16–18 h. Following this initial propagation, the expanded Agrobacterium suspension was added to 20 mL of liquid LB medium (kanamycin: 50 $\mu g \cdot mL^{-1}$; rifampicin: 25 $\mu g \cdot mL^{-1}$) and further cultured in a constant-temperature shaker under identical conditions (220 rpm, 30°C) for an additional period ranging from 16 to 20 h.

The resulting *Agrobacterium* solution was centrifuged, after which the supernatant was discarded. A volume of antibiotic solution (25 mL) was added to resuspend the bacterial pellet, followed by shaking in a constant-temperature shaker (220 rpm, 30°C) for approximately 18 h until the bacterial suspension exhibited a yellow flocculent appearance. The cultured bacterial solution underwent another round of centrifugation at 10,000 rpm. After discarding the supernatant once more, an additional volume of IM medium (25 mL) was introduced into each centrifuge tube to re-disperse the colonies.

For detection using a microplate reader, an absorbance value targeting approximately 0.3 is required. TRV1 bacterial solution is mixed with both TRV2 empty Agrobacterium and TRV2-*CaIEA-DC3* solutions in equal ratios (1:1). This well-mixed bacterial suspension is then stored overnight at 4°C before proceeding with infection via injection on pepper plants.

Pepper seedlings chosen for infection should be undamaged, free from other diseases, exhibit vigorous growth characteristics, and possess between five to seven true leaves demonstrating uniform growth vigor. Post-infection care involves maintaining these chili plants in dark and moist conditions over a span of 72 h.

Detection of plant efficiency with gene silencing

About 3 to 5 d following the injection of Agrobacterium solution into pepper plants, leaves from both TRV2 empty vector plants and *CaLEA-Dc3* silenced plants were collected and temporarily stored in 1.5 mL centrifuge tubes. RNA extracted from pepper leaves was subsequently subjected to reverse transcription using a kit obtained from Beijing Qingke Biotechnology Co., Ltd., converting it into cDNA for semi-quantitative gene expression analysis. The sequences of *CaLEA-Dc3* gene and the internal reference gene, *CaUBI3*, are presented in Table 2.

Salt stress treatment of pepper plants

Pepper plants exhibiting successful gene silencing were selected for salt stress treatment, while wild-type plants and TRV2 empty vector control plants were used as controls. The control group plants and gene-silenced pepper plants were subjected to stress treatment by exposure to a 150 mmol.L^{-1} NaCl solution. Leaves from corresponding positions of wild-type, TRV2 empty vector, and gene-silenced plants were harvested separately and immediately stored at -80°C. Each treatment was performed in triplicate to ensure experimental reproducibility.

Measurement of OD, CAT, POD, MDA, and PRO Levels

Physiological indicators associated with plant stress resistance, including SOD, CAT, POD, MDA, and PRO, were determined according to the manufacturer's instructions of the reagent kits (Beijing Qingke Biotechnology Co., Ltd.). These measurements were conducted on leaf samples from wild-type control plants, TRV2 empty vector control plants, and *CaLEA-Dc3* gene-silenced pepper plants.

Observation of reactive oxygen species staining

Pepper leaves were stained at the time point exhibiting the most pronounced phenotypic differences. Leaves from the control group and gene-silenced pepper plants were separately incubated in DAB and NBT solutions, respectively, under dark conditions for 10 h. After incubation, the DAB and NBT solutions were discarded, and 30 mL of anhydrous ethanol was added to each sample. The samples were then heated in a water bath at 100°C for 12 min with shaking every 3 min. If decolorization was incomplete, leaves were briefly rinsed with fresh anhydrous ethanol. Finally, fully cleared leaves were carefully transferred using tweezers onto a white test bench or white A4 paper for observation, documentation, and photography.

Table 1
Primer sequence of CaLEA-Dc3 silenced

Gene	Accession numbers:	Forward primer(5'→3')	Reverse primer(5'→3')
VIGS-CaLEA-Dc3	CA03g31580	cggaattcatggcttcccacgagcagag	cgggatccctatggttctctgggtgtttttg

Table 2
Primer sequences of CaLEA-Dc3 gene expression

Gene	Accession numbers:	Forward primer(5'→3')	Reverse primer(5'→3')
CaLEA-Dc3	CA03g31580	TTCCCACGAGCAGAGTT	TCAGCAGTATCCGCCAT
CaUbi3	AY486137.1	GTTGTCTCTCGTCTACCTGT	CAGAATAACACGAACCCCAC

Results

Transcriptome sequencing results

Venn diagrams were used to visualize the shared and unique gene expression patterns across different samples or treatment groups, as well as the overlapping and distinct expression profiles of differentially expressed genes between groups. Transcriptome sequencing revealed a large number of differentially expressed genes. Among them, 2,986 genes were commonly expressed under salt stress in the three chili pepper materials (Fig. 2), with *CaLEA-Dc3* (CA03g31580) gene exhibiting the most significantly upregulated expression level among the three materials (Table 3).

Table 3
List of up-regulated and down-regulated genes

Gene ID	SC-C vs SC-T	Expression level	SW-C vs SW_T	Expression level	PO-C vs PO-T	Expression level
CA03g31580	up	8.92	up	8.86	up	8.22
CA10g20950	up	8.34	up	6.38	up	7.73
CA05g06460	up	10.00	up	8.09	up	7.41
CA12g06250	up	6.77	up	8.72	up	7.28
CA09g05580	up	7.19	up	5.96	up	7.19
CA03g17780	up	8.50	up	8.37	up	7.17
CA08g01060	up	8.53	up	7.86	up	7.16
CA09g03370	up	8.69	up	9.63	up	6.73
CA03g22980	up	8.19	up	8.27	up	6.05
CA09g18740	up	5.52	up	7.67	up	6.02
CA03g08390	up	6.38	up	6.75	up	5.76
CA06g20630	up	7.10	up	8.60	up	5.65
CA11g08310	up	7.02	up	7.89	up	5.60
CA10g08820	up	6.11	up	5.26	up	5.55
CA07g20810	up	6.51	up	6.40	up	5.51
CA08g08650	up	6.43	up	6.83	up	5.42
CA03g03740	up	8.62	up	7.60	up	5.40
CA06g08180	up	6.70	up	8.90	up	5.31
CA07g16930	up	7.02	up	8.59	up	5.15
CA03g17990	up	10.13	up	10.36	up	5.02
CA12g15790	up	7.36	up	6.69	up	5.01
CA03g36920	up	9.19	up	8.74	up	4.95
CA03g28600	down	-6.27	down	-9.59	down	-8.70
CA09g05000	down	-6.09	down	-10.81	down	-8.63
CA09g02850	down	-5.34	down	-9.74	down	-8.18
CA07g14100	down	-5.83	down	-6.34	down	-7.92

Gene ID	SC-C vs SC-T	Expression level	SW-C vs SW_T	Expression level	PO-C vs PO-T	Expression level
CA04g16070	down	-6.55	down	-8.72	down	-7.15
CA05g01990	down	-7.31	down	-10.22	down	-6.39
CA02g25740	down	-7.01	down	-9.13	down	-6.37
CA11g00010	down	-6.38	down	-6.52	down	-6.16
CA06g24320	down	-5.90	down	-7.32	down	-6.03
CA07g12090	down	-7.41	down	-9.65	down	-5.62
CA06g04570	down	-6.39	down	-6.51	down	-5.50
CA06g17620	down	-8.13	down	-5.04	down	-5.29
CA03g09910	down	-7.02	down	-6.41	down	-5.20
CA09g01620	down	-6.51	down	-6.31	down	-4.87

Gene fragment amplification and VIGS vector construction

As shown in the gel image in Fig. 3A, distinct bands corresponding to 28S, 18S, and 5S rRNA are clearly visible. The micro-spectrophotometer measurement yielded an OD260/OD280 ratio of 2.0, indicating high-quality RNA that meets the requirements for downstream experiments. Figure 3B presents the restriction enzyme digestion results of the constructed TRV2-*CaLEA-Dc3* expression vector. Two bands were observed in each lane: one corresponds to the linearized TRV2 vector after excision of the insert, and the other represents the inserted target gene fragment with a size between 500 and 750 bp, consistent with the expected length. Figure 3C shows the PCR amplification results from bacterial colonies following transformation of *CaLEA-Dc3* into Agrobacterium, revealing a product of approximately 500–750 bp, which aligns with the anticipated size of the target gene. Collectively, these results confirm the successful construction of TRV2-*CaLEA-Dc3* gene silencing vector, enabling progression to the subsequent infiltration experiment.

Phenotypic observation of gene-silenced leaves following stress treatment

As shown in Fig. 4, compared with the empty vector control and wild-type control leaves, *CaLEA-Dc3* gene-silenced leaves exhibited yellowing and color fading as early as 3 d after treatment. By day 6, both the gene-silenced group and the empty vector control group showed visible yellowing, and by day 9, all leaves displayed pronounced loss of green pigmentation (Fig. 4). These observations indicate that silencing of *CaLEA-Dc3* gene leads to an earlier onset of reduced salt tolerance.

Phenotypic observation of gene-silenced plants under stress treatment

As shown in Fig. 5, compared with the empty vector control and wild-type control plants, *CaLEA-Dc3* gene-silenced plants exhibited root whitening and inhibited root growth, displaying significant phenotypic differences relative to the control groups.

Detection of gene expression levels in gene-silenced plants following stress treatment

As shown in Fig. 6, there was no significant difference in the expression level of *CaLEA-Dc3* gene between wild-type control plants and TRV2 empty vector control plants. In contrast, the expression level of *CaLEA-Dc3* in gene-silenced plants was significantly lower than that in the control groups. RT-PCR band corresponding to *CaLEA-Dc3* in silenced plants was nearly undetectable, indicating effective gene silencing.

Physiological parameter measurements in gene-silenced plants following stress treatment

The SOD assay results are presented in Fig. 7. Following stress treatment, no significant difference in SOD activity was observed between *CaLEA-Dc3* gene-silenced plants and control plants at 0 d or 3 d. However, by day 7, the SOD activity in silenced plants was significantly lower than that in the control groups, suggesting that silencing of *CaLEA-Dc3* gene impairs SOD enzyme function. Meanwhile, CAT enzyme activity showed minimal variation between gene-silenced and control plants at 0 d. Starting at 3 d, CAT activity in silenced plants increased significantly and remained higher than in controls throughout the treatment period, indicating that *CaLEA-Dc3* gene silencing enhances CAT activity under stress conditions. With regard to POD activity, a transient increase followed by a decrease was observed in silenced plants. Specifically, on day 3 of salt stress, POD activity was significantly elevated compared to controls, but declined by day 7 and fell below control levels (Fig. 7). MDA content measurements are also shown in Fig. 8. In *CaLEA-Dc3* gene-silenced plants, MDA levels initially decreased relative to controls, then increased markedly at later stages, exhibiting significant differences over time.

As shown in Fig. 8, proline content changed significantly following *CaLEA-Dc3* gene silencing. Specifically, on day 3 of treatment, proline levels in gene-silenced plants were significantly lower than those in the control group and continued to decline by day 7. Concurrently, root activity in silenced plants decreased markedly, and chlorophyll content also dropped compared to controls.

Reactive oxygen species staining results

The DAB staining results following salt stress treatment are shown in Fig. 9. No significant difference in leaf coloration was observed between control plants and the empty vector group, whereas a higher number of reddish-brown spots appeared in the mid-regions of leaves from *CaLEA-Dc3* gene-silenced plants.

Figure 10 showed the staining situation of NBT solution under salt stress treatment. Figure 10 showed the NBT staining results under salt stress treatment. Following salt stress, extensive and more intense staining was observed in the leaves of *CaLEA-Dc3* gene-silenced plants, indicating that silencing *CaLEA-Dc3* gene impairs the ability of pepper plants to scavenge reactive oxygen species.

Discussion

Under salt stress conditions, pepper yield decreases significantly, making the study of salt-tolerant genes in peppers particularly important. This experiment validated the role of *CaLEA-Dc3* gene in salt stress tolerance, providing a new theoretical foundation for understanding the physiological mechanisms of pepper salt tolerance and for breeding novel salt-tolerant varieties. The LEA protein family has been widely reported in recent studies. Yang et al. (2022) found that the PIN gene family in Chinese hickory (*CcPINs*) was a total of 11 *CcPINs*, and transient transformation in tobacco leaves demonstrated that *CcPIN1a*, *CcPIN3*, and *CcPIN4* were localized in the plasma membrane. There were varying phylogenetic relationships between *CcPINs* and homologous genes in different species. *CcPIN1a* and *CcPIN8a* were related to the regulation of salt stress in Chinese hickory. Wang et al. (2025) identified a total of 83 MsLEA proteins in M. sativa and conducted a comprehensive analysis to elucidate their functions in response to abiotic stresses, and these proteins could be classified into seven groups and were distributed across eight chromosomes. Furthermore, the promoters of *MsLEA* genes were found to be enriched with cis-elements associated with various stress responses. Through transcriptome and qRT-PCR analysis, nine *MsLEA* genes related to drought, salinity, and cold stress were identified. Geng et al. (2022) utilized RNA sequencing and quantitative PCR analyses to demonstrate that *NtLEA* genes were significantly induced under two distinct abiotic stresses in *Nicotiana tabacum*. These genes exhibited different expression patterns, with 35 *NtLEA* genes responding to abscisic acid (ABA) and three *NtLEA* genes responding to NaCl-induced abiotic stress.

In this study, *CaLEA-Dc3* gene was silenced using VIGS technology and validated by semi-quantitative RT-PCR analysis. The results showed that *CaLEA-Dc3* gene expression was significantly upregulated in wild-type control plants and TRV2 empty vector control plants. VIGS-based phenotypic analysis of detached leaves revealed that, compared to controls, *CaLEA-Dc3*-silenced leaves developed significant chlorosis at 3 days post-treatment, suggesting that suppression of *CaLEA-Dc3* gene compromises pepper salt tolerance. Physiological parameter analyses indicated that after 3 days of salt stress, *CaLEA-Dc3*-silenced plants exhibited significant differences in multiple indices relative to both TRV2 empty vector and wild-type control plants, demonstrating that downregulation of *CaLEA-Dc3* expression substantially affects pepper salt tolerance. Following stress treatment, no significant difference in SOD activity was observed between silenced and control plants at 0 d or 3 d; however, SOD activity in silenced plants was significantly lower than in controls at day 7. CAT enzyme activity began to increase significantly from day 3 onward and remained higher than in control plants throughout the experiment. POD activity in silenced plants showed a transient increase followed by a decrease. MDA levels in *CaLEA-Dc3*-silenced plants initially decreased and then increased, showing clear divergence from control plants. Reactive oxygen species (ROS) are toxic byproducts generated under adverse conditions, primarily including O^{2-} and H_2O_2 (Wells et al. 2017). H_2O_2 accumulation is commonly detected by DAB staining, in which DAB reacts with hydrogen peroxide to form a reddish-brown polymer; the intensity of coloration reflects the level of H_2O_2 in tissues. Superoxide anion (O^{2-}) accumulates as blue formazan deposits following NBT staining. The extent of leaf staining directly correlates with cellular damage.

After salt stress, staining intensity in silenced pepper leaves was markedly higher than in controls. Combined DAB and NBT staining results indicate that silencing C*aLEA-Dc3* leads to increased ROS accumulation and reduced salt tolerance in peppers. These findings demonstrate that *CaLEA-Dc3* gene plays a critical role in mediating salt stress tolerance in peppers. This study provides a theoretical basis for the development of salt-tolerant pepper varieties.

Conclusions

This study utilized transcriptome sequencing analysis to investigate the molecular responses of pepper plants subjected to salt stress. The results indicated that the expression levels of numerous genes were altered to varying degrees following exposure to salt stress. Notably, *CaLEA-Dc3* gene (CA03g31580) was significantly upregulated under salt stress, suggesting its potential involvement in salt stress response in peppers. Further functional analysis employing virus-induced gene silencing (VIGS), along with measurements of key physiological and biochemical indicators, demonstrated that silencing *CaLEA-Dc3* gene resulted in reduced activities of superoxide dismutase (SOD) and peroxidase (POD), increased catalase (CAT) activity and malondialdehyde (MDA) content, decreased proline accumulation, diminished root activity, lower chlorophyll content, impaired root system development, enhanced reactive oxygen species (ROS)-induced leaf damage, and an overall reduction in salt tolerance among pepper plants. These findings indicated that *CaLEA-Dc3* gene plays a critical role in mediating responses to salt stress in peppers. The results provided valuable theoretical insights for breeding salt-tolerant pepper varieties and elucidating the molecular mechanisms underlying salt tolerance.

Abbreviations

VIGS: virus-induced gene silencing; SOD: superoxide dismutase; POD: peroxidase; CAT: catalase; MDA: malondialdehyde; NBT: Nitro blue tetrazolium; DBA: Diaminobenzidine.

Declarations

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SLT and LL designed the research, analyzed data and drafted the manuscript. SLT and JYL designed primers for the RT-qPCR assays and collected virus samples. JYL and YW carried out virus vector construction and VIGS technology. AMS and JFL extracted RNA from pepper fruits. SLT wrote the paper, and all the authors read and approved the final mnuscript.

Acknowledgements

The following organizations are acknowledged for their financial support: Key research and development special of Zhumadian city (ZMDSZDZX2023009); Key research and development special of Zhumadian city (ZMDSZDYF2025017); The innovation scientists and technicians troop construction projects of Henan province (C20150054); The zhumadian international joint laboratory of pepper modern breeding. Language editing was done by MogoEdit (Xi'an) Company Ltd. (http://www.mogoedit.com).

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Figures

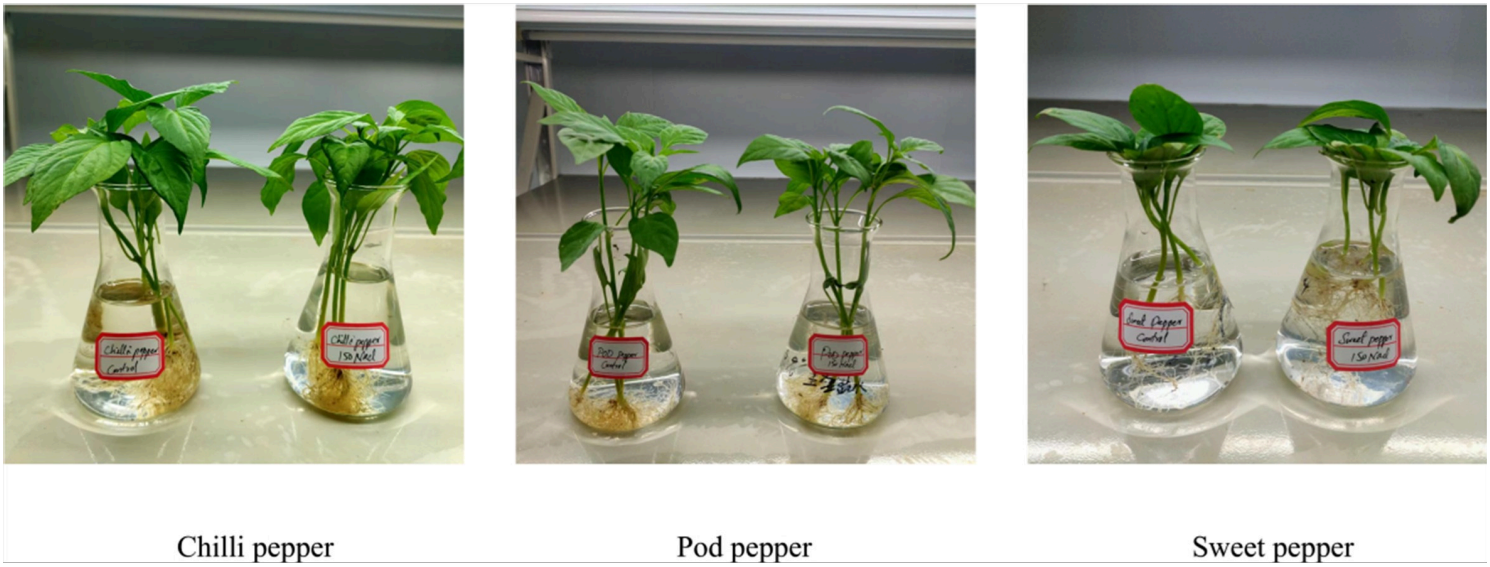

Figure 1

Salt stress treatment of three types of pepper materials

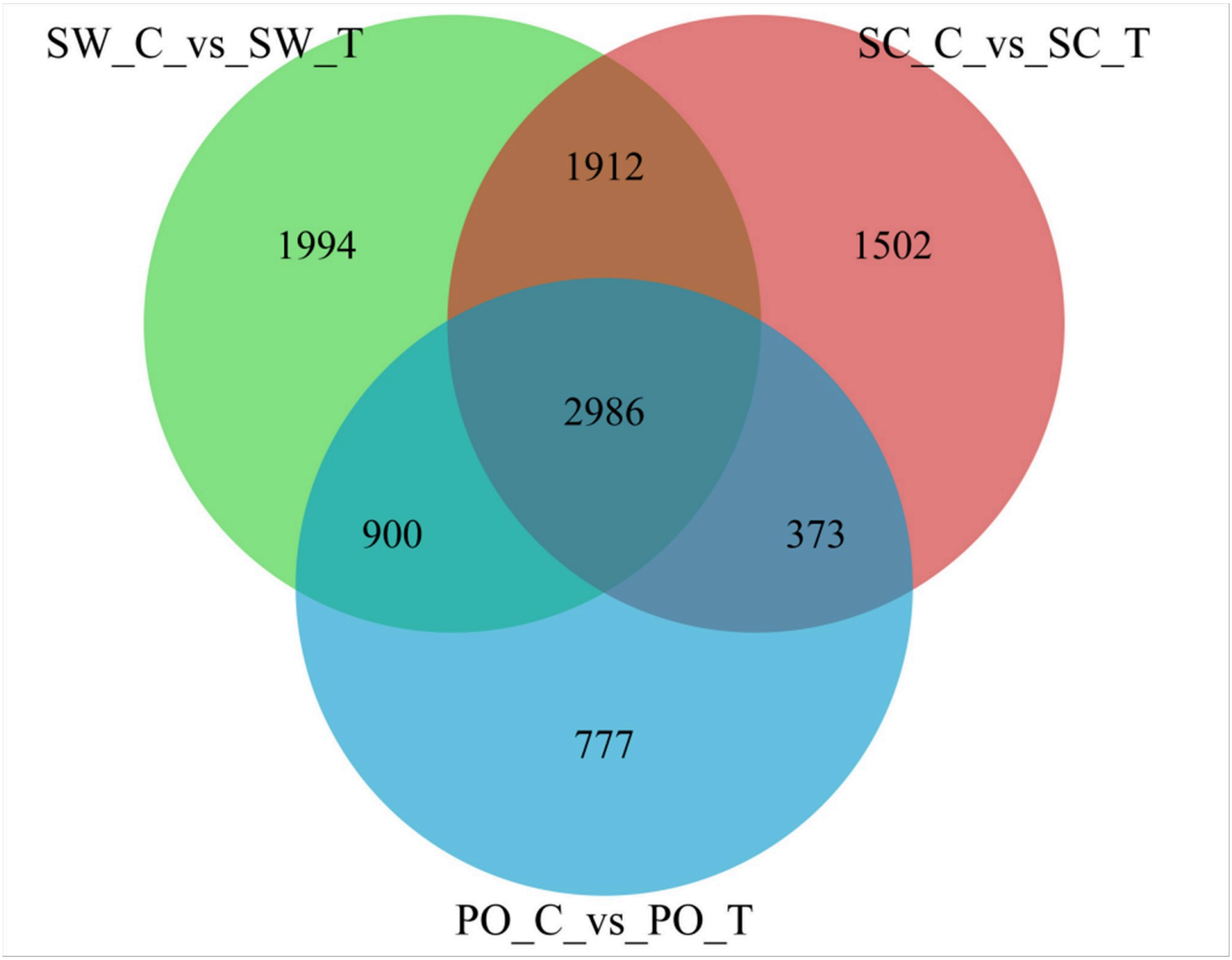

Figure 2

Venn diagrams under three types of pepper salt stress

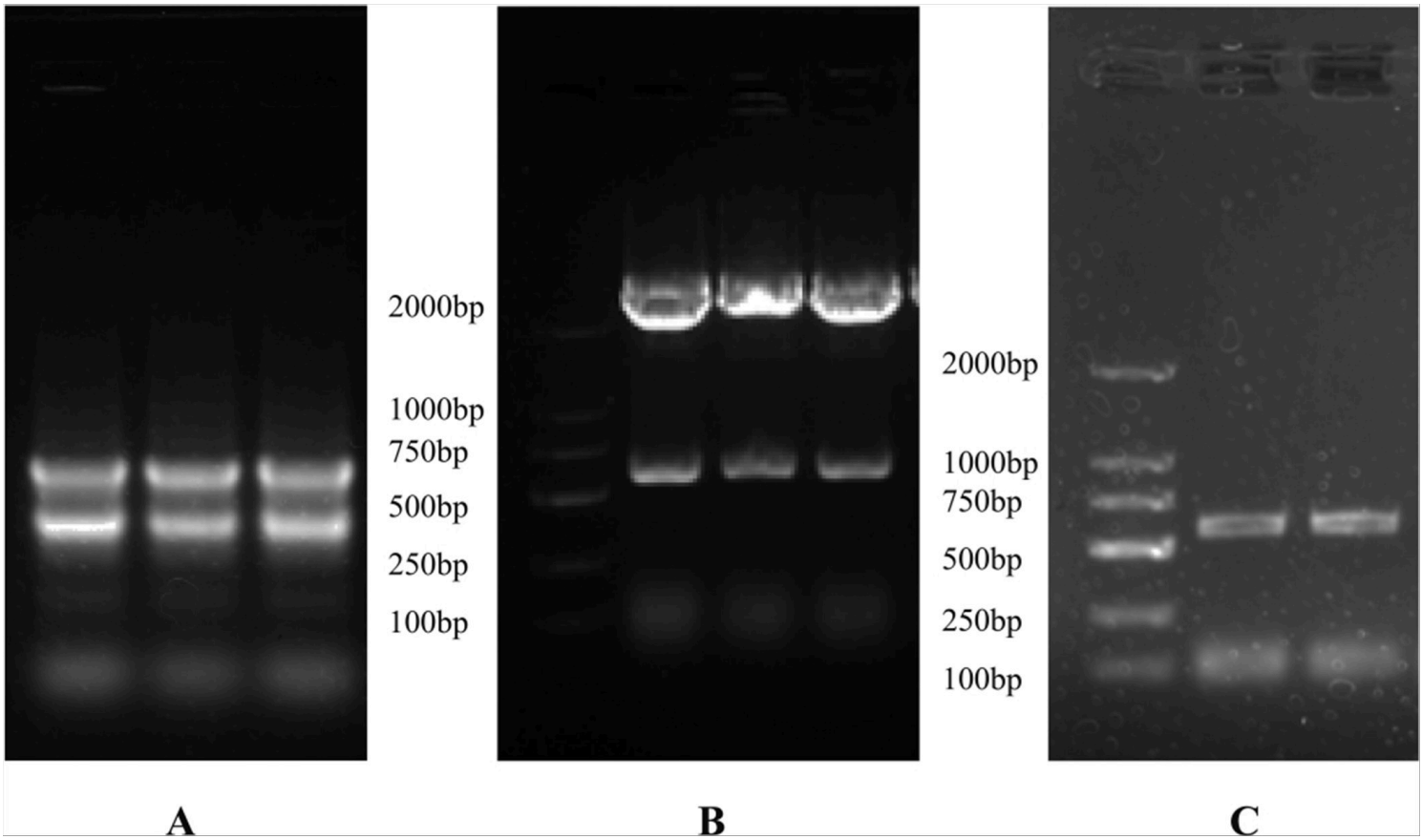

Figure 3

Vector construction for VIGS

A: RNA extraction; B: Construction of TRV2 vector for double enzyme digestion test; C: PCR detection after Agrobacterium transformation

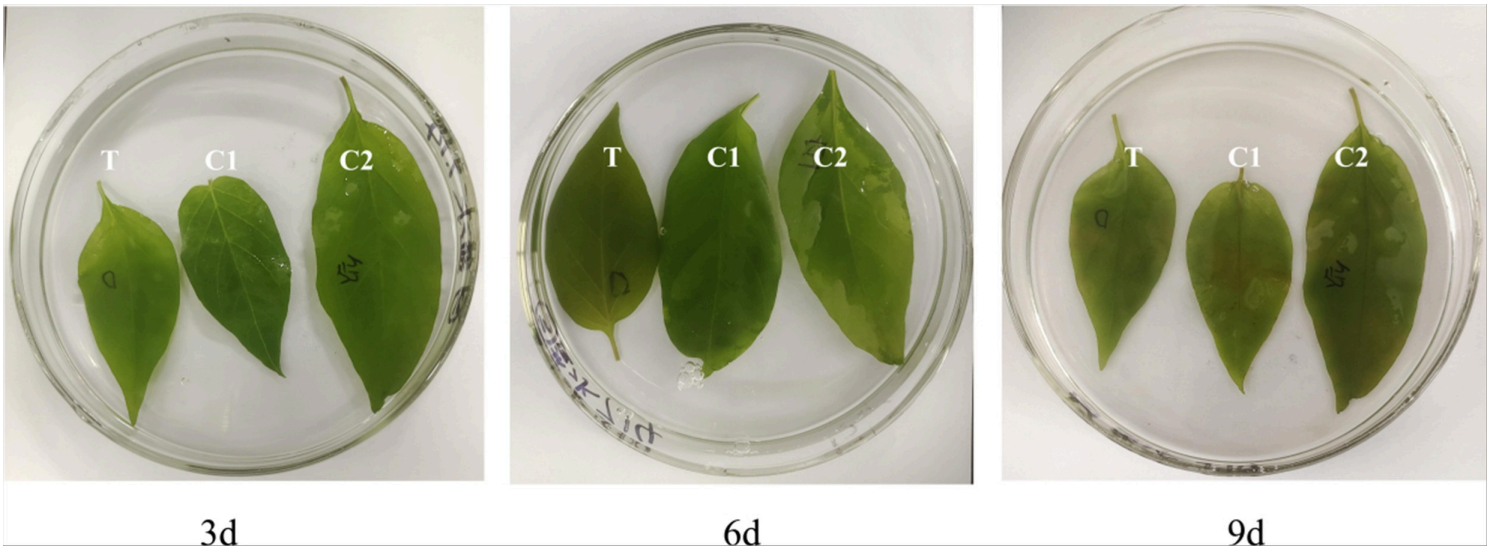

Figure 4

Gene-silenced leaves of peppers under salt stress

T：TRV/CaLEA-Dc3；C1：WT； C2：TRV/00

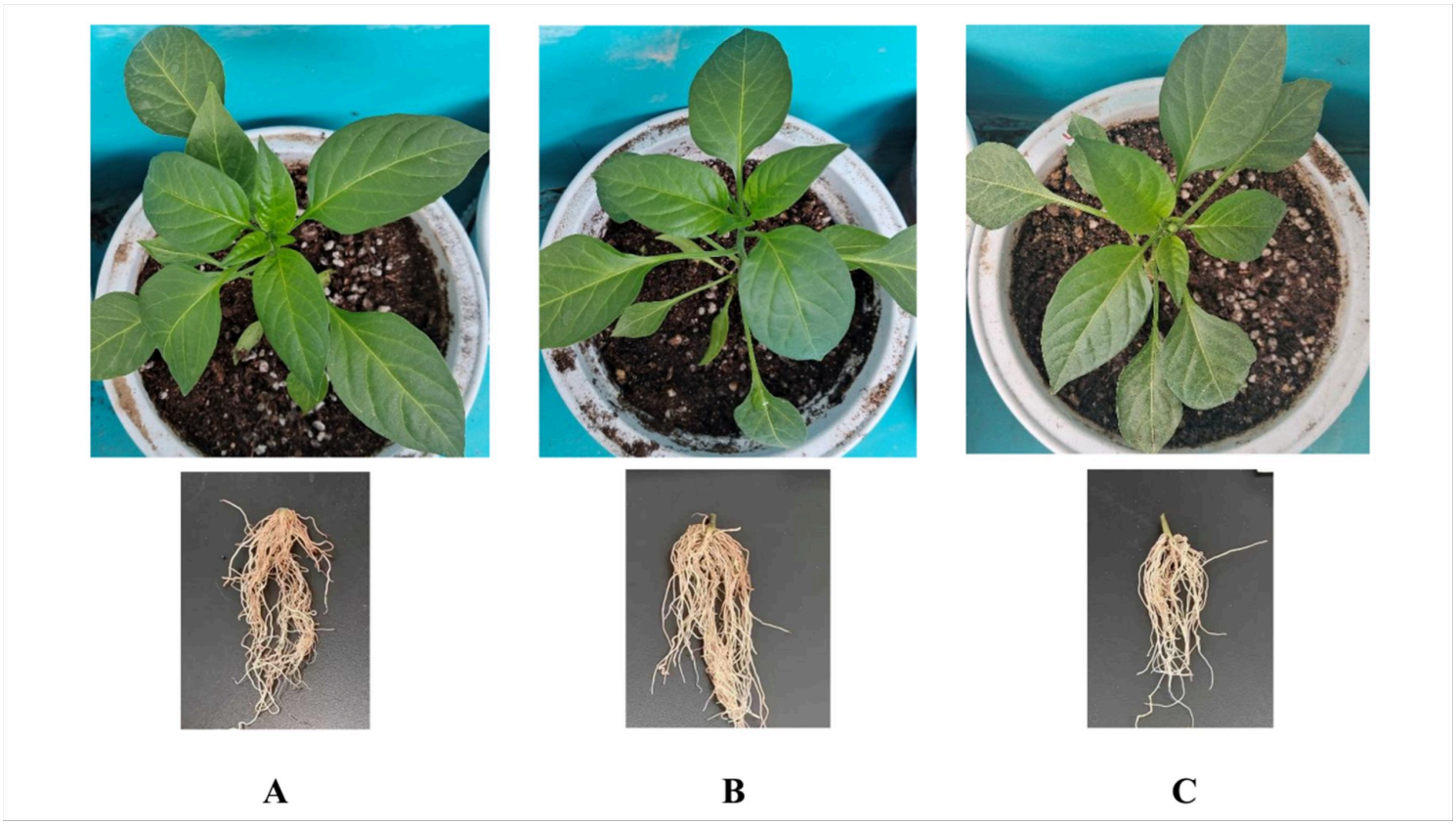

Figure 5

Comparison of phenotypes between silenced plants and their roots under salt stress treatment

A: Plants and roots in clear water control; B: Empty carrier control plants and roots; C: CaLEA-Dc3 gene silenced plants and roots.

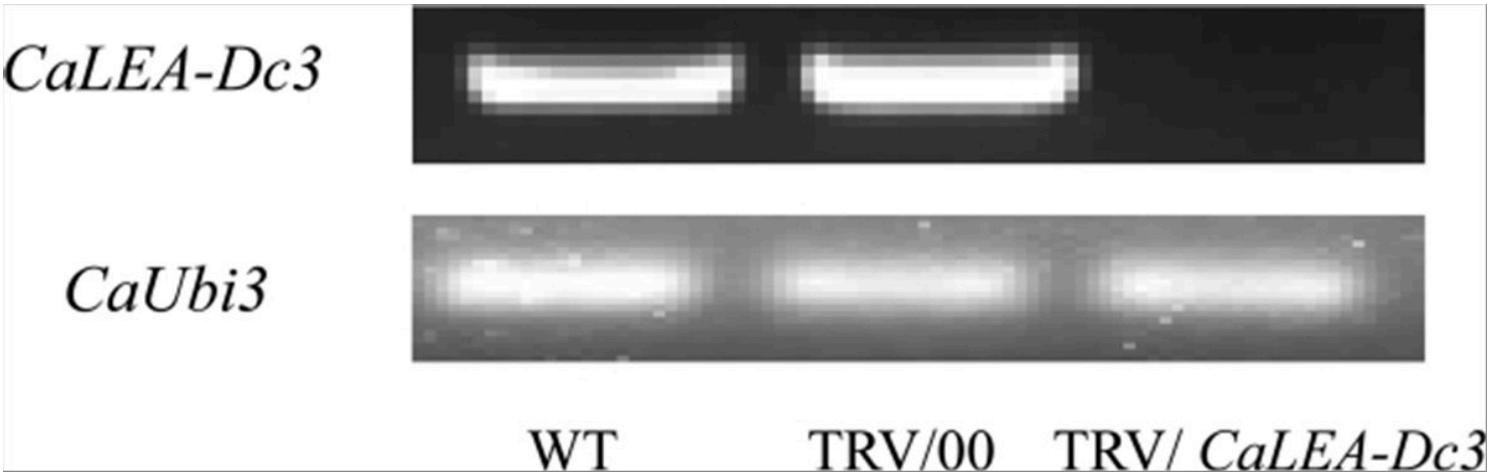

Figure 6

Gene expression levels in gene-silenced plants following salt stress treatment

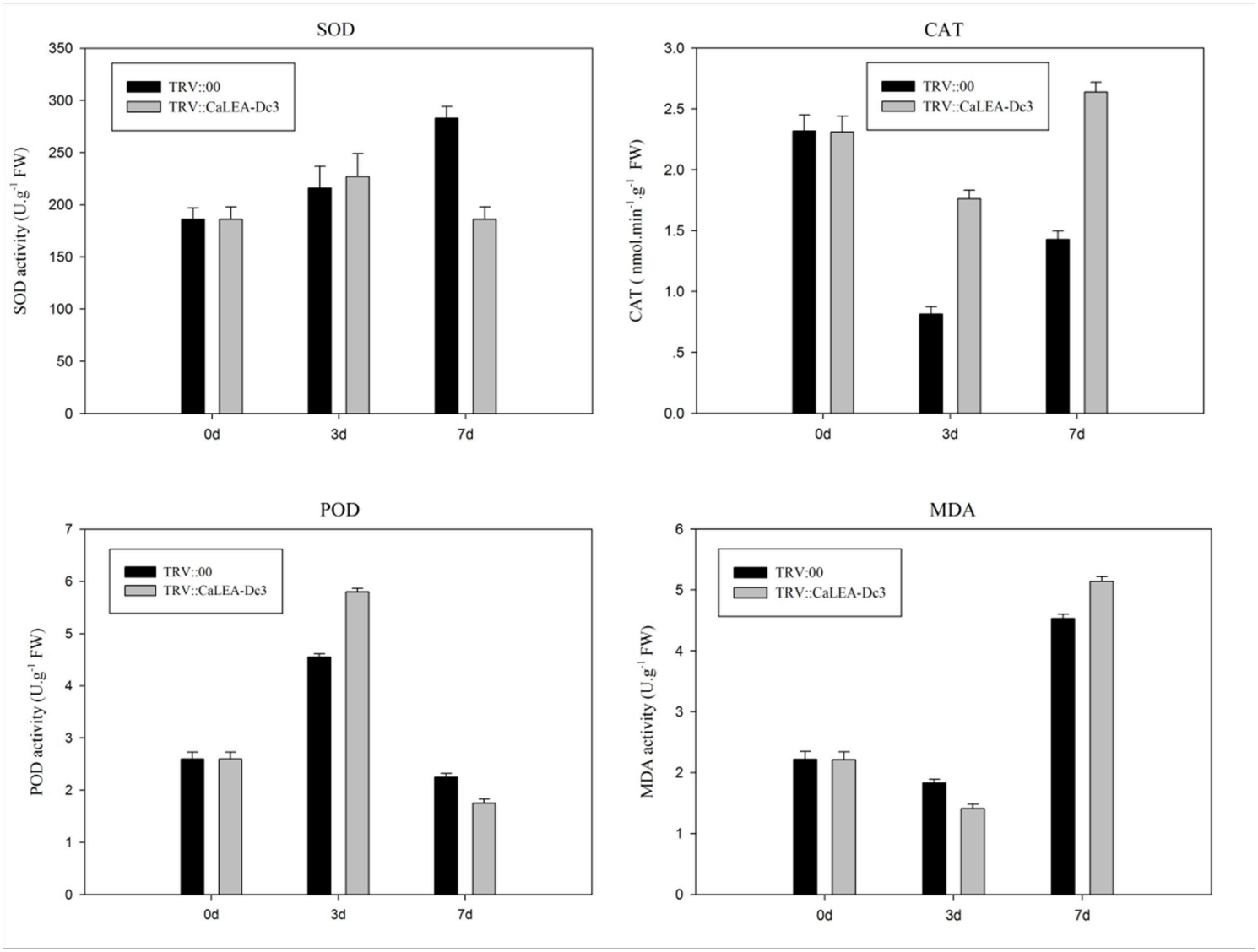

Figure 7

Changes in stress-related enzyme activities

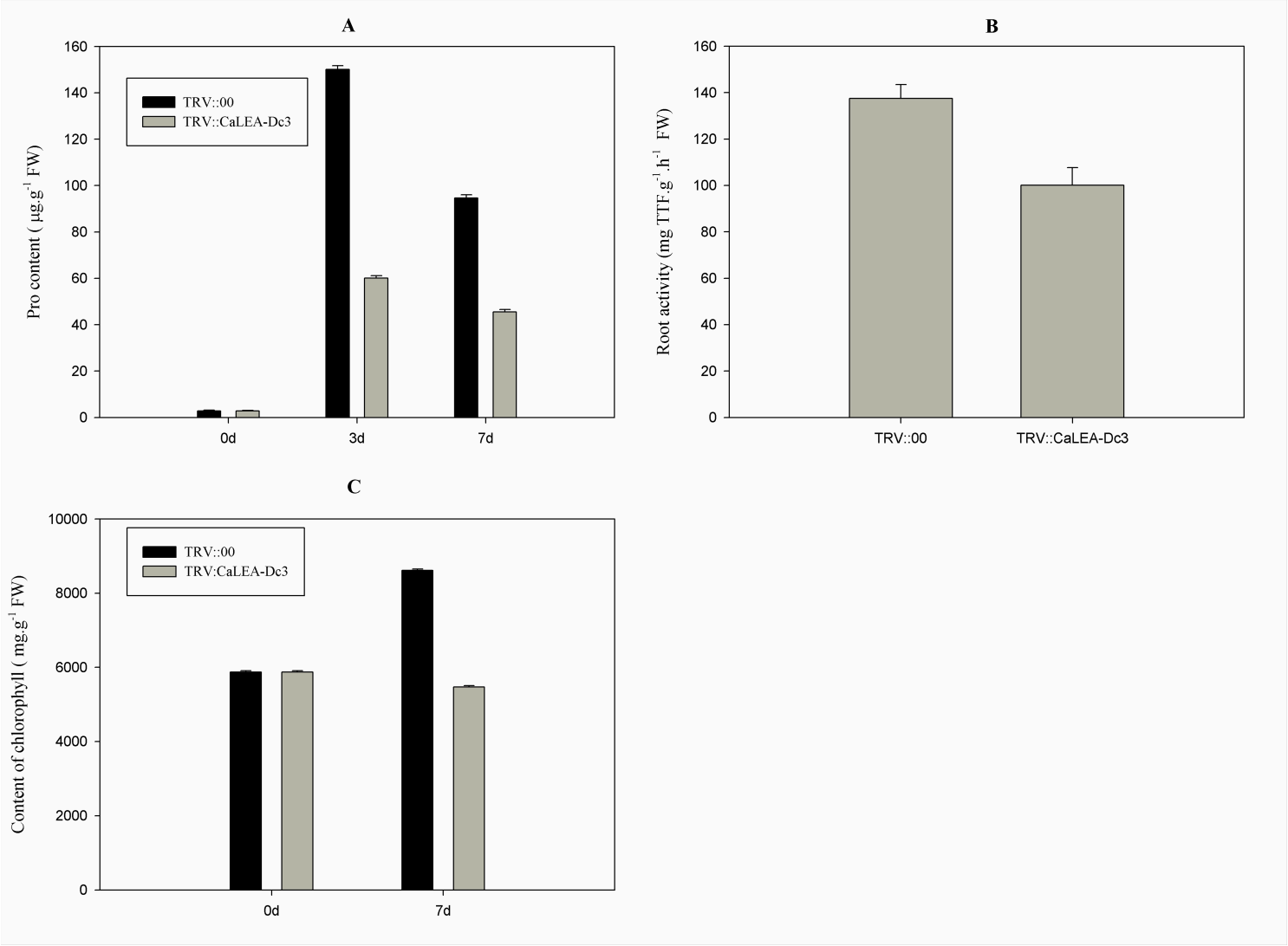

Figure 8

Changes in proline content, root activity, and chlorophyll content

Figure 9

DAB staining

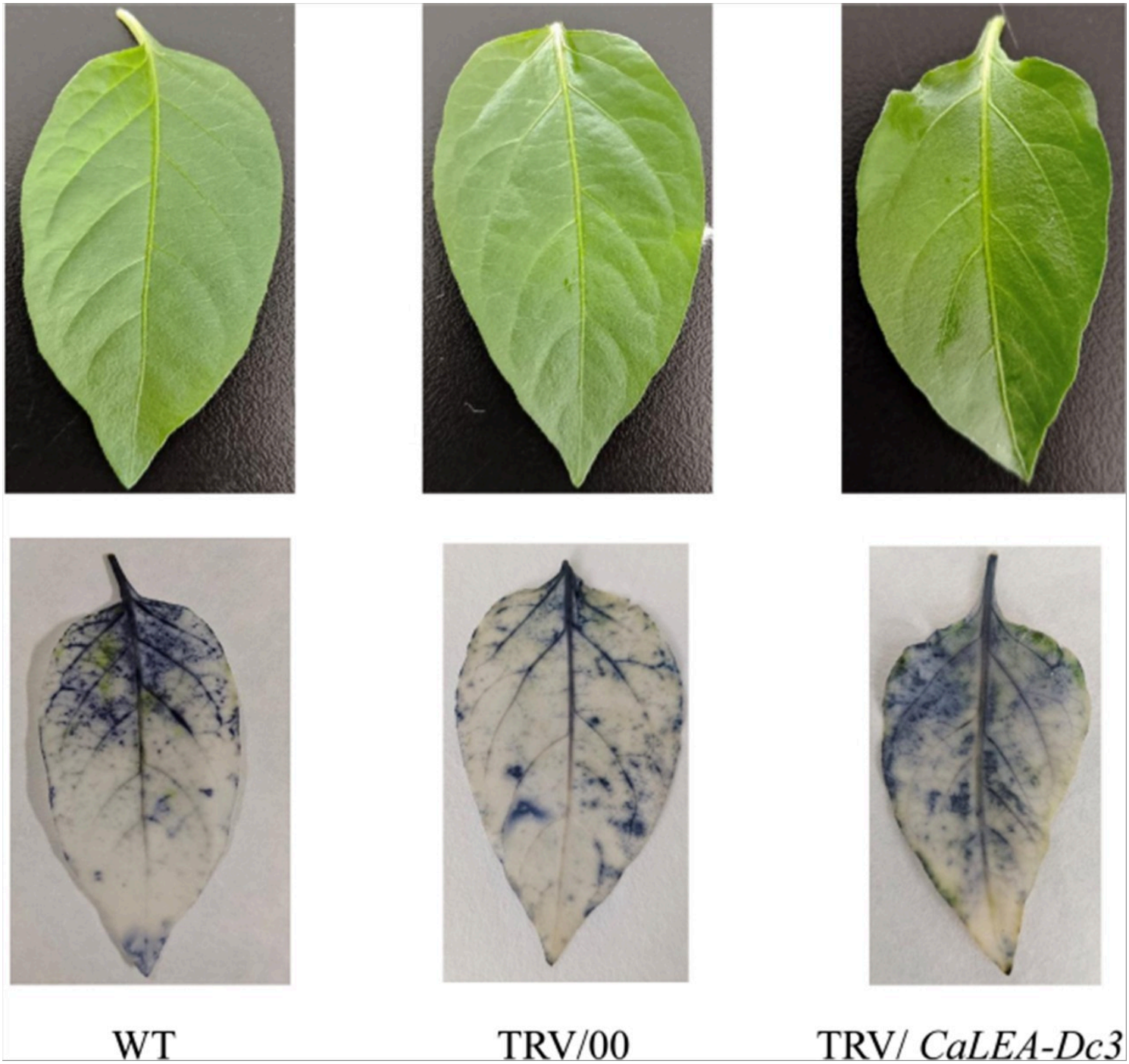

Figure 10

NBT staining