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Overexpression of a sugarcane *ScCaM* gene negatively regulates salinity and drought stress responses in transgenic *Arabidopsis thaliana*

Jinxian LIU[1]; Jingfang FENG[2]; Chang ZHANG[2]; Yongjuan REN[2]; Weihua SU[2]; Guangheng WU[1]; Xianyu FU[1]; Ning HUANG[3]; Youxiong QUE[2]; Hui LING[3,*]; Jun LUO[2,*]

[1] College of Tea and Food Science; Fujian Provincial Key Laboratory of Eco-Industrial Green Technology, Wuyi University, Nanping, 354300, China
[2] Key Laboratory of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture; Key Laboratory of Genetics, Breeding and Multiple Utilization of Crops, Ministry of Education, College of Agriculture, Fujian Agriculture and Forestry University, Fuzhou, 350002, China
[3] College of Agriculture, Yulin Normal University, Yulin, 537000, China

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Abstract: Calmodulin (CaM) proteins play a key role in signal transduction under various stresses. In the present study, the effects of a sugarcane *ScCaM* gene (NCBI accession number: GQ246454) on drought and salt stress tolerance in transgenic *Arabidopsis thaliana* and *Escherichia coli* cells were evaluated. The results demonstrated a significant negative role of *ScCaM* in the drought and salt stress tolerance of transgenic lines of *A. thaliana*, as indicated by the phenotypes. In addition, the expression of *AtP5CS* and *AtRD29A*, two genes tightly related to stress resistance, was significantly lower in the overexpression lines than in the wild type. The growth of *E. coli* BL21 cells expressing *ScCaM* showed weaker tolerance under mannitol and NaCl stress. Taken together, this study revealed that the *ScCaM* gene plays a negative regulatory role in both mannitol and NaCl stresses, and it possibly exerts protective mechanisms common in both prokaryotes and eukaryotes under stress conditions.

Introduction

Calmodulin (CaM) is an important calcium-binding protein ubiquitous in eukaryotes and is the most important receptor for Ca^{2+} (Nelson and Chazin, 1998). CaM consists of 149 amino acid residues and belongs to the classical EF-hand family with a similar EF-hand domain at both the N- and C-terminals (Gifford *et al.*, 2007). The spherical end of every EF-hand can bind to two Ca^{2+}, and therefore, one CaM can bind to four Ca^{2+}. CaM itself does not have enzymatic activity and cannot play a direct regulatory role but may interact with the downstream CaM-binding protein (CaMBP) after binding to Ca^{2+} to activate enzymatically active proteins, thereby regulating the development of plant cells and their response to external stimuli (Hoeflich and Ikura, 2002; Poovaiah *et al.*, 2013; Rhoads and Friedberg, 1997).

To date, *CaM* genes have been identified and analyzed in different plants such as *Arabidopsis thaliana* (McCormack and Braam, 2003), rice (*Oryza sativa*) (Chinpongpanich *et al.*, 2012), tomato (*Solanum lycopersicum)* (Munir *et al.*, 2016), wild tomato (*S. pennellii)* (Shi and Du, 2020), cabbage (*Brassica rapa* L. ssp. *pekinensis*) (Nie *et al.*, 2017), and apple (*Malus x domestica*) (Boonburapong and Buaboocha, 2007). *CaM* genes respond to external stimuli, such as high and low temperatures, pathogens, and hormones (Park *et al.*, 2004; Townley and Knight, 2002), and there has been rapid progress in research on the involvement of Ca^{2+}/CaM in plant stress physiology. Currently, Ca^{2+}/CaM research has received extensive attention, and research progress in various crops has been achieved. CaM in *Arabidopsis thaliana* responds to high-temperature stress (Zhang *et al.*, 2009). Ca^{2+}-CaM is involved in abscisic acid (ABA)-induced antioxidant defense, and the cross-talk between Ca^{2+}-CaM and hydrogen peroxide (H_2O_2) plays a pivotal role in the ABA signaling pathway (Hu *et al.*, 2007). In rice, CaM was found to regulate glutamate decarboxylase (GAD) activity to respond to anoxia (Reggiani *et al.*, 1995). Zhu *et al.* (2021) found that the mildew resistance locus O4 interacts with CaM/calmodulin-like (CML) and is involved in root gravity response. In plants, when responding to salt stress, CaM-dependent calcineurin functions within transduction pathways required for salt stress adaptation (Snedden and Fromm, 2001). Specific CaM isoforms are involved in response to pathogens in plants (Heo *et al.*, 1999). In planta

*Address correspondence to: Hui Ling, linghuich@163.com; Jun Luo, sisluojun@126.com
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expression disclosed that CaM is localized to the cytoplasm and nuclei of the plant cell and suppresses plant defenses against such as hydrogen peroxide (H_2O_2) accumulation and callose deposition (Fu *et al*., 2022).

Sugarcane is not only the most important cash crop globally, but it is also an important energy crop. It accounts for 92% of the total sugar production in China, and its production and yield rank 4th after that of grains, oilseeds, and cotton, conferring a special economic status (Su *et al*., 2020a; Zhang *et al*., 2018). In China, 85% of sugarcane is planted on saline-alkali soil and early sloping land, and frequent droughts, frost damage, and increasing incidences of pest infestations and diseases in recent years, especially smut, have resulted in low yields of sugarcane in many parts of China. Therefore, the tolerance or resistance of sugarcane to abiotic environmental stresses can be enhanced to improve the yield of sugarcane.

Our group previously cloned the *ScCaM* gene (NCBI accession number: GQ246454) from sugarcane and confirmed that this gene may receive calcium ion signals on the cell membrane and may be involved in the responses to plant growth and development (Liu *et al*., 2021). However, no further study of this *CaM* gene has been carried out yet. In the present study, we successfully transferred this gene into *Arabidopsis* and obtained five T_3 transgenic *Arabidopsis* lines. Hence, we intended to screen the lines of positive transgenic *ScCaM Arabidopsis* and evaluate the function of *ScCaM* overexpressing transgenic *Arabidopsis* in salinity and drought stress tolerance from the perspectives of seed germination, phenotypic observation, and also at the molecular level. We performed prokaryotic expression analysis and plate stress experiments on the ScCaM protein expressed in transgenic *Arabidopsis*. Furthermore, the role of *ScCaM* genes and their potential underlying mechanisms in response to high salinity and drought stresses were examined. This study is expected to provide reference values for functionally validated gene resources for the genetic improvement of sugarcane for stress resistance.

Materials and Methods

Acquisition of ScCaM-overexpressing transgenic Arabidopsis thaliana

Plant material and growth conditions: the *Arabidopsis* species used were *Colombia* ecotype. Seed culture conditions were 22°C, 16 h/d light, 8 h/d dark cycle, 60% relative humidity, and 2200 L× light intensity, and plants were grown in pots at 22°C, 12 h/d light, 12 h/d dark cycle, and 60% relative air humidity.

Genetic transformation of the sugarcane *ScCaM* gene into *A. thaliana* and screening of homozygous plants: the eukaryotic expression vector pBWA(V)*HS-35S-ScCaM* was constructed by introducing the *ScCaM* coding region into the overexpression vector pBWA(V)*HS-35S* using the in-fusion cloning method. The recombinant plasmid was validated and then transformed into *Agrobacterium tumefaciens* GV3101. After *Arabidopsis thaliana* had bolted, *Agrobacterium* containing the pBWA(V)*HS-35S-ScCaM* recombinant plasmid was titrated and used to infect wild-type Col-0 flowers. Next, the flowers were stored for 24 h in the dark, followed by a long period of irradiation (15 h light, 10,000–12,000 L×, 50%–60% relative humidity, and 21°C–24°C temperature) until pod maturity. T_3 generation homozygous strains of transgenic *ScCaM Arabidopsis* were finally obtained using hygromycin B screening and polymerase chain reaction (PCR) amplification assays.

Screening and characterization of T_3 generation ScCaM-overexpressing Arabidopsis plants

Screening culture was performed by using Murashige and Skoog (MS) medium (Murashige and Skoog 4.4 g/L, agar 8 g/L, adjusted to pH5.8) containing 100 g/L of hygromycin B, and three seedlings were transplanted to one planting pot when they grew to the 3–4-leaf stage. On reaching the 5–8-leaf stage, RNA from plant leaf was extracted to detect whether the transgenic plants were successfully transformed to overexpress *ScCaM*. HYG-F/R. For performing RT-PCR, pBWA(V)*HS-35S-ScCaM*-F/R were used as primers, pBWA (V)*HS-35S-ScCaM* plasmid was used as a positive control, while water was used as a blank control. cDNA obtained following RNA reverse transcription was used as a template for PCR amplification and electrophoresis. The PCR procedure was as follows: pre-denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, denaturation at 55°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 10 min, and holding at 4°C. The primer sequences and RT-PCR system are shown in Suppl. Tables S1 and S2, respectively.

Salinity stress treatment of ScCaM-overexpressing Arabidopsis thaliana

Seeds of T_3 generation transgenic overexpressing *Arabidopsis thaliana* and wild-type Col-0 *Arabidopsis thaliana* were sterilized using 2% sodium hypochlorite, and the seeds were grown on MS plates containing 0, 100, or 150 mM sodium chloride (NaCl) after sterilization and incubated continuously for 12 d, during which germination potential was observed, and germination rate was determined. A line graph of germination rate was plotted using Origin 8.0 software. *Arabidopsis* plants were grown for about 4 weeks, and those with uniform growth were selected and subjected to salinity stress. Specifically, 15 mL of 300 mM NaCl was poured on the *Arabidopsis* plants once every two days for 12 d. Wild-type Col-0 plants were used as controls, their phenotypes were observed, and photographs were acquired. At 12 d of treatment, *Arabidopsis* leaf RNA was extracted using Trizol and reversed transcribed into cDNA using the Novozymes HiScript® Q RT SuperMix for qPCR (+gDNA wiper) kit for further expression analysis of resistance-related genes.

Drought stress treatment of ScCaM-overexpressing Arabidopsis thaliana

Sterilized *ScCaM*-overexpressing T_3 generation *Arabidopsis* and wild-type Col-0 *Arabidopsis* seeds were spotted on MS medium plates containing 0, 200, or 300 mM mannitol and incubated continuously for 12 d. Germination potential was observed, and the germination rate was determined. A line graph of germination rate was plotted using Origin 8.0 software. When the *Arabidopsis* plants were grown for about 4 weeks, the plants with uniform growth were selected for drought stress treatment. Specifically, the conditions were

natural drought (no watering) for 11 d and rehydration for 5 and 11 d, and wild-type Col-0 plants were used as the controls, during which their phenotypes were observed and photographs were acquired. At 11 d of treatment, *Arabidopsis* leaf RNA was extracted and reverse transcribed into cDNA for further expression analysis of resistance-related genes.

Expression analysis of resistance-related genes in ScCaM-overexpressing Arabidopsis thaliana

Quantitative reverse transcription-PCR (RT-qPCR) was used to analyze the expression of some *Arabidopsis* stress-related genes under salinity and drought stress, including genes related to reactive oxygen species (ROS) scavenging (*AtSOD*, *AtCAT*, and *AtAPX*) (Yang *et al.*, 2015), ABA response (*AtNCED3*, *AtP5CS*, and *AtRD29A*) (Qiu *et al.*, 2020), salinity stress (*AtSOS3*), and drought stress (*AtCDPK1*) (Yang *et al.*, 2015), with *Atactin2* as an internal reference gene (Zhu, 2002). Primer sequences are listed in Suppl. Table S1. The qRT-PCR steps were as follows: 50°C, 2 min; 95°C, 2 min; 40 cycles of 95°C, 10 min; 95°C, 15 s; 60°C, 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Significance ($p < 0.05$) was calculated using one-way ANOVA. Duncan's new multiple range test was calculated using DPS 9.50 software, and data are expressed as the mean ± standard error (SE). Histograms were constructed using Origin 8.0 software.

Expression analysis of ScCaM in Escherichia coli cells under salinity and drought stress

We constructed the prokaryotic expression vector *ScCaM-pGEX-4T-1* and determined the optimal conditions for *ScCaM* expression in *E. coli* cells as 16°C, 20 h, and induced by 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) (Su *et al.*, 2020b). In this study, we first tested whether the optimal temperature and IPTG concentration could induce the expression of ScCaM at the protein level. Following the successful induction of *ScCaM-pGEX-4T-1*, recombinant *E. coli* cells were subjected to salinity and drought stress experiments to analyze the tolerance of ScCaM protein to salinity and drought in *E. coli* cells. The procedure was as follows: pGEX-4T-1 and *pGEX-4T-1-ScCaM* were transformed in prokaryotic expression strain *E. coli* BL21 (DE3), and single colonies of recombinant bacteria were cultured in ampicillin (70 μg/mL)-containing LB broth. The cells were incubated at 37°C with shaking at 200 rpm until OD_{600} = 0.6. Subsequently, 0.5 mM IPTG (to analyze the expression of TCGT transcript) was added, and the cells were incubated at 16°C in a 200 rpm shaking incubator for induction. The bacterial suspensions were collected at intervals of 0, 2, 8, and 20 h. The bacteria were then collected via centrifugation at 10,000 g for 10 min at 26°C, mixed with 6× protein loading buffer, boiled at 100°C for 5 min, and centrifuged again at 10,000 g for 5 min at room temperature. Next, 10 μL of supernatant was resolved by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, followed by coomassie brilliant blue staining and photograph acquisition. The above bacterial broth collected after 20 h of IPTG induction was diluted to the same concentration using LB broth, i.e., OD_{600} = 0.7, following which the broth was diluted to 10^{-3} and 10^{-4} and used as a stock solution. The diluted broth was spotted onto LB agar plates containing 70 μg/mL ampicillin, incubated overnight at 37°C, and photographed for retention.

Results

Screening and characterization of T3 generation ScCaM-overexpressing Arabidopsis plants

As shown in Suppl. Fig. S1, T_3 generation *ScCaM*-overexpressing *Arabidopsis* seeds were essentially homozygous by screening cultures on MS medium plates exhibiting hygromycin resistance.

Fig. 1 shows the amplification products of *HYG* and *ScCaM* genes in *Arabidopsis* plants. As shown in Figs. 1A and 1C, a single band of approximately 750 bp in size was amplified when the overexpression plasmid was used as a template and generally matched the length of the vector *HYG* tag. A single band consistent with the overexpression plasmid was identified in samples 4 to 9, and no band was identified in samples 1 to 3. As shown in Figs. 1B and 1D, a single band was amplified when the overexpression plasmid was used as a template, which was approximately 450 bp in size and generally matched the length of the *ScCaM* open reading frame. The results indicate that pBWA(V)*HS-35S-ScCaM* was successfully expressed in the *Arabidopsis* transgenic strain, and the selected *Arabidopsis* plants could be used for the next step of analysis for salinity and drought tolerance.

Germination status of T_3 generation transScCaM Arabidopsis seeds under salinity and drought stresses

Selected transgenic *ScCaM* T_3 generation lines, T_3-1 and T_3-5, and wild-type Col-0 *Arabidopsis* seeds were grown on MS blank medium plates and MS medium plates containing 100 mM NaCl, 150 mM NaCl, 200 mM mannitol, and 300 mM mannitol, respectively. The germination rate and growth after 12 days of continuous incubation are shown in Fig. 2, according to which both transgenic lines T_3-1 and T_3-5 were able to germinate on MS medium plates. The germination rate of Col-0 seeds was 95.24%, and all germinated plants were green, suggesting that they were able to grow normally (Figs. 2A and 2B). The germination rates differed in T_3-1, T_3-5, and wild-type Col-0 seeds on MS medium plates containing different concentrations of NaCl and mannitol, but the differences were generally small (Figs. 2C–2F). Thus, overexpression of the *ScCaM* gene did not affect the tolerance of transgenic *Arabidopsis* to salinity and drought stresses during seed germination.

Phenotypic analysis of ScCaM transgenic Arabidopsis lines under salinity and drought stresses

To investigate the salinity and drought tolerance of transgenic *ScCaM Arabidopsis* plants, we treated the plants with 300 mM NaCl solution in water and performed natural drought treatments. As shown in Fig. 3A, there was no significant difference in the growth of Col-0, T_3-1, and T_3-5 *Arabidopsis* plants before salinity stress, and after 7 days and 12 days of salinity stress, chlorosis was observed in the leaves of Col-0, T_3-1, and T_3-5 plants; however, it was more severe in the leaves of T_3-1 and T_3-5 plants than in the leaves of Col-0 plants. As shown in Fig. 3B, there was no

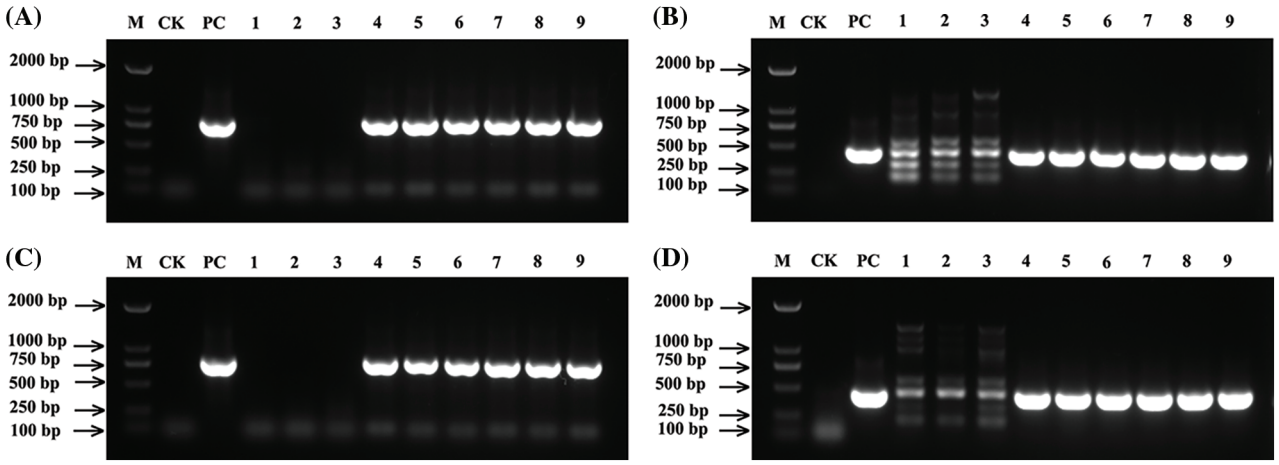

FIGURE 1. Identification of *ScCaM* gene positive transgenic *Arabidopsis thaliana*.
Note: (A and C) PCR detection electropherogram of overexpression vector pBWA(V)HS-35S *HYG* tag; (B and D) PCR detection electropherogram of *ScCaM* gene; M: 2000 bp marker; CK: blank control; PC: PCR amplification product of recombinant plasmid pBWA (V)HS-35S-*ScCaM*; lanes 1~3: PCR amplification products of wild-type *Arabidopsis thaliana* plants; lanes 4~6: PCR amplification products of transgenic *Arabidopsis thaliana* T_3-1 with *ScCaM* gene; lanes 7~9: PCR amplification products of transgenic *Arabidopsis thaliana* T_3-5 with *ScCaM* gene.

significant difference in the growth of Col-0, T_3-1, and T_3-5 *Arabidopsis* plants before drought treatment, and after 11 days of drought treatment, chlorosis was observed in the leaves of Col-0 and *ScCaM*-transformed T_3-1 and T_3-5 plants; however, chlorosis was more severe in the leaves of T_3-1 and T_3-5 plants than in the leaves of Col-0 plants. Thus, the transgenic *ScCaM Arabidopsis* plants were less tolerant to salinity and drought stress than the Col-0 plants.

Expression analysis of stress resistance-related genes in ScCaM overexpressing transgenic Arabidopsis plants under salinity and drought stresses

We analyzed the expression of relevant stress resistance genes to further clarify the molecular mechanisms underlying drought and salinity tolerance in *ScCaM* overexpressing transgenic *Arabidopsis*.

As shown in Fig. 4A, the expression of *AtNCED3, AtP5CS*, and *AtRD29A* was significantly downregulated in *ScCaM* overexpressing transgenic *Arabidopsis* T_3-1 and T_3-5 lines after 12 d of salinity stress, which was lower than that of the wild type, respectively. However, the expression of the ROS scavenger gene, *AtSOD*, and salinity stress response-related gene, *AtSOS3*, was significantly upregulated in the T_3-1 line, which was 4.28-and 2.04-fold higher than that of the control, respectively. Interestingly, the expression of this gene was not significantly different in the T_3-5 line compared with the control, and the expression of *AtCAT, AtAPX*, and *AtCDPK1* was not significantly different in Col-0, T_3-1, and T_3-5 plants.

As shown in Fig. 4B, after 12 d of drought stress, the expression of *AtP5CS* and *AtRD29A* was significantly downregulated in both T_3-1 and T_3-5 plants, which was lower than that of the control, respectively. The ROS scavenging enzyme-coding genes, *AtSOD* and *AtCAT*, were significantly upregulated in both the T_3-1 and T_3-5 lines, which were higher than that of the wild type, respectively. The ABA pathway-related gene, *AtNCED3*, was significantly upregulated in the T_3-1 line, which was 5.11-fold higher than that of the wild type, and the expression of this gene was not significantly different in the T_3-5 line compared with the wild type. The expression of *AtAPX, AtSOS3*, and *AtCDPK1* was not significantly different in Col-0, T_3-1, and T_3 -5 plants.

Overall, the expression of *AtNCED3, AtP5CS*, and *AtRD29A* was significantly downregulated, and the expression of *AtSOD* and *AtSOS3* was significantly upregulated in the transgenic lines under salinity stress. However, the expression of *AtP5CS* and *AtRD29A* was significantly downregulated, and the expression of *AtSOD*, *AtCAT*, and *AtNCED3* was significantly upregulated in the transgenic lines under drought stress. Notably, the expression of *AtP5CS* and *AtRD29A* was suppressed in both T_3-1 and T_3-5 lines under salinity and drought stresses, and it is speculated that *ScCaM* attenuated the tolerance of the transgenic plants to salinity and drought mainly by reducing the expression of *AtP5CS* and *AtRD29A* genes.

Expression of ScCaM in Escherichia coli BL21 (DE3) strain

To find the role of the *ScCaM* gene in salinity and drought resistance in prokaryotes, *ScCaM* protein expression was induced as previously described (Liu *et al.*, 2021), and stress experiments in LB plates with different concentrations of NaCl and mannitol were performed. As shown in Fig. 5, the accumulation of recombinant protein was observed after 8 h and 20 h induction by 0.5 mM IPTG. Based on EXPASY ProtParam, the estimated molecular weight of ScCaM protein was 16.83 kDa, and the molecular weight of GST protein was 26 kDa; the target protein of GST-ScCaM of about 43 kDa molecular weight was induced and was used for further plate stress experiments.

Analysis of ScCaM expression in Escherichia coli cells under salinity and drought stresses

Plate stress experiments for the prokaryotic expression of the recombinant bacterium BL21 with pGEX-4T-1-*ScCaM* are shown in Fig. 6. According to Fig. 6A, after 12 h of incubation at 37°C, there was no significant difference in the growth of pGEX-4T-1-*ScCaM* and pGEX-4T-1 cells on LB plates containing 70 μg/mL ampicillin. Besides,

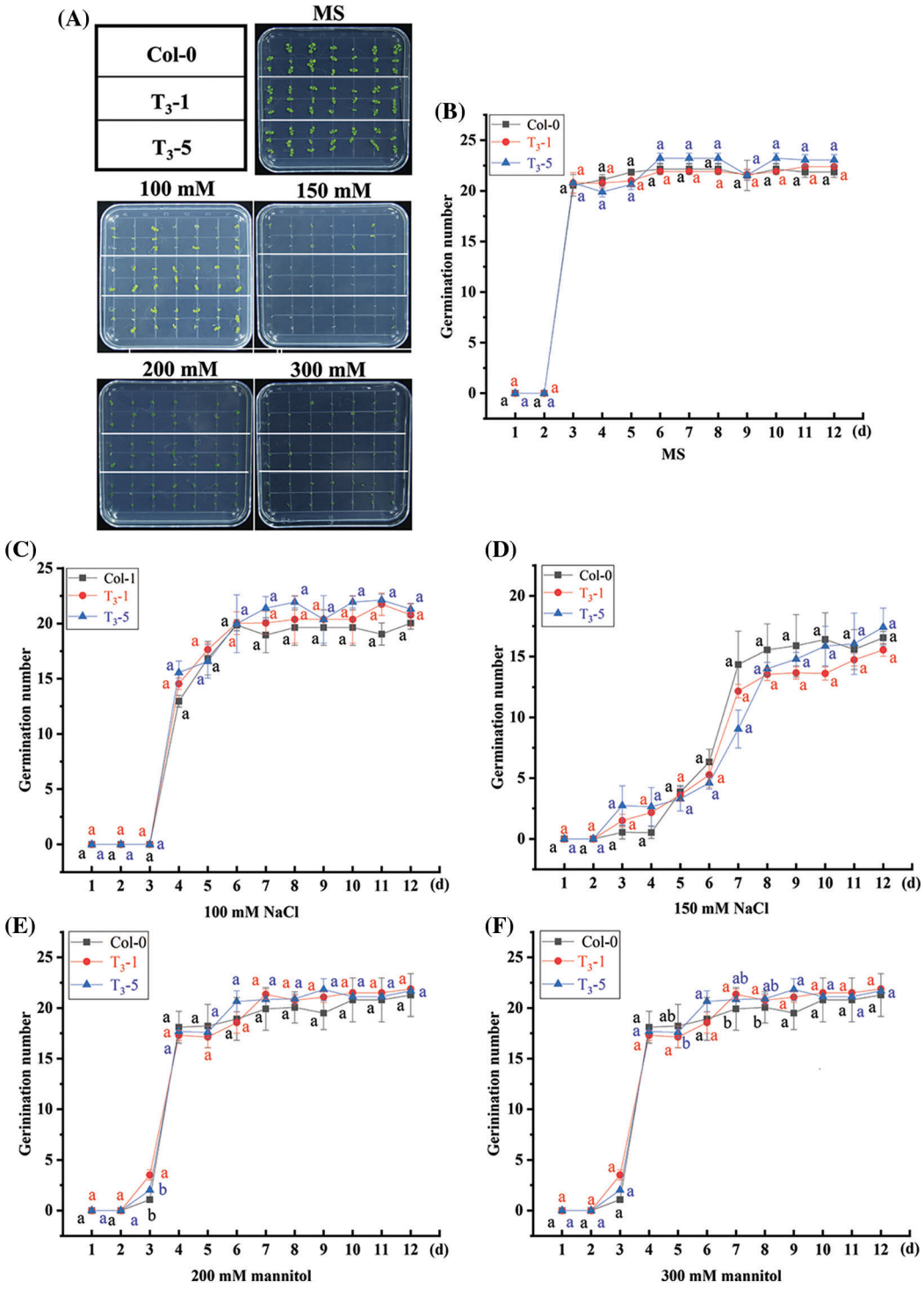

FIGURE 2. Germination of T_3 generation seeds of transgenic *Arabidopsis thaliana* overexpressing *ScCaM* gene under drought and salt stress. Note: (A) The growth status of *Arabidopsis thaliana* seeds on MS blank medium plates and MS medium plates containing 100 mM NaCl, 150 mM NaCl, 200 mM mannitol, and 300 mM mannitol for 12 days; (B), (C), (D), (E) and (F) are line graphs of germination rate of *Arabidopsis* seeds cultured on MS blank medium plates and MS medium plates containing 100 mM NaCl, 150 mM NaCl, 200 mM mannitol, and 300 mM mannitol for 12 days, respectively. The experiments were randomized, two replicates were used to avoid plate effects, and the germination number was calculated from the multiple plates. Significance ($p < 0.05$) was calculated using one-way ANOVA. Duncan's new multiple range test was calculated using DPS 9.50 software, and data are expressed as the mean ± standard error (SE).

pGEX-4T-1-*ScCaM* and pGEX-4T-1 cells did not differ in growth on 100 mM NaCl and 500 mM NaCl stress plates, i.e., both could grow at 100 mM NaCl with no significant difference in growth, whereas neither could grow on 500 mM NaCl stress plates. However, the growth of both pGEX-4T-1 and pGEX-4T-1-*ScCaM* cells was inhibited on 300 mM NaCl stress plates, with the growth status of pGEX-4T-1-*ScCaM* being significantly weaker than that of pGEX-4T-1. It is thus hypothesized that *ScCaM* attenuated the tolerance of *E. coli* to NaCl stress.

Fig. 6B showed that the growth of pGEX-4T-1-*ScCaM* and pGEX-4T-1 cells on LB plates with 70 μg/mL ampicillin

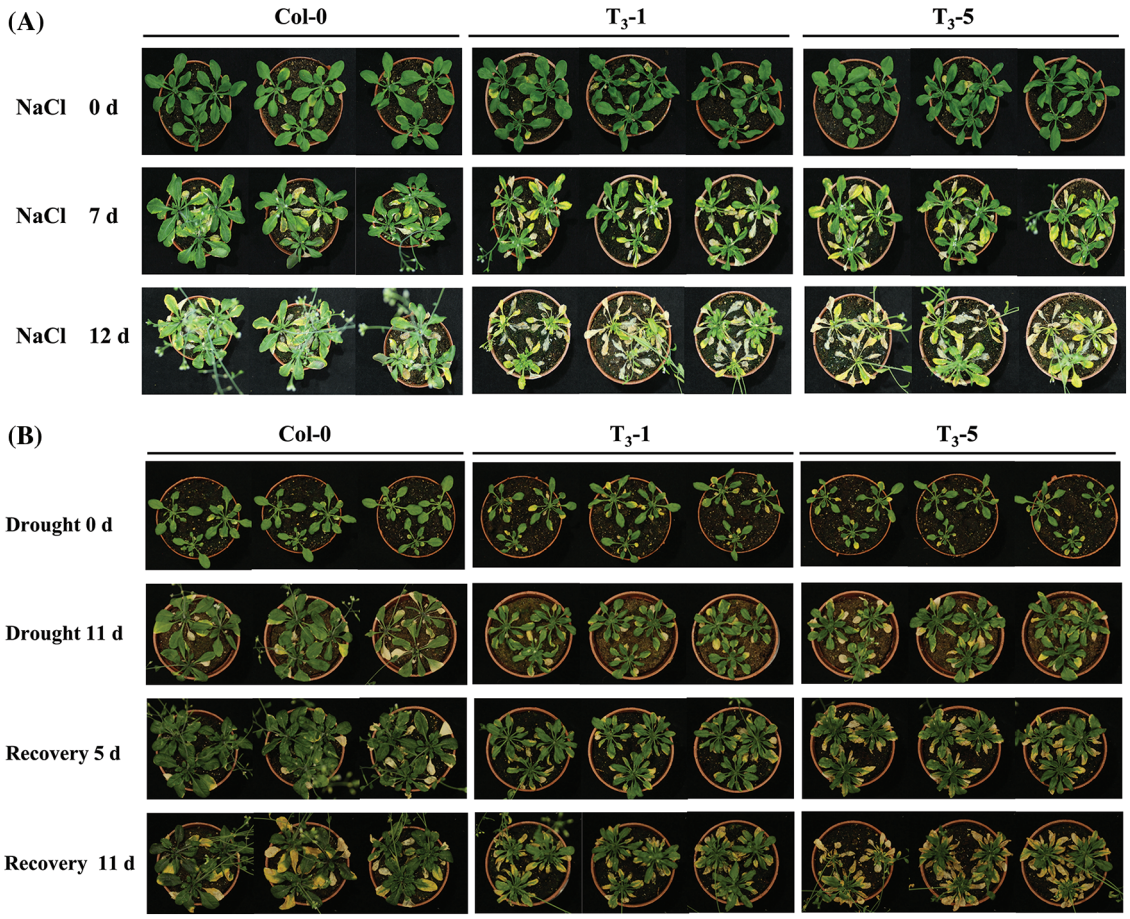

FIGURE 3. Phenotypic analysis of T_3 lines *ScCaM* overexpressing transgenic *Arabidopsis thaliana* under salt and drought stress.
Note: (A) Phenotypes of 4-weeks-old plants of the transgenic line (T_3-1 and T_3-5) and the wild type (Col-0) before and after NaCl treatment. (B) Phenotypes of 4-weeks-old plants of the transgenic line (T_3-1 and T_3-5) and the wild type (Col-0) before and after drought treatment. Each plot contained three *Arabidopsis thaliana* plants.

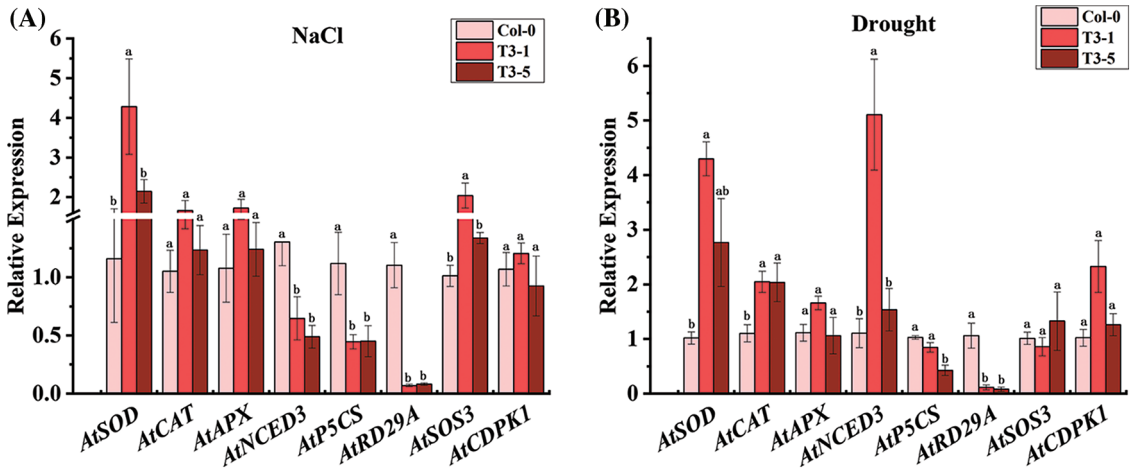

FIGURE 4. Expression analysis of stress-related genes by qRT-PCR in transgenic *Arabidopsis thaliana* plants overexpressing *ScCaM* under NaCl and drought stress.
Note: *Atactin2* was used as the reference gene. All data points shown are mean ± SE (n = 3). Different lowercase letters indicate a significant difference, as determined by Duncan's new multiple range test ($p < 0.05$).

did not differ much after 12 h of incubation at 37°C. Non-recombinant pGEX-4T-1 could grow on 500, 750, and 1000 mM mannitol stress plates. In contrast, pGEX-4T-1-*ScCaM* cells only grew on 500 mM mannitol stress plates, and there was no colony on 750 mM mannitol or 1000 mM mannitol stress plates; besides, the growth of pGEX-4T-1-*ScCaM* cells on 500 mM mannitol stress plates was weaker than that of pGEX-4T-1 cells. Thus, it was hypothesized that *ScCaM* also attenuated the tolerance of *E. coli* to mannitol stress.

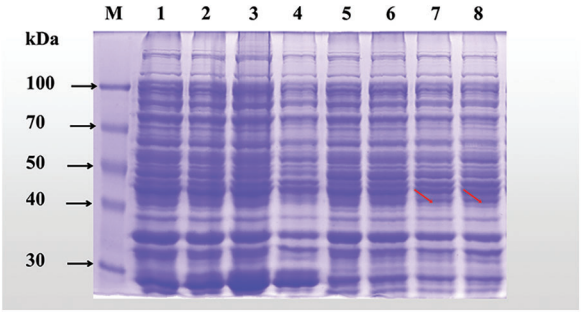

FIGURE 5. Prokaryotic expression of the pGEX-4T-1-ScCaM fusion protein.
Note: M: Protein marker; 1~4: control induction for 0, 2, 8, and 20 h; 5~8: recombinant strain pGEX-4T-1-ScCaM induction for 0, 2, 8, and 20 h.

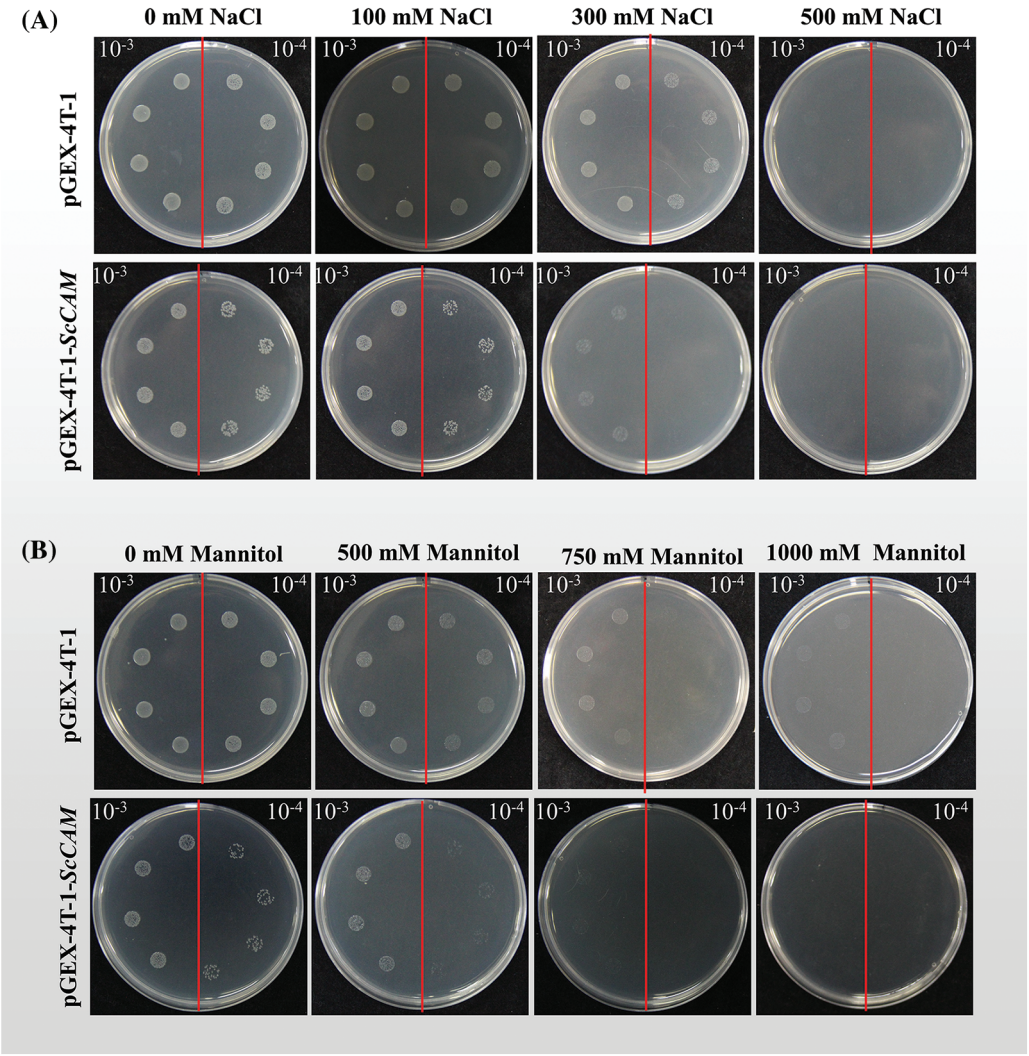

FIGURE 6. The spot assays used for monitoring the growth performance of BL21/pGEX-4T-1 and BL21/pGEX-4T-1-*ScCaM* transformed *E. coli* cells on LB plates under NaCl and mannitol stress.
Note: The cultures were adjusted to OD_{600} = 0.7 after IPTG induction; 10 μL from 10^{-3} (left side of the red line on the plate) and 10^{-4} (right side of the red line on plate) dilutions of the culture were spotted onto LB medium plate. The growth performance of BL21/pGEX-4T-1 and BL21/pGEX-4T-1-*ScCaM* cells on LB plates without supplements or stresses were used as controls. (A) The growth performance of BL21/pGEX-4T-1 and BL21/pGEX-4T-1-*ScCaM* cells on LB plates with 100 mM, 300 mM, and 500 mM NaCl supplements are used as treatments. (B) The growth performance of BL21/pGEX-4T-1 and BL21/pGEX-4T-1-*ScCaM* cells on LB plates with 500, 750, and 1000 mM mannitol supplements are used as treatments.

Discussion

Calcium (Ca^{2+}), as an important intracellular second messenger, was related to various biological programs (Perochon *et al.*, 2011). CaMs proteins, which contain four EF-hand, are vital transducers of Ca^{2+} signals. It is worth noting that CaMs lack effector domains and they function as sensor relays via Ca^{2+}-dependent interaction with the downstream effector proteins (Perochon *et al.*, 2011). Current studies have shown that CaMs bind and regulate a vast array

of target proteins in plants, including transcription factors, metabolic enzymes, kinases, and so on (Astegno *et al.*, 2016; Vandelle *et al.*, 2018). In addition, CaM proteins are involved in many cellular responses through binding to Ca^{2+} and play an important role, especially in abiotic stresses (Galon *et al.*, 2010; Poovaiah *et al.*, 2013).

Previous studies have shown that drought and salt stresses changed the concentration of Ca^{2+} (Knight *et al.*, 1997). Calcium signaling has been implicated in the transduction of drought- and salt-stress signals in plants and may play a role in a number of responses to drought stress and water potential changes (Johansson *et al.*, 1996; Takahashi *et al.*, 1997). CaM is considered to regulate several intracellular proteins involved in modulating intracellular Ca^{2+} levels and provides a negative feedback mechanism for calcium signaling (Kovacs *et al.*, 2010). Shen *et al.* (2020) found that *HvCaM1* negatively regulates salt tolerance via modulation of the expression of *HvHKT1s* and *HvCAMTA4*. On the contrary, *OsCaM1-1* overexpression in the transgenic rice mitigated salt-induced oxidative damage (Kaewneramit *et al.*, 2019; Yuenyong *et al.*, 2018). In this study, the experiments of eukaryotic overexpression and prokaryotic expression were conducted to explore the function of *ScCaM* under NaCl and drought stresses. The above results indicated that *ScCaM* plays a negative regulatory role in response to NaCl and drought stresses. CaMs play both positive and negative roles in plant immunity (Cheval *et al.*, 2013; Poovaiah *et al.*, 2013). Similarly, calcium/calmodulin-dependent protein kinase is negatively and positively regulated by CaM (Miller *et al.*, 2013). In *Arabidopsis*, *CaM2*, *CaM3*, *CaM5*, and *CaM7* were found to negatively regulate Ca^{2+} influx under heat shock conditions (Niu *et al.*, 2020). Many studies have also suggested a positive regulatory role for CaM. For instance, a previous study found that overexpression of *GmCaM4* in soybean enhanced resistance to pathogens and tolerance to salinity stress (Rao *et al.*, 2014). Zhou *et al.* (2016) demonstrated that the expression of *AtCaM1/4* was upregulated under salt stress, and it resulted in enhanced salt tolerance in transgenic *A. thaliana*. Overexpression of *EcCaM* in *A. thaliana* was found to make the plant more tolerant to polyethylene glycol (PEG)-induced drought and salt stress (NaCl) (Jamra *et al.*, 2021). Noman *et al.* (2019) even thought that *GmCaMTA12* may enhance the tolerance of *Arabidopsis* plants under drought stress through *Ca-CaM-CAMTA*-mediated stress regulatory mechanisms.

Interestingly, when the germination rate of *Arabidopsis* seeds overexpressing *GmCaMTA12* was determined after treatment with mannitol, it was observed that the germination rate of transgenic *Arabidopsis* seeds was significantly higher than that of the wild type (Noman *et al.*, 2019). In the present study, the growth potential of *ScCaM* transgenic *Arabidopsis* seeds under NaCl and mannitol stress was weaker than that of the wild type, and *ScCaM overexpressing* transgenic *Arabidopsis* plants were less tolerant to salinity and drought stresses than the wild type, whereas the expression of *AtP5CS* and *AtRD29A*, two genes tightly related to stress resistance, were also significantly lower than that in the wild type. High expression of *AtSOD* and *AtCAT* genes mitigates the negative effects of ROS and enhances the tolerance of plants under abiotic stress (Ju *et al.*, 2020; Wang *et al.*, 2021). CaM can regulate ROS-scavenging enzymes (SOD and CAT) (Ming and Zhong-Guan, 1995; Yang and Poovaiah, 2002). However, in this study, a sensitive phenotype was observed. Based on these results, we thus suppose that high expression of *SOD/CAT* protects cells against ROS, but low expression of the *P5CS* gene synthesizes less proline as osmotic substances (Jain *et al.*, 2015). The sensitivity of plants is mainly due to the lack of osmotic substances. *AtRD29A* was found to function in ABA-related response to stress (Liu *et al.*, 2020). We thus speculated that overexpression of *ScCaM* may inhibit the expression of *AtRD29A* by inhibiting the ABA signal transduction. Our previous study showed that overexpression of the allogenic *ScCaM* gene had effects on the growth of transgenic plants (Liu *et al.*, 2021). In this study, we found that the transgenic plants also had a specific phenotype after stress. Hence, a working model was thus proposed in this study (Fig. 7). Based on the model, we can notice that *ScCaM* may influence the catabolism of osmotic substances and the ABA signal transduction. However, the relationship between growth and stresses in transgenic plants still requires further studies. This may be the reason why *ScCaM* transgenic *Arabidopsis* plants were less tolerant to salinity and drought stresses. Interestingly, overexpression of *ScCaM* in *E. coli* cells also expressed weaker tolerance under salinity and drought stresses.

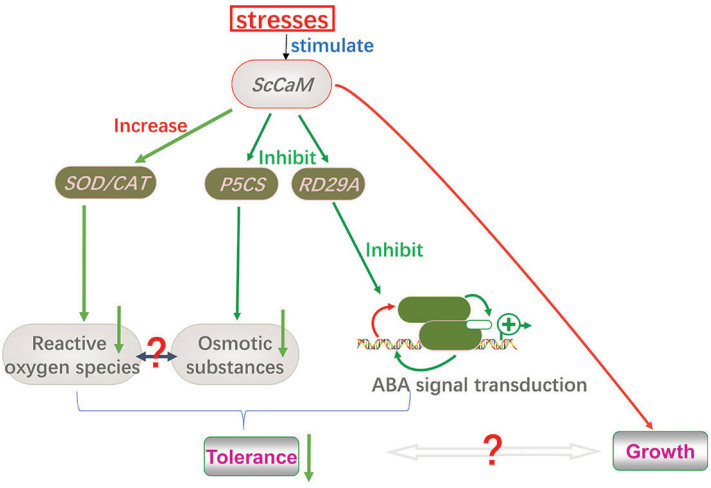

FIGURE 7. A working model of the function of sugarcane calmodulin (ScCaM) in negatively regulating the salinity and drought stress in *Arabidopsis* (Jain *et al.*, 2015).

Taken together, the present study indicates that the *ScCaM* gene plays a negative regulatory role under salinity and drought stresses, possibly through influencing the protective mechanisms common in both prokaryotes and eukaryotes under stress conditions.

Conclusion

In summary, *ScCaM* is a negative regulator in response to salinity and drought stress, and it is speculated that *ScCaM* mainly reduces the expression of *AtP5CS* genes, thus weakening the tolerance to salinity and drought stresses in transgenic plants and *E. coli* cells. However, the specific regulatory mechanism still needs to be investigated. The present study lays the foundation for further analysis of the functional role of *ScCaM* and its regulatory mechanism in sugarcane. Once this regulatory mechanism is understood, it should facilitate the application of transgenic *ScCaM* sugarcane in the near future.

Availability of Data and Materials: All relevant data supporting the conclusions of this article are mentioned in this article.

Author Contribution: J.L., Y.R., J.F., and J.L. conceived, designed, and initiated the project. J.L., C.Z., G.W., and X.F. prepared materials. J.L., Y.R., J.F., C.Z., G.W., X.F., and N.H. performed experiments and contributed to data analysis and validation. J.L., J.L., H.L., W.S., and Y.Q. drafted the manuscript. J.L., J.L., H.L., W.S., and Y.Q. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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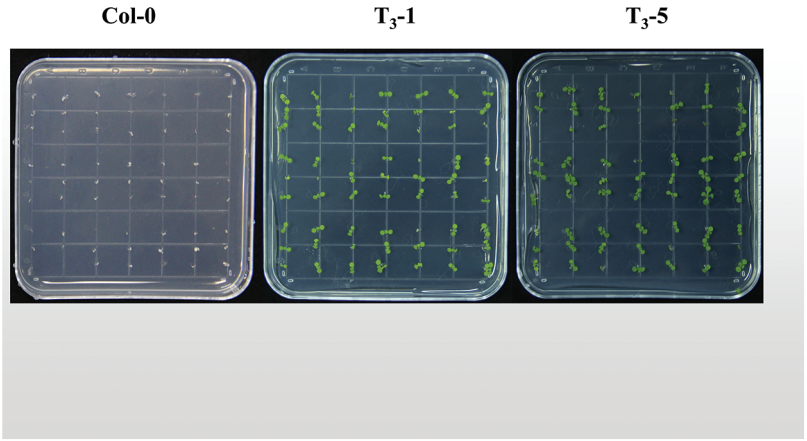

SUPPLEMENTARY FIGURE S1. Selection of T3 generation transgenic overexpressing *ScCaM* *Arabidopsis* seeds on plates containing hygromycin.

TABLE S1

Primers used in this study

Primer	Forward primer (5′-3′)	Reverse primer (5′-3′)	Strategy
HYG	TAGGAGGGCGTGGATATGTC	TACACAGCCATCGGTCCAGA	RT-PCR analysis
pBWA(V)HS-35S-ScCaM	CGGGATCCATGGCGGACCAGCTCACC	CGGAATTCTCACTTGGCCATCATCACCTTG	RT-PCR analysis
AtSOD1-Q	AACTGCCACCTTCACAATCACT	ATGGACAACAACAGCCCTACC	qRT-PCR analysis
AtCAT1-Q	TCCTGTTATCGTTCGTTTCTCA	CAAAGTTCCCCTCTCTGGTGTA	qRT-PCR analysis
AtAPX1-Q	AAATACGCTGCTGATGAAGATG	GGAGACACACACACACACACAG	qRT-PCR analysis
AtNCED3-Q	CGAGCCGTGGCCTAAAGTCT	GCTCCGATGAATGTACCGTGAA	qRT-PCR analysis
AtP5CS-Q	GGTGGACCAAGGGCAAGTAAGATA	TCGGAAACCATCTGAGAATCTTGT	qRT-PCR analysis
AtRD29A-Q	GATAACGTTGGAGGAAGAGTCGG	TCCTGATTCACCTGGAAATTTCG	qRT-PCR analysis
AtSOS3-Q	ATCGAGCGAGAAGAATTGAAAG	CGAAAGCCTTATCCACCATTAC	qRT-PCR analysis
AtCDPK-Q	ATCCCTATGAGCAATCAAACT	AATCGCTACCTCACGACGAAC	qRT-PCR analysis
AtActin2-Q	GGAAGGATCTGTACGGTAAC	TGTGAACGATTCCTGGACCT	qRT-PCR analysis

TABLE S2

RT-PCR system

Reaction system	Volume (μL)
ddH_20	9.5
2 × Es Taq MasterMix(Dye)	12.5
Forward primer	1
Reverse primer	1
cDNA templates	1
Total volume	25