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Research Article

CONSTANS-LIKE 1a positively regulates salt and drought tolerance in soybean

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C.X., J.S., and T.L. performed the gene cloning, soybean transformation, phenotype observations and measurements. Y.Z. performed dual-luciferase assay. C.X. performed the ChIP assays, ChIP-qPCR, and EMSA. Q.W., N.X., Y.J., and M.W. performed the expression analysis and data analysis. C.X. and L.Z. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.
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Abstract

Salt and drought stresses are major factors limiting soybean (*Glycine max* [L.] Merr.) growth and development; thus, improving soybean stress tolerance is critical. In this study, both salt stress and drought stress induced mRNA levels of *CONSTANS-like 1a* (*GmCOL1a*) and stabilized the GmCOL1a protein. Transgenic *35S:GmCOL1a* soybean plants exhibited enhanced salt and drought tolerance, with higher relative water content in leaves, greater proline content, lower malondialdehyde (MDA) content, and less reactive oxygen species (ROS) production compared with wild-type plants; the *GmCOL1a* knockout *co-9* mutant showed opposite phenotypes. In addition, *GmCOL1a* promoted the expression of genes related to salt tolerance, effectively reducing the Na^+/K^+ ratio in soybean plants, especially in stems and leaves of *35S:GmCOL1a* soybean. Chromatin immunoprecipitation sequencing (ChIP-seq) analysis identified two potential direct targets of GmCOL1a, late embryogenesis abundant (*GmLEA*) and Δ^1-pyrroline-5-carboxylate synthetase (*GmP5CS*) genes, which were verified by chromatin immunoprecipitation quantitative polymerase chain reaction (ChIP-qPCR), electrophoretic mobility shift assay (EMSA), and transient transcriptional activation assays. GmCOL1a bound directly to the Myc(bHLH)-binding and Che-binding motifs of *GmLEA* and *GmP5CS* promoters to stimulate mRNA expression. Analysis of transgenic hairy-root *GmP5CS:GmP5CS* soybean plants in wild type, *co-9*, and *35S:GmCOL1a* backgrounds further revealed that GmCOL1a enhances salt and drought tolerance by promoting GmP5CS protein accumulation in transgenic soybean hairy roots. Therefore, we demonstrate that GmCOL1a plays an important role in tolerance to abiotic stress in soybean.

Introduction

Soybean (*Glycine max*) plants are important economic oil crops that provide a major source of oil and protein. When plants are subjected to abiotic stressors, such as salt and drought, they affect seed germination, crop growth, and yield (Farooq et al., 2009; Zelm et al., 2020). Currently, saline soils cover more than 800 million hectares, accounting for about 6% of the world's total soil area (Rengasamy, 2002; Munns and Tester, 2008). Drought stress is one of the most severe constraints that inhibit plant growth and development and limits plant productivity (de Brito et al., 2021). Therefore, it is important to understand the genetic and regulatory mechanisms of salt and drought tolerance in soybean and breed soybean varieties with excellent salt and drought tolerance.

Salt stress is usually caused by high concentrations of sodium and chloride ions in the soil (Ismail et al., 2014). Salt stress includes three pathways: osmotic, ionic, and

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secondary stress. A high salt concentration disrupts the dynamic balance of water potential and ion distribution in plant cells, causing loss of greenery, dehydration, and cell necrosis and preventing normal plant growth (Munns and Tester, 2008; Xie et al., 2008). For plants, sodium ions (Na^+), the major ion in saline soils, are absorbed from the root system and accumulate in photosynthetic tissues, resulting in ion imbalance and reduced productivity. In response to salt stress, plants have a range of salt tolerance mechanisms, including enhanced Na^+ rejection, limited Na^+ uptake, and regulation of cellular ion balance (especially Na^+/K^+ ratio; Zhu, 2002; Ishikawa and Shabala, 2019). When plants are stimulated by abiotic stress, the balance between the production and scavenging of reactive oxygen species (ROS) in vivo is disrupted, and ROS levels are elevated, resulting in oxidative damage in plants (Fahnenstich et al., 2008). In addition to the plant's ability to balance intracellular ROS through enzymatic degradation and the use of small molecules, such as ascorbic acid, mannitol, and flavonoids, for reduction (Lu et al., 2013; Yan et al., 2014), ROS accumulation can also be limited by enhancing the antioxidant system and regulating the expression of defense-related genes to protect plant cell membranes from damage (Ketehouli et al., 2021). The *E2* knockout mutant $e2^{CR}$ not only has a shortened flowering time but also positively regulates the transcription level of ROS scavenging-related genes, which enhances the salt tolerance of soybean, laying a foundation for the selection of early maturing and salt-tolerant soybean varieties (Dong et al., 2022).

Transcription factors (TFs) can be activated in response to specific abiotic stressors (Song et al., 2016; Li et al., 2019) and have been widely used in transgenic plants to enhance plant tolerance under abiotic stress (Wang et al., 2016). NAC (NAM, ATAF, and CUC) proteins are one of the largest families of TFs involved in abiotic stress. *GmNAC8* overexpression lines promote the production of proline content and superoxide dismutase (SOD) activity, and *GmNAC8* improves tolerance under drought stress (Yang et al., 2020). *Stress associated proteins 16* (*GmSAP16*) overexpression in transgenic soybean hairy roots enhances salt and drought tolerance in soybean seedlings and increases the transcript levels of the stress-related genes drought response element-binding factors (*GmDREB1B; 1*, and *GmDREB2*), 9-cis-epoxycarotenoid dioxygenase 3 (*GmNCED3*), responsive to dehydration protein 22 (*GmRD22*), a vacuolar Na^+/H^+ antiporter gene (*GmNHX1*), and *Salt Overly Sensitive 1* (*GmSOS1*; Zhang et al., 2019). Therefore, the identification of key genes is essential to understand the complex molecular mechanisms of salt and drought tolerance in soybean.

Late embryogenesis abundant (LEA) proteins are involved in plant stress tolerance mechanisms and play a crucial role in adversity resistance (Abdul Aziz et al., 2021). In rice (*Oryza sativa*), five *LEA* genes (*OsLEA1*, *OsLEA2*, *OsLEA3*, *OsLEA4*, and *OsLEA5*) were substantially up-regulated under drought stress and provided better physiological adaptation to drought stress (Kamarudin et al., 2019). The GmLEA2-1 protein enhances salt and drought tolerance in Arabidopsis (*Arabidopsis thaliana*; Putterill et al., 1995; Robson et al., 2001). Notably, GmLEA is a soybean PM2 protein (Accession No. M80664), belonging to the LEA3 protein family (Hsing et al., 1992), that has been shown to enhance tolerance to high salt stress in *Escherichia coli* (*E. coli*) with some hydrophilic and thermal stability, protecting proteins and enzyme activities against freeze–thaw or high-temperature stress (Min et al., 2015; Song et al., 2018). In *Arabidopsis*, the gene encoding Δ^1-pyrroline-5-carboxylate synthetase (P5CS) is induced by salt stress, drought stress, and abscisic acid (ABA; Liu et al., 2016). Proline biosynthesis is catalyzed by P5CS, and the resulting Δ^1-pyrroline-5-carboxylate (P5C) is reduced by P5CR to proline (Hu et al., 1992). Overexpression of P5CS in transgenic *Nicotiana benthamiana* (*N. benthamiana*) plants increases proline content, improves tolerance to osmotic stress, and induces participation in the proline biosynthetic pathway in plants (Kishor et al., 1995).

The *CONSTANS-like* (*COL*) gene was first isolated in *Arabidopsis*, and the COL protein encoded by the *COL* gene contains two homologous domains: the B-box domain (zinc-finger domain) with a unique crystal structure (Dahal et al., 2022) and the CCT (CO, CO-like, and TOC1) domain. To date, only a few members of the CO family have been involved in abiotic stress responses. *AtCOL4* and *ZmCOLs* in maize (*Zea mays L.*) might be dependent on ABA signaling to participate in salt and drought stress responses (Min et al., 2015; Song et al., 2018). In rice, a *COL* TF, *Ghd7*, is a negative regulator involved in developmental and drought-induced leaf senescence pathways (Liu et al., 2016). In *Tamarix hispida*, *ThCOL2* overexpression enhances ROS clearance to reduce cell damage and death caused by salt stress (Lei et al., 2021). Zinc-finger protein B-BOX 7/CONSTANS-LIKE 9 (MdBBX7/MdCOL9), which formed a module with MdMIEL1, played an active role in drought resistance in apple (*Malus domestica*; Chen et al., 2022). In mango (*Mangifera indica L.*), *MiCOL1A* and *MiCOL1B* are involved in both the flowering pathway and enhanced drought tolerance in *Arabidopsis* (Guo et al., 2022). Therefore, we also aimed to identify whether the floral suppressor *GmCOL1a* was involved in the regulation of stress responses in soybean.

In this study, *35S:GmCOL1a* transgenic soybeans had enhanced salt and drought tolerance by increasing water absorption, Na^+/K^+ ratio, and proline content, decreasing MDA content, and reducing ROS production, whereas the *co-9* mutant showed the opposite phenotypes. GmCOL1a directly bound to Myc(bHLH)-binding and Che-binding motifs in the promoters of stress-related genes *GmLEA* and *GmP5CS* to promote mRNA expression and suppress ROS production, achieving salt and drought tolerance. These results provided important insights into the functional diversity of the GmCOL1a protein, a positive regulator involved in salt and drought tolerance pathways.

Results

Subcellular localization of the GmCOL1a protein and spatial expression patterns

GmCOL1a, belonging to the BBX family of zinc-finger TFs, contained one or two B-box patterns and sometimes a CCT domain, which played important roles in plant growth and development, including the photoperiodic regulation of flowering and responses to biotic and abiotic stress (Putterill et al., 1995; Robson et al., 2001). To investigate the phylogenetic relationships among soybean *GmCOL1a* genes, 105 CO protein sequences from *Glycine max*, *Arabidopsis thaliana*, *Zea mays*, tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*), and *Oryza sativa* were compared, and a phylogenetic tree was constructed using a neighbor-joining (NJ) algorithm, indicating that these neighboring genes may exhibit similar functions, with most genes containing B-box patterns and a CCT domain and a few genes containing only a CCT domain (Figure 1A).

To determine the subcellular localization of GmCOL1a, its localization was analyzed by expressing a gene encoding a green fluorescent protein (GFP)-GmCOL1a fusion protein under the control of the 35S promoter. The fluorescence microscopic observations showed that GFP fluorescence was dispersed throughout the cells, bombarded with the control plasmid 35S:GFP. In contrast, the 35S:GFP-GmCOL1a fusion protein was localized exclusively to the nucleus of *Nicotiana benthamiana* (*N. benthamiana*) cells (Figure 1B).

To investigate the tissue expression patterns in soybean "DongNong 50" (DN50), the transcript abundance of the *GmCOL1a* gene in soybean root, stem, leaf, flower, pod, and seed tissues was also detected by reverse-transcription quantitative PCR (RT-qPCR). The expression of *GmCOL1a* was detected in all tissues, with the highest expression in the leaves (Figure 1C).

mRNA level of GmCOL1a was induced by salt, drought, and ABA

To further confirm the function of *GmCOL1a* in soybean salt and drought tolerance, we generated three T_3 generation *GmCOL1a-ox* (overexpression) transgenic lines under the control of the CaMV35S promoter. The expression of the GmCOL1a-FLAG protein with a size of 58 kDa was detected by western-blot analysis in three *GmCOL1a-ox* transgenic soybean lines (Figure 1D). RT-qPCR showed that the transcript levels of *GmCOL1a* were induced in leaves at 1, 3, and 6 h after salt and drought treatment (Figure 1E). The expression of the GmCOL1a protein immediately decreased after salt and drought treatment and gradually stabilized with a longer treatment time, as shown by western-blot analysis of *GmCOL1a-ox*-2 transgenic leaves (Figure 1, F and G). In addition, the transcript level of *GmCOL1a* was especially induced after 3 and 12 h of ABA treatment (Figure 1H).

GmCOL1a promoted salt tolerance in soybean

To clarify the biological functions of *GmCOL1a* in soybean, we generated an independent CRISPR/Cas9-mediated loss-of-function mutant (named *co-9*) in the DN50 background. DNA sequencing confirmed that seven bases (ATGGCTC) upstream of the PAM site were deleted in the *co-9* mutant, which resulted in amino acid conversions that ultimately stopped translation at 684 bp (Figure 2A).

For germination assays, the seeds of three *GmCOL1a-ox* transgenic lines, *co-9*, and the wild type (WT) were sown on germination medium [1/2 Murashige and Skoog basic nutrient salts with B5 vitamins (MSB) with 2% sucrose] with various concentrations of NaCl. There was no difference in germination rates on 0 mM NaCl medium; however, the germination of WT and *co-9* seeds was inhibited to a greater extent than that of *GmCOL1a-ox* seeds in the medium containing 250 mM NaCl (Figure 2B). Furthermore, the hypocotyl length of the three *GmCOL1a-ox* transgenic lines was significantly longer than that of the WT and *co-9* seedlings after 250 mM NaCl treatment, although salt stress also repressed the growth rate of *GmCOL1a-ox* transgenic lines to a lesser extent (Figure 2C). We further examined the effect of salt treatments on soybean root growth; seedlings grown under normal growth conditions in a bag showed no significant difference in primary roots. However, the WT and *co-9* produced shorter primary roots, and the three *GmCOL1a-ox* transgenic lines showed longer roots and more and longer lateral roots under 100 mM NaCl treatment (Figure 2D). For seedlings grown in soil treated with salt, *co-9* and WT plants began to wilt substantially 8 days after treatment with 200 mM NaCl. Although the three *GmCOL1a-ox* transgenic lines were shorter than the WT seedlings when not treated with salt, the three *GmCOL1a-ox* transgenic lines showed higher heights and healthier growth than the *co-9* and WT plants. Statistical analysis of survival rates further showed that the three *GmCOL1a-ox* transgenic lines had 62%–85% survival rates, while the WT and *co-9* plants had 30% and 20% survival rates, respectively (Figure 2E).

It is important to maintain the water balance in plants under stress conditions. To investigate the ability of transgenic soybean plants to regulate osmotic stress, the leaf relative water content (RWC) was assessed during salt treatment. After 8 days of salt treatment, RWCs decreased by 31% and 48% in WT and *co-9* plants, respectively, but only decreased by 14%–15% in the three *GmCOL1a-ox* transgenic lines (Figure 2F).

Taken together, the overexpression of *GmCOL1a* increased salt tolerance, and the *co-9* mutant increased salt sensitivity.

GmCOL1a enhanced salt tolerance by maintaining the Na+/K + ratio

To assess the functional properties of *GmCOL1a*, we compared the accumulation of K^+ and Na^+ in the roots, stems, and leaves of different transgenic plants. Under normal growth conditions, *GmCOL1a-ox-2* transgenic soybean

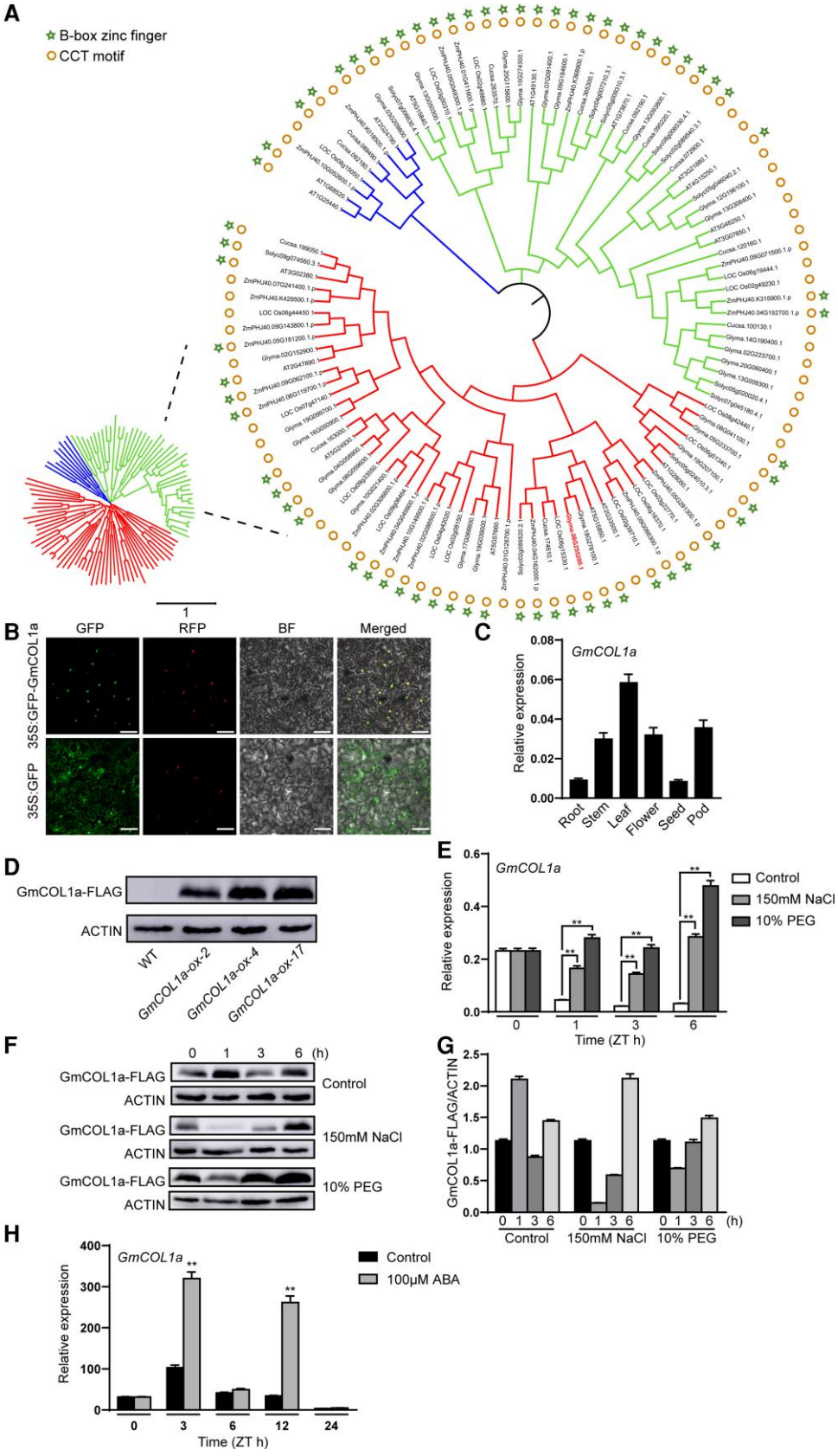

Figure 1 Subcellular localization of GmCOL1a and spatial expression patterns. A, Phylogenetic tree was constructed using the NJ method (with a bootstrap of 1000) with CONSTANS. The DIR nomenclature is as follows: *Glycine max* (Glyma), *Arabidopsis thaliana* (At), *Zea mays* (Zm),

(continued)

showed a higher K^+ content and lower Na^+ content than WT and *co-9* in roots, stems, and leaves. However, under salt stress, the Na^+ content in the roots, stems, and leaves of WT, *co-9*, and *GmCOL1a-ox-2* was obviously increased compared to each of the nontreated plants, which led to a substantial increase in the Na^+/K^+ ratio. Although there were no significant differences in the Na^+/K^+ ratio in the roots of different transgenic plants under salt stress conditions, Na^+/K^+ ratios in the stems and leaves of *GmCOL1a-ox-2* transgenic soybean were significantly lower than those of the WT and *co-9* under salt stress (Figure 3A).

To further understand whether *GmCOL1a* transgenic plants maintained ion homeostasis by regulating the acquisition and distribution of K^+ and Na^+ under salt stress, the expression levels of *GmSOS1* in Na^+ efflux transporters (Zhang et al., 2022) and *GmSALT3* (*salt tolerance-associated gene on chromosome 3*; Guan et al., 2014) were analyzed. Both *GmSOS1* and *GmSALT3* were induced at 0, 1, 3, and 6 h after salt treatment in the WT plants (Figure 3B). We found that the expression of *GmSOS1* and *GmSALT3* was more upregulated in different transgenic soybean plants than the control under 150 mM NaCl treatment for 3 h. Therefore, the transcript levels of *GmSOS1* and *GmSALT3* were studied in *GmCOL1a* transgenic plants under salt stress for 3 h. The transcript levels of *GmSOS1* and *GmSALT3* were higher in *GmCOL1a-ox-2* transgenic soybean plants than those in WT. The opposite result was observed in *co-9* transgenic soybean plants (Figure 3C). *GmCOL1a* increased the expression of *GmSOS1* and *GmSALT3* by maintaining the Na^+/K^+ ratio to improve salt tolerance.

GmCOL1a promoted drought tolerance in soybean

In the drought treatment, the green coloration of the three overexpression transgenic lines persisted longer than that in the WT and *co-9* plants. After re-watering for 4 days, most WT and *co-9* plants failed to recover (33% and 23% survival, respectively), whereas the three overexpression transgenic lines showed higher recovery (74%–85% survival; Figure 4, A and B). Additionally, RT-qPCR assays showed that the transcript levels of *GmCOL1a* were induced in leaves after 1, 3, and 6 h of drought treatment (Figure 1E). The RWC was then measured during the water deficit treatment. The RWCs decreased by 52% and 63% in WT and *co-9* plants, respectively, but the RWCs of the three overexpression transgenic lines decreased by only 13%–17% on day 5 of the water deficit treatment. In contrast, the RWCs of WT and *co-9* soybeans decreased by 77% and 82%, respectively, while those of the three overexpression transgenic lines decreased by only 55%–58% on day 8 of the drought treatment (Figure 4C).

ABA plays an important role in regulating stomatal movement and is closely related to water loss under drought stress (Verslues et al., 2006; Tai et al., 2014). We further verified that ABA treatment resulted in reduced stomatal opening in the three overexpression transgenic lines, and *co-9* showed a larger stomatal aperture than the WT (Figure 4D). These results suggest that *GmCOL1a* overexpression increases drought tolerance by ABA-mediated stomatal movement.

Physiological index analysis under salt and drought stress

To further investigate the role of *GmCOL1a* in regulating salt stress and drought tolerance, we measured the proline, MDA, and H_2O_2 content, as well as catalase (CAT), SOD, and peroxidase (POD) activities, to reflect the response ability of plants for resilience. First, we stained soybean root tips and leaves with nitro blue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB) to detect H_2O_2 content under normal or treatment conditions in three *GmCOL1a-ox* transgenic lines, *co-9*, and WT plants. No substantial difference was observed under normal conditions with NBT and DAB staining; however, the color depth of WT and *co-9* plants was substantially higher than that of the three *GmCOL1a-ox* transgenic lines under water deficit or 250 mM NaCl treatment (Figure 5A). In contrast, the leaf color depth of the three *GmCOL1a-ox* transgenic lines was substantially lower than that of the *co-9* and WT plants (Figure 5, C, D, F, G). Finally, we selected the best phenotypic *GmCOL1a-ox-2* line for H_2O_2 content measurement and found that the H_2O_2

Figure 1 (Continued)

Solanum lycopersicum (Solyc), *Cucumis sativus* (Cucsa), and *Oryza sativa* (Os). The 1 scales show substitution distance. B, Subcellular localization of the GmCOL1a protein. After infiltration, the *Nicotiana benthamiana* leaves were grown for 2 days, and the GFP signal was detected by fluorescence microscopy. A red nuclear marker plasmid (H2B-RFP) was used to confirm the location of the cell nucleus. GFP, green fluorescent protein; RFP, red fluorescent protein; BF, bright field; Merge, GFP, RFP, and bright-field images. Scale bars = 100 μm. C, Tissue-specific expression of *GmCOL1a* under LDs. Roots, stems, and leaves were collected from 20-day-old plants. Flowers were collected from 52-day-old plants. Seeds and pods were collected from 68-day-old plants. All data were normalized with the soybean *GmActin4* gene as an internal reference. D, Immunoblot analysis of the *35S:GmCOL1a* transgenic soybean. The GmCOL1a-FLAG protein was expressed in transgenic seedlings grown for 20 days under LDs. Actin was used as a control. E, Expression of *GmCOL1a* in leaves of WT under salt (150 mM) and PEG 6000 (10%) treatments determined by RT-qPCR. Asterisks indicated values significantly different from 150 mM NaCl or 10% PEG compared to control. F, Expression of GmCOL1a protein in *GmCOL1a-ox-2* seedlings grown in LDs for 14 days and treated with 150 mM NaCl or 10% PEG 6000 for 0, 1, 3, and 6 h. Leaf samples were collected (ZT0 is 7:00 a.m.), western blots of protein extracts are shown. G, FLAG-tagged proteins were detected using an anti-FLAG antibody. Actin served as the loading control. The levels of the GmCOL1a protein were determined by normalization of the GmCOL1a signals to ACTIN signals and were presented as GmCOL1a/ACTIN. H, RT-qPCR analysis of the mRNA level of *GmCOL1a* in the leaves of 14-day-old WT plants under ABA (100 μM) treatment. Asterisks indicated values significantly different between the control and ABA in each group. For each experiment, three technical replicates were conducted. Data shown are the mean ± SD of three independent experiments (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test).

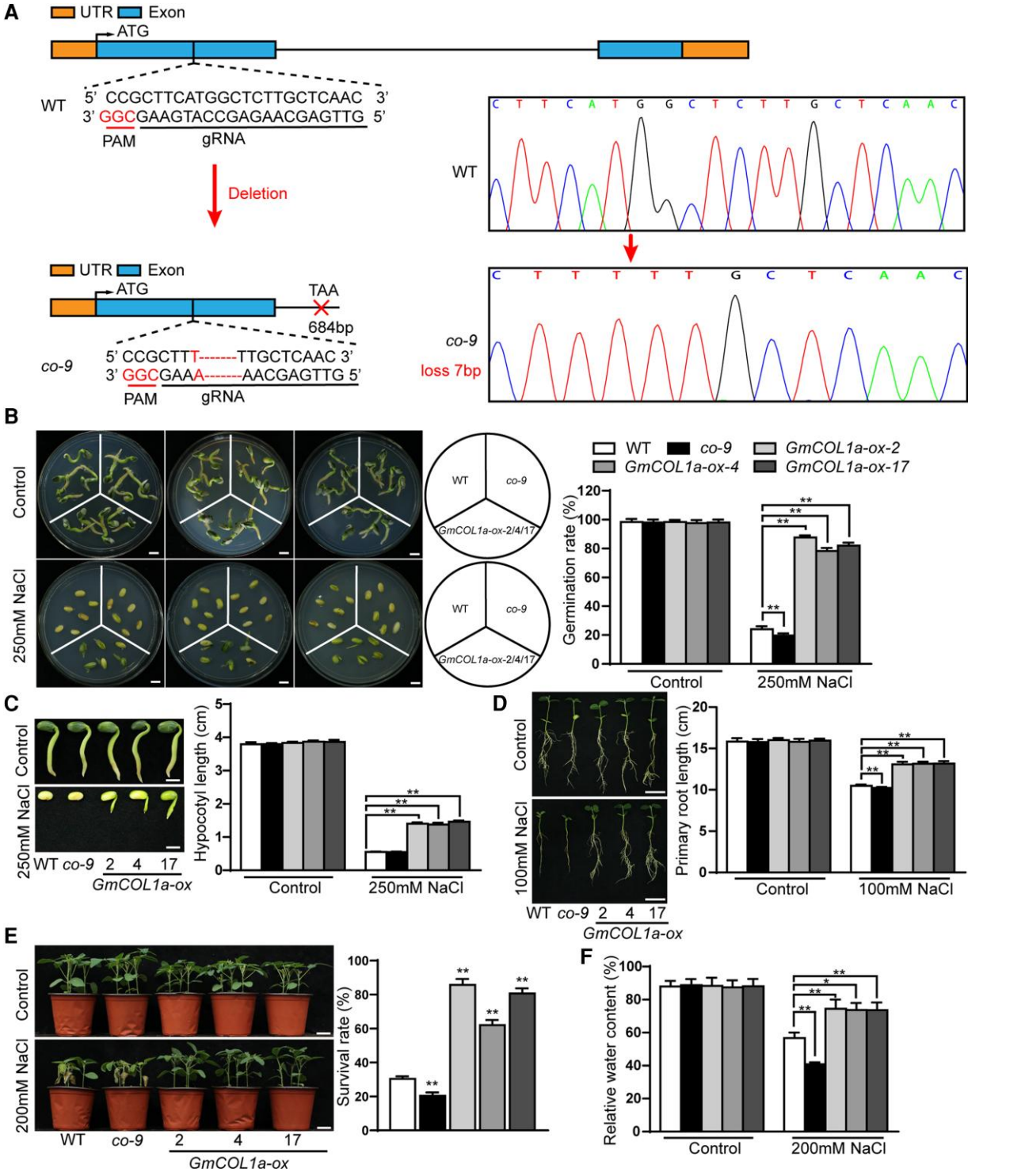

Figure 2 GmCOL1a positively regulated salt tolerance in soybean. A, Gene structures of *GmCOL1a* with a CRISPR/Cas9 target site designed in the exon. Black lines, orange strips, and blue strips indicate introns, untranslated regions (UTRs), and exons, respectively. Nucleotide sequences indicate regions targeted by the gRNA designed in this study, and nucleotides in red indicate proto-spacer adjacent motifs (PAMs). B, Images of 6-day-old seedlings were taken, and germination rates were recorded ($n \geq 20$). Scale bars = 1 cm. C, Images of whole seedlings were taken. Scale bars = 1 cm. Hypocotyl length of WT and transgenic lines under 0 (control) or 250 mM NaCl treatment. Each column represents the hypocotyl length ($n \geq 20$). D, Primary root length of 4-day-old soybean seedlings treated with 0 (control) or 100 mM NaCl. Images were taken 21 days after stress treatment. Each column represents the primary root length ($n \geq 20$). Root growth bags from seedlings grown in 10 plastic bags were collected and treated similarly. Each bag contained two seedlings from three *GmCOL1a-ox* transgenic lines, the *co-9* mutant, and the WT soybeans. Scale bar = 5 cm.

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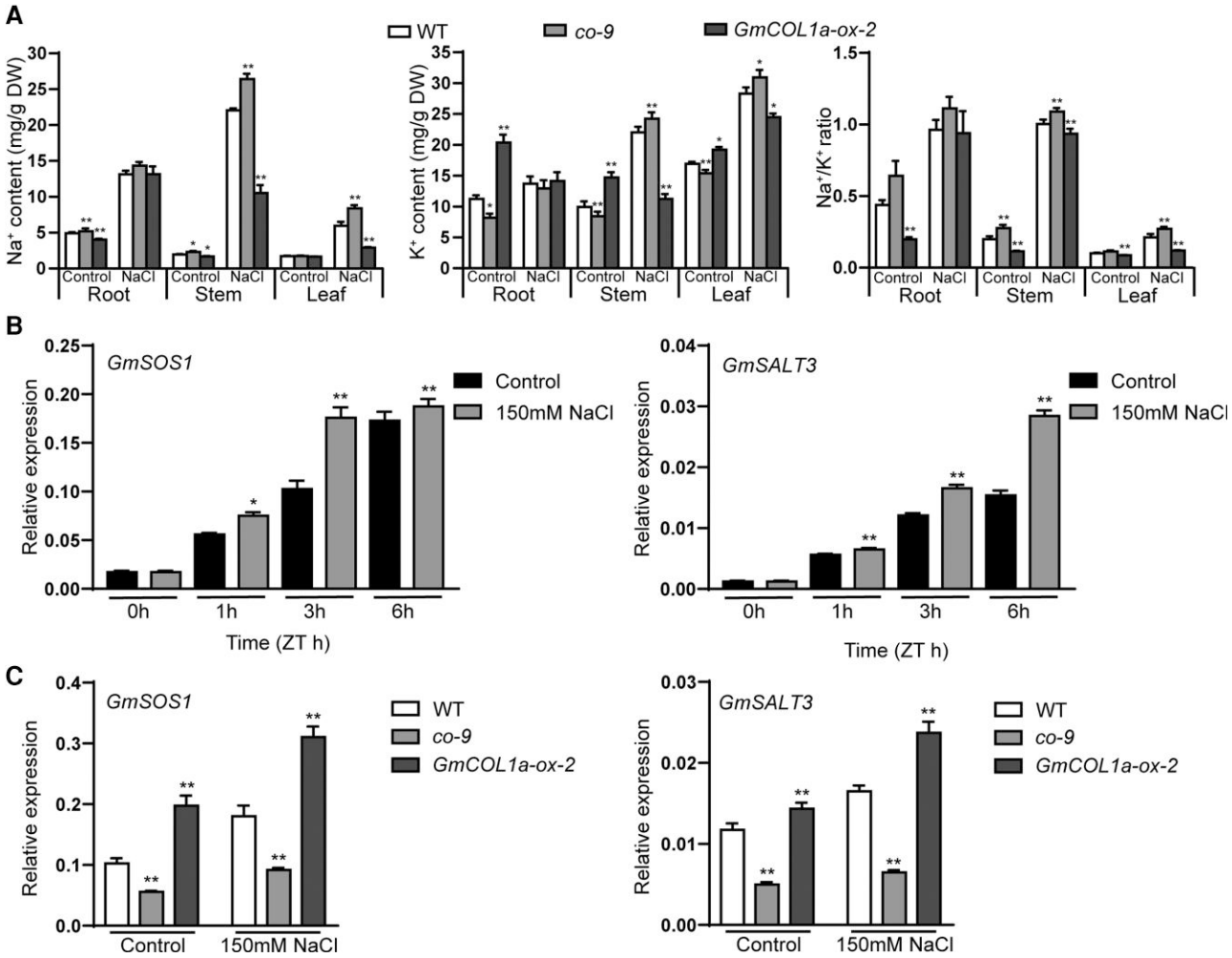

Figure 3 Salt sensitivity of different *GmCOL1a-ox* plants. A, Concentrations of Na^+ and K^+ and Na^+/K^+ ratio of roots, stems, and leaves in WT, *co-9*, and *GmCOL1a-ox* soybean materials grown under 0 mM NaCl or 150 mM NaCl treatments for 8 days, respectively. Asterisks indicated values significantly different between the transgenic lines and the WT in each group. B, Transcripts of *GmSOS1* and *GmSALT3* in the WT under control and salt (150 mM NaCl) conditions. Asterisks indicated values significantly different between the control and 150 mM NaCl in each group. C, RT-qPCR analysis of the mRNA levels of *GmSOS1* and *GmSALT3* in 14-day-old leaves from *GmCOL1a-ox* and *co-9* plants under 150 mM NaCl treatments for 3 h, determined by RT-qPCR. Asterisks indicated values significantly different between the transgenic lines and the WT in each group. For each experiment, three technical replicates were conducted. Data shown are the mean ± SD of three independent experiments (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test).

content was the highest in both root tips and leaves of the *co-9* mutant and the lowest in the *GmCOL1a-ox*-2 soybean (Figure 5, B, E, H).

Proline accumulation enhances plant stress tolerance (Ashraf and Foolad, 2007). The proline accumulation rate among the different transgenic soybean plants showed no significant differences under control conditions; however, under salt or drought stress, the proline content was significantly higher in the three *GmCOL1a-ox* transgenic lines. The MDA content is used to indicate lipid peroxidation products and to reflect the extent of plant damage caused by adversity (Cao et al., 2007). Compared with the *co-9* and WT plants, the MDA content in *GmCOL1a-ox* transgenic plants significantly decreased after salt and drought stress. The enzymatic activities of SOD, POD, and CAT scavenge intracellular ROS and reduce H_2O_2 production, thereby enhancing salt

Figure 2 (Continued)
E, Phenotypes and survival rates of three *GmCOL1a-ox* transgenic lines, the *co-9* mutant, and the WT soybeans after 200 mM salt treatment for 8 days. Scale bars = 5 cm. Survival rates were calculated ($n \geq 20$). F, Relative leaf water content was measured from three *GmCOL1a-ox* transgenic lines, the *co-9* mutant, and the WT soybeans ($n \geq 10$) grown under various concentrations of NaCl for 8 days. Asterisks indicated values significantly different between the transgenic lines and the WT in each group. For each experiment, three technical replicates were conducted. Data shown are the mean ± SD of three independent experiments (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test).

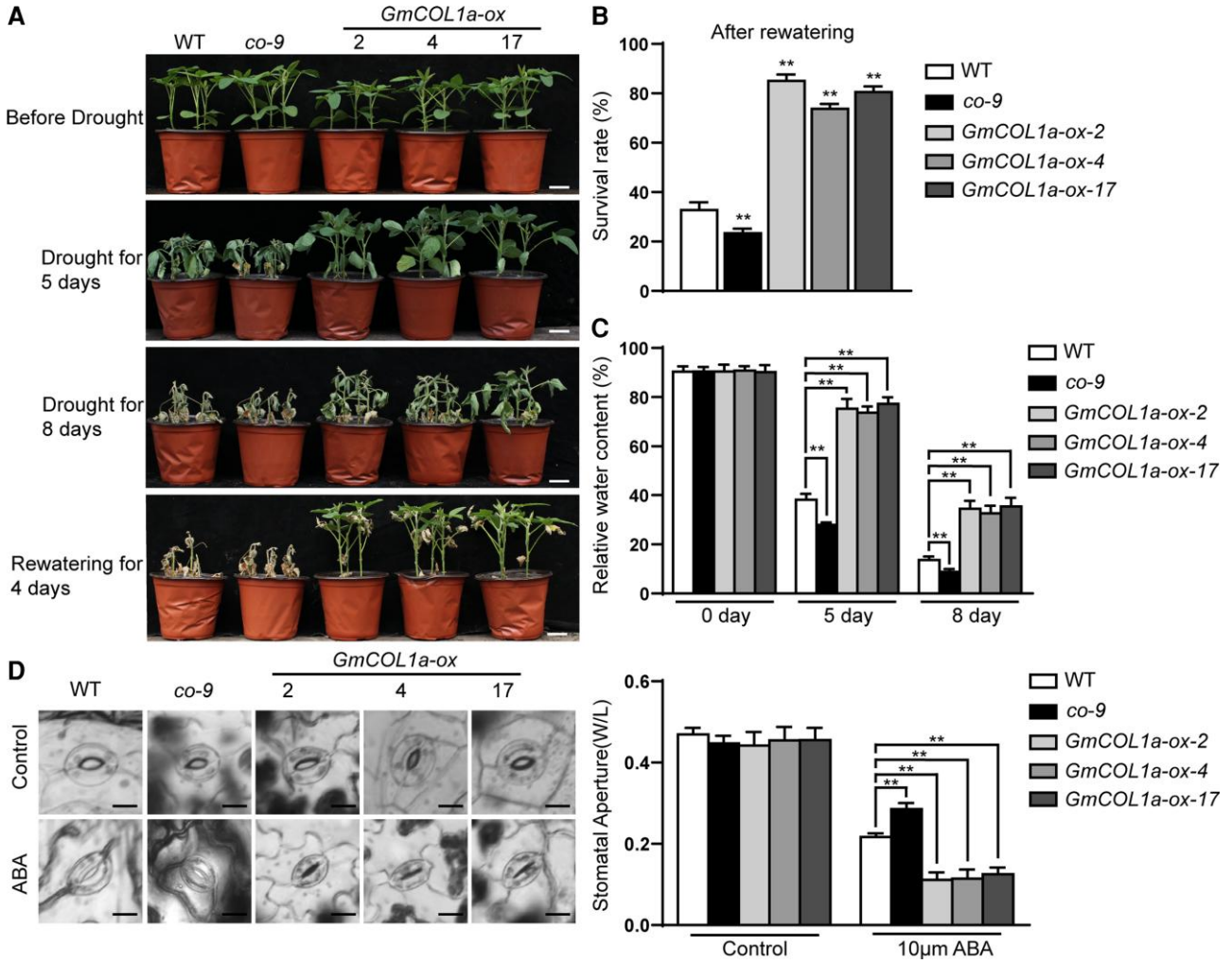

Figure 4 Analyses of drought stress phenotypes in different soybean plants. A, B, Phenotypes and survival rates of three *GmCOL1a-ox* transgenic lines, *co-9* mutant, and WT soybeans ($n \geq 20$) after dehydration for 5 days, 8 days, and recovery for 4 days. The control group was watered with half-strength Hoagland solution every 3 days when the first trifoliate leaves of the plants were fully expanded. Scale bars = 5 cm. C, Relative leaf water content of three *GmCOL1a-ox* transgenic lines, *co-9* mutant, and WT soybean seedlings ($n = 10$) after drought treatment. The water content was measured at 0, 5, and 8 days after drought treatment. D, Left panel: Images of epidermal stomata after 10 μm ABA treatment observed with a bright field microscope. Scale bars = 10 μm. Right panel: Stomatal aperture of the second trifoliate leaf at the top after ABA treatment; width/length of the stomatal aperture was measured using ImageJ ($n = 10$). Asterisks indicated values significantly different between the transgenic lines and the WT in each group. Data are the means ± SD of three independent experiments (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test).

and drought tolerance (Han et al., 2020). The SOD, POD, and CAT activities of the *co-9* and WT plants were significantly lower than those of the three *GmCOL1a-ox* transgenic lines (Figure 5I). These results suggest that *GmCOL1a* overexpression in soybeans enhanced salt and drought tolerance by increasing the proline content, reducing the MDA content, and increasing the enzyme activities of SOD, POD, and CAT to reduce ROS production.

GmCOL1a directly bound to the promoters of GmLEA and GmP5CS

To identify the DNA-binding sites and the downstream target genes of GmCOL1a in soybean and further elucidate the potential mechanism by which GmCOL1a promotes salt and drought tolerance, we performed ChIP-seq using *GmCOL1a-ox-2* soybean leaves. Based on the sequencing data, two promoter regions of the GmCOL1a target genes, *GmLEA* and *GmP5CS*, were putatively directly bound by GmCOL1a (Figure 6A; Supplemental Table S2).

Then, Myc(bHLH)-binding, and Che-binding motifs that bind to GmCOL1a were identified (Figure 6B). We further performed ChIP-qPCR assays to verify the potential enrichment of *GmLEA* and *GmP5CS* promoters by GmCOL1a binding using *GmCOL1a-ox-2* transgenic soybean plants expressing a GmCOL1a-FLAG fusion protein under salt and drought treatments with a WT sample as a negative control. The promoter fragments of *GmLEA* and *GmP5CS* carrying the identified GmCOL1a-binding sites were highly enriched in DNA chromatin immunoprecipitation (ChIPed) at 0 and

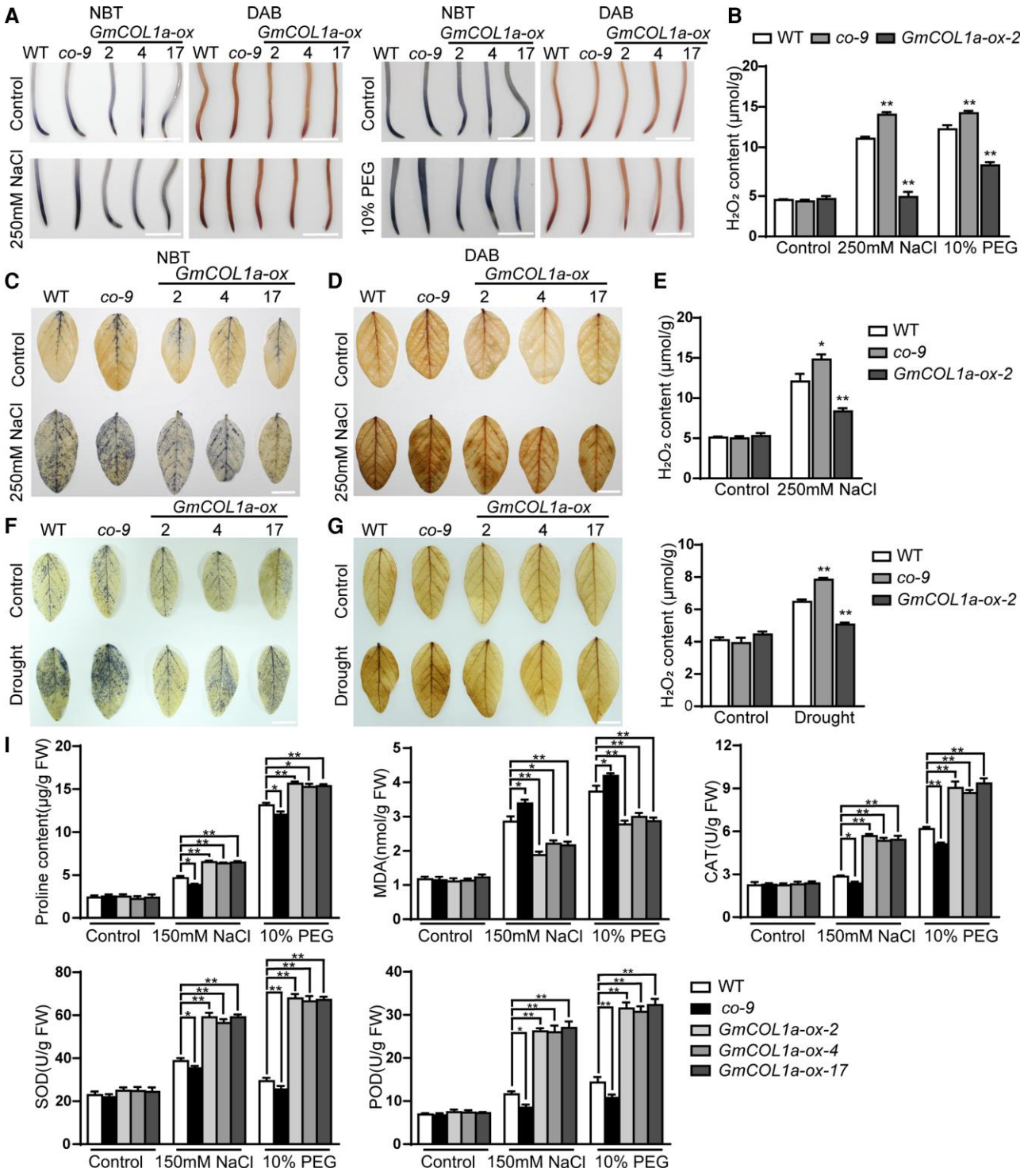

Figure 5 H_2O_2 levels and analysis of physiological parameters of different soybean plants under salt and drought treatment. A, Hairy roots of 6-day-old seedling plants of three *GmCOL1a-ox* transgenic lines, *co-9*, and WT plants at 3 h after 250 mM NaCl or 10% PEG 6000 treatment were stained by NBT or DAB, and half-strength Hoagland nutrient solution was used as a control. Scale bars = 0.5 cm. B, Determination of H_2O_2 content in the roots of *GmCOL1a-ox-2*, *co-9*, and WT plants under 250 mM NaCl or 10% PEG 6000 treatment for 3 h. C, NBT and D, DAB staining of the leaves of 14-day-old three *GmCOL1a-ox* transgenic lines, *co-9*, and WT plants exposed to 250 mM NaCl for 6 h. F, NBT and G, DAB staining of the leaves of 14-day-old three *GmCOL1a-ox* transgenic lines, *co-9*, and WT plants subjected to drought (no irrigation) treatment for 4 days. Scale bars = 0.5 cm. H_2O_2 content of 14-day-old seedlings of *GmCOL1a-ox-2*, *co-9*, and WT plants under E, salt or H, drought treatment. I, Proline and MDA content and CAT, POD, and SOD activities of 2-week-old seedlings of three *GmCOL1a-ox* transgenic lines, *co-9*, and WT plants. All values were measured after 6 h of exposure to salt or drought stress (150 mM NaCl or 10% PEG 6000). Asterisks indicated values significantly different between the transgenic lines and the WT in each group. For each experiment, three technical replicates were conducted. Data shown are the means ± SD (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test).

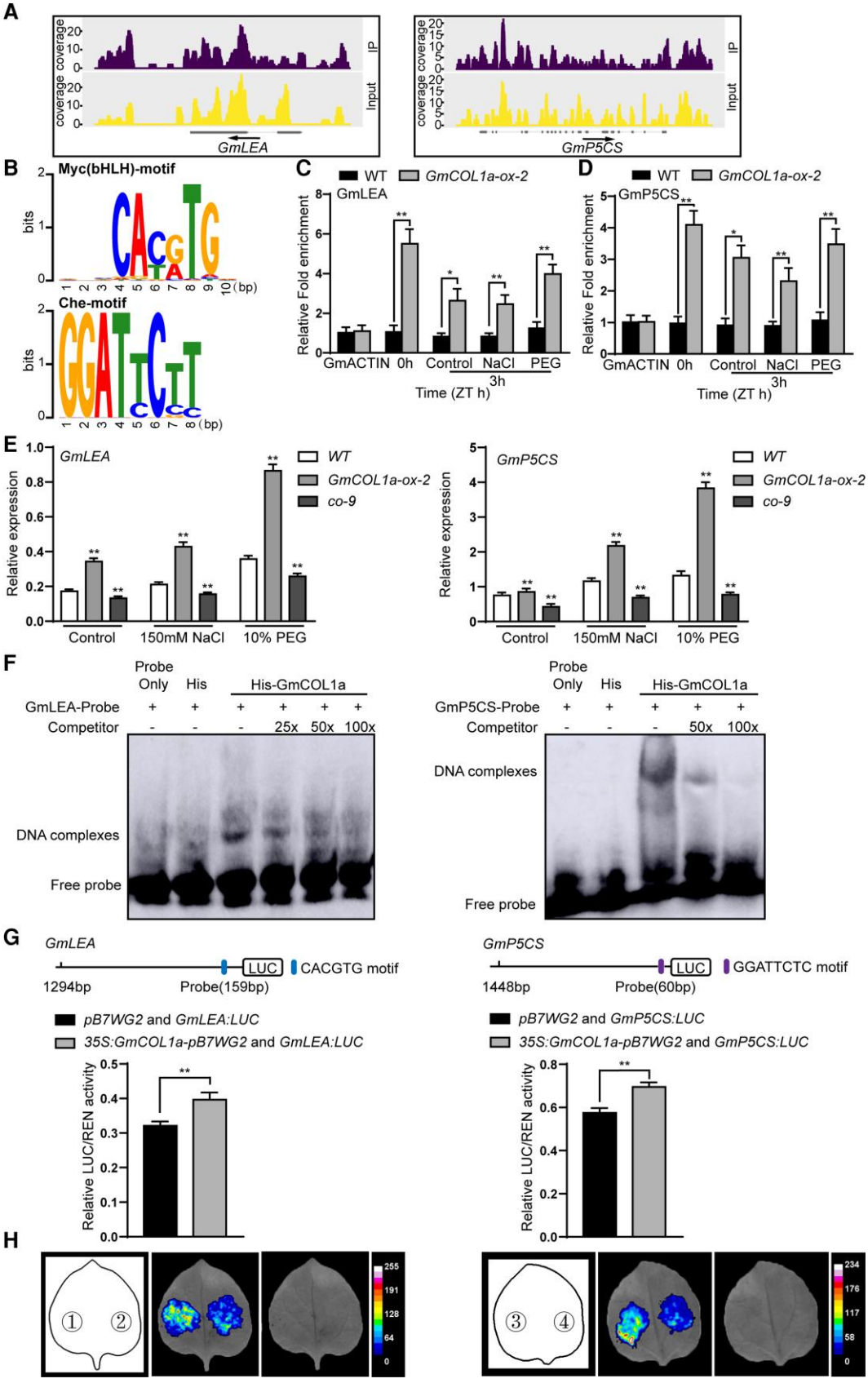

Figure 6 Binding of GmCOL1a protein to the promoters of *GmLEA* and *GmP5CS*. A, Peak graphs showing the ChIP-seq raw reads at the indicated gene loci in Integrative Genomics Viewer. The arrow indicated the direction of transcription, and the gray bars indicated the transcripts of the gene.

(continued)

3 h of salt and drought stress, indicating that *GmLEA* and *GmP5CS* promoters containing Myc(bHLH)-binding and Che-binding motifs, respectively, were bound by GmCOL1a, despite the decreasing enrichment after 3 h of salt or drought treatment compared with the nontreated (0 h; Figure 6, C and D). We then examined the transcript levels of *GmLEA* and *GmP5CS* in different transgenic soybean plants under salt and drought stress. RT-qPCR showed that the transcript levels of *GmLEA* and *GmP5CS* were significantly elevated in *GmCOL1a-ox-2* and decreased in *co-9* soybean plants at 3 h of salt and drought treatment (Figure 6E).

To determine whether GmCOL1a could directly bind to the *GmLEA* promoter Myc(bHLH) motif (CACGTG) and the *GmP5CS* promoter Che motif (GGATTCTC), in vitro binding was analyzed using electrophoretic mobility shift assay (EMSA). HIS-GmCOL1a dramatically reduced the migration of the 40-bp and 32-bp probes, indicating that GmCOL1a directly bound to both CACGTG and GGATTCTC motifs (Figure 6F). Reciprocal competitive EMSA showed strong and specific binding of GmCOL1a to the two motifs.

Moreover, the reporters *GmLEA:LUC* and *GmP5CS:LUC* were constructed with *GmLEA* and *GmP5CS* promoters containing GmCOL1a-binding sites, the CACGTG motif and the GGATTCTC motif, respectively, driving the LUC reporter gene (Figure 6G). When co-infiltrating *Agrobacterium* expressing 35S:GmCOL1a-pB7WG2 effectors together with *GmLEA:LUC* and *GmP5CS:LUC* reporters into the leaves of *N. benthamiana*, LUC activity was elevated by GmCOL1a protein expression, demonstrating that GmCOL1a could promote the transcriptional activation activities of both *GmLEA* and *GmP5CS* (Figure 6H).

Overall, the results suggest that the GmCOL1a protein directly promotes the expression of *GmLEA* and *GmP5CS* by binding directly to their promoters.

GmP5CS improved salt and drought stress tolerance in transgenic soybean hairy roots

Notably, GmCOL1a bound directly to the promoters of *GmLEA* and *GmP5CS*. *GmLEA* has been shown to be one of the major genes regulating salt stress (Liu and Zheng, 2005; Liu et al., 2010). However, the function of *GmP5CS* under salt or drought stress has not been reported. To explore the molecular mechanism of *GmP5CS* involved in salt and drought tolerance in soybean, transgenic soybean plants expressing *GmP5CS:GmP5CS-GFP* were generated by *Agrobacterium rhizogene* (*A. rhizogene*)-mediated hairy-root transformation in the WT, *co-9*, and *GmCOL1a-ox-2* backgrounds, forming "composite" transgenic plants. The plasmid carrying *pCAMBIA1302-no35S* (control) produced by *A. rhizogenis* strain *K599* was used as a control hairy root for the soybean plants. Transgenic hairy roots were identified and selected using GFP under a confocal microscope (Figure 7A), and the remaining non-GFP hairy roots and main roots were removed.

After salt treatment, the "composite" transgenic soybean plants with *GmP5CS* hairy roots in the WT background showed healthier leaves and larger root elongation than the control plants. *GmP5CS* also increased the survival rates of "composite" plants with *GmP5CS* hairy roots under salt stress (Figure 7B). In addition, after drought treatment, the *GmP5CS* hairy roots retained life processes, unlike the control plants. After rewatering for 2 days, the majority of *GmP5CS* transgenic soybean plants were rejuvenated (87% survival), but most of the control plants were not (31% survival; Figure 7C). Then, we measured the RWC of *GmP5CS* "composite" transgenic soybean plants after salt and drought treatment. Under salt and drought treatment, the RWC of transgenic *GmP5CS* "composite" soybean plants decreased significantly compared to that of control plants (Figure 7D). *GmP5CS* enhanced tolerance to both salt and drought stress in the *GmP5CS:GmP5CS-GFP* "composite" transgenic soybean with *A. rhizogene*-mediated hairy roots.

We also measured the proline content in hairy roots after salt and drought stress. *GmP5CS* transgenic soybean hairy roots contained higher levels of proline compared to soybean control hairy roots, and the proline content of *GmP5CS* transgenic soybean hairy roots increased under salt and drought conditions (Supplemental Figure S1, A). To determine whether *GmP5CS:GmP5CS-GFP* "composite" transgenic

Figure 6 (Continued)
B, Motif analysis of GmCOL1a-binding sequences. The distribution of a single pattern in the sequences, the parameter was set to zero or one per sequence. ChIP-qPCR assay of GmCOL1a binding to the *GmLEA* promoter and *GmP5CS* promoter using the soybean leaf samples treated with 150 mM NaCl and 10% PEG 6000 for 0 or 3 h. 0 h sample collection time was the same in C and D. The *GmActin4* locus was used as a negative control. The asterisk indicated a significant difference between the WT and *GmCOL1a-ox-2*. Data shown are the means ± SD of three independent experiments (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test). E, The soybean seedlings were treated with 150 mM NaCl and 10% PEG 6000 for 3 h to collect samples. *GmLEA* and *GmP5CS* expressions in 21-day-old leaves from WT, *GmCOL1a-ox-2* and *co-9* plants. Values were means ± SD ($n = 3$). Asterisks indicated a significant change compared with WT. (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test). F, EMSA assays showing the specific binding of GmCOL1a to the *GmLEA* and *GmP5CS* promoter fragments containing CACGTG and GGATTCTC motifs, respectively. The unlabeled competitive probe is 25-, 50-, 100-fold excess of the labeled probe. HIS served as a negative control. G, The effect of GmCOL1a protein on the promoter activities of *GmLEA* and *GmP5CS*. Relative luciferase activity of co-transfected effector and reporter genes in *Nicotiana benthamiana* leaves was detected in LDs ZT 12 h. The activities of firefly LUC were normalized by the activities of 35S:Renilla LUC. Upper panel: physical locations of fragments harboring putative motifs were shown in the schematic diagram. Asterisks indicated values significantly different the line indicated. Results represented means ± SD of eight independent samples (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test). H, Luciferase activities of *GmLEA* and *GmP5CS* under LDs. Panel: ①: *35S:GmCOL1a-pB7WG2 + GmLEA:LUC* ②: *pB7WG2 + GmLEA:LUC* ③: *35S:GmCOL1a-pB7WG2 + GmP5CS:LUC* ④:*pB7WG2 + GmP5CS:LUC*. The two leaf images were digitally extracted for comparison under the same imaging conditions.

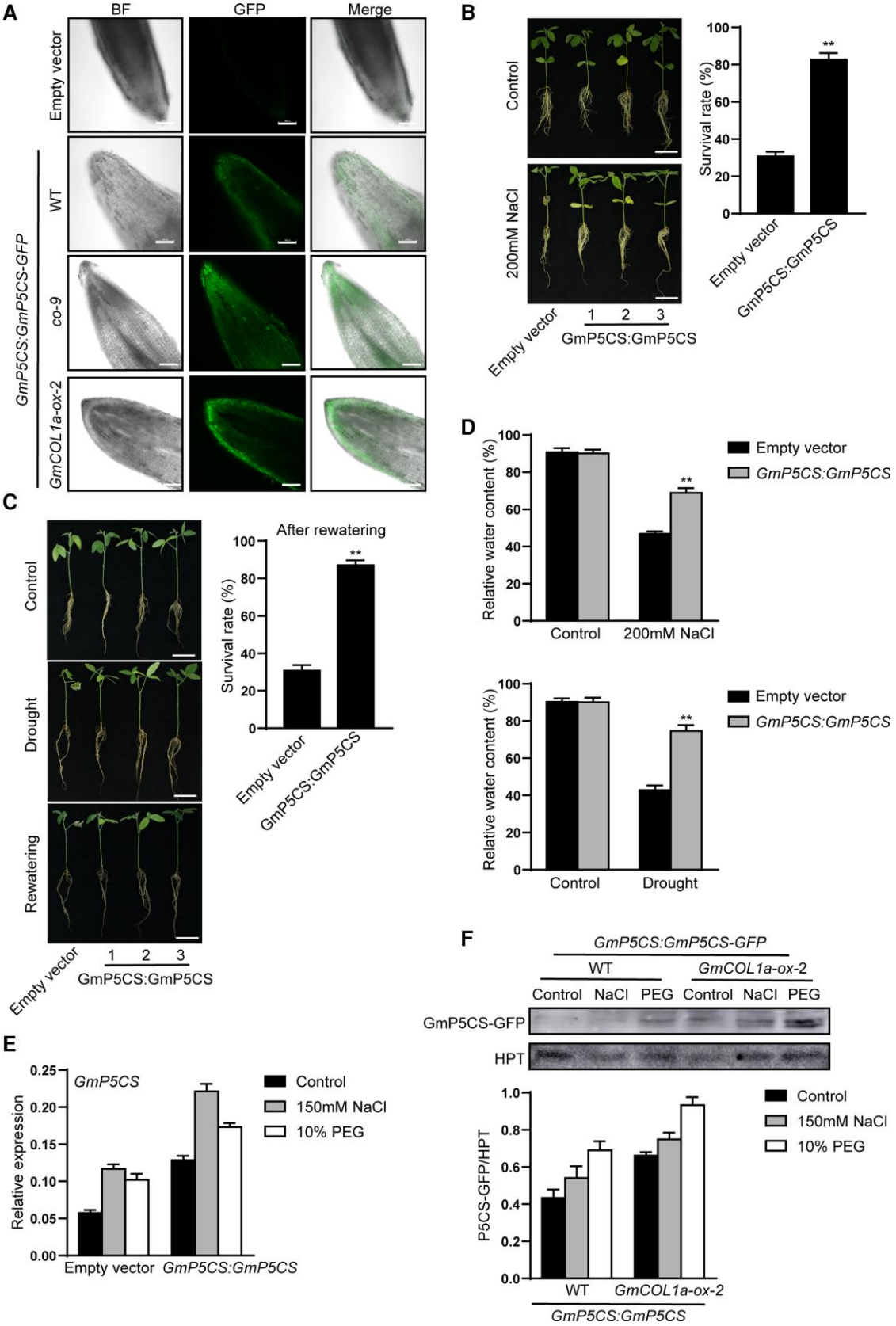

Figure 7 Assessment of the effects of salt and drought stress on GmP5CS transgenic soybean hairy roots. Empty vector indicated soybean hairy roots carrying the pCAMBIA1302-no35S vector and produced by A, *rhizogenis* strain *K599*. *GmP5CS:GmP5CS* indicated soybean plants with transgenic

(continued)

plants regulated salt and drought stress in relation to ROS, we measured CAT, SOD, and POD activities. The CAT, SOD, and POD activities of *GmP5CS:GmP5CS-GFP* "composite" transgenic plants were significantly higher than those of empty vector plants (Supplemental Table S1, B–D). The results suggest that the *GmP5CS:GmP5CS-GFP* "composite" transgenic soybean plants enhanced salt and drought tolerance by increasing SOD, POD, and CAT enzyme activities to reduce ROS production.

The mRNA levels of *GmP5CS* were higher in *GmP5CS:GmP5CS-GFP* transgenic hairy roots than in empty vectors under salt and drought conditions (Figure 7E). *GmP5CS:GmP5CS* was transformed into WT and *GmCOL1a-ox-2* soybean backgrounds. After 6 h of treatment with 150 mM NaCl or 10% PEG 6000, the protein levels of GmP5CS-GFP in transgenic seedlings were observed. As expected, the GmP5CS-GFP protein was induced in roots by salt and drought stress in both the WT and *GmCOL1a-ox-2* backgrounds, and GmP5CS-GFP protein expression was higher in the *GmCOL1a-ox-2* background than in the WT background (Figure 7F), which further validated that GmCOL1a promoted GmP5CS expression.

Thus, the evidence above strongly suggests that *GmP5CS* was a downstream gene of *GmCOL1a*, which was required to promote the stress-responsive GmP5CS protein in soybean and improved salt tolerance and drought resistance by increasing the expression of *GmP5CS* to increase the RWC, proline content, and SOD, POD, and CAT enzyme activities.

Overexpression of *GmP5CS* rescued the salt- and drought-sensitive phenotypes of *co-9* plants

Since *GmP5CS* has been proven to increase salt and drought tolerance, we further investigated whether the transformation of *GmP5CS:GmP5CS-GFP* into the *co-9* mutant background by *A. rhizogene*-mediated hairy-root transformation could rescue the salt- and drought-sensitive phenotype of *co-9* plants (Figure 8, A and B). Under salt and drought stress, the survival rates of "composite" plants with *GmP5CS* hairy roots increased by 48.33% and 50%, respectively (Figure 8C). Similarly, the RWCs of *GmP5CS* "composite" transgenic soybean plants increased by 27.66% and 38.18% after salt and drought treatment, respectively (Figure 8D).

Based on all experimental evidence, we proposed that GmCOL1a was a nuclear-localized protein that regulated salt and drought tolerance in soybean. GmCOL1a promoted drought tolerance dependent on the ABA signaling pathway. Our proposed model clarified that GmCOL1a regulates salt and drought stress responses by directly binding to Myc(bHLH) and Che motifs in the promoters of the stress resistance-related genes *GmLEA* and *GmP5CS*, respectively, to up-regulate their transcription. GmCOL1a enhanced salt and drought tolerance by promoting GmP5CS accumulation, which increased the proline content and reduced ROS production to protect soybean plants from salt and drought stress damage. Meanwhile, GmCOL1a might also directly inhibit ROS production independent of downstream *GmP5CS* (Figure 9).

Discussion

GmCOL1a was involved in the stress resistance of soybean plants

Currently, the molecular mechanisms involved in the photoperiodic pathway of *CO* in plants are relatively well established. For example, the TF *CO* is a crucial gene affecting the circadian rhythm and flowering time in plants, regulated by the circadian and biological clock, which promotes flowering by affecting the expression of the flowering hormone *FLOWERING LOCUS T* (*FT*; Suárez-López et al., 2001; Hayama et al., 2003). The four cis-elements (CORE1, CORE2, P1, and P2) of the *FT* promoter region are direct targets of CO, forming a CO-NF-Y complex that binds to the *FT* promoter with high overall binding specificity and affinity, further revealing the importance of CO in the photoperiodic pathway (Lv et al., 2021). A few studies have shown the involvement of the *CO* family in abiotic stress responses in *Arabidopsis*, maize, rice, apple, mango, and *Tamarix hispida* plants. However, the analysis of *CO* gene expression in soybean under abiotic stress has not been reported. In this study,

Figure 7 (Continued)
hairy roots expressing *GmP5CS*. GFP fluorescence was detected in hairy roots infected by *A. rhizogenis* strain K599 harboring *pCAMBIA1302-no35S* (control) and *GmP5CS:GmP5CS-GFP-pCAMBIA1302* plasmids, in WT, *co-9*, and *GmCOL1a-ox-2* background, respectively. Scale bar = 100 μm. B, Phenotypes and survival rates of "composite" plants with *GmP5CS:GmP5CS* transgenic hairy roots in WT background after 200 mM NaCl treatment for 6 days. Scale bar = 5 cm. Survival rates were quantified ($n \geq 20$). Asterisks indicated a significant change compared with the empty vector. Data shown are means ± SD (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test). C, Phenotypes and survival rates of "composite" plants with *GmP5CS:GmP5CS* transgenic hairy roots in WT background after dehydration for 5 days and recovery for 2 days. Scale bar = 5 cm. Survival rates were quantified ($n \geq 20$). Asterisks indicated a significant change compared with the empty vector. Data shown are means ± SD (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test). D, RWCs of leaves were measured from "composite" plants with *GmP5CS:GmP5CS* transgenic hairy roots in WT background after 200 mM NaCl treatment for 6 days or dehydration for 5 days. RWCs of leaves were calculated ($n \geq 10$). Asterisks indicated a significant change compared with the empty vector. Data shown are means ± SD (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test). E, The transcript level of *GmP5CS* in transgenic hairy roots in WT background. Data shown are means ± SD of three independent experiments. F, Immunoblotting showing GmP5CS protein levels of *GmP5CS:GmP5CS-GFP* using GFP-Tag antibodies, Anti-HPT served as the loading control. Protein expression profiles of GmP5CS-GFP exposed to 150 mM NaCl or 10% PEG treatment for 6 h. Roots of GmP5CS-GFP protein calculated by normalization of the GmP5CS-GFP signals to Anti-HPT signals and presented as GmP5CS-GFP/HPT. Data shown are means ± SD of three independent experiments.

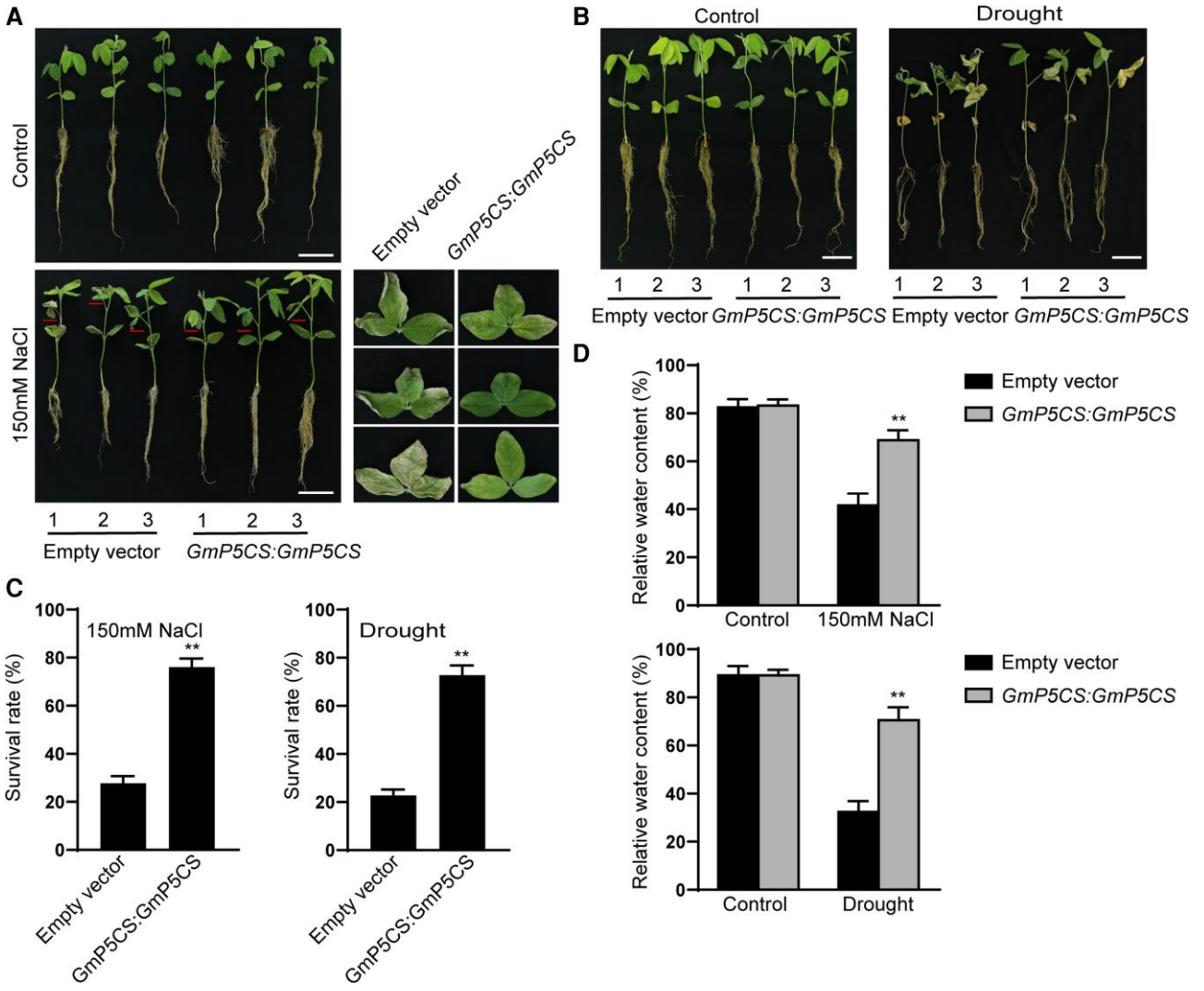

Figure 8 Overexpression of *GmP5CS* in transgenic soybean hairy roots rescued the salt and drought-sensitive phenotype of the *co-9* plants. A, Phenotypes of "composite" plants with *GmP5CS:GmP5CS* transgenic hairy roots in *co-9* background after 150 mM NaCl treatment for 5 days. Scale bar = 5 cm. B, Phenotypes of "composite" plants with *GmP5CS:GmP5CS* transgenic hairy roots in *co-9* background after dehydration for 6 days. Scale bar = 5 cm. C, Survival rates of "composite" plants with *GmP5CS:GmP5CS* transgenic hairy roots in *co-9* background under salt and drought stress. Survival rates were quantified ($n \geq 20$). D, RWCs of leaves were measured from "composite" plants with *GmP5CS:GmP5CS* transgenic hairy roots in *co-9* background under salt and drought stress. RWCs of leaves were calculated ($n \geq 10$). Asterisks indicated a significant change compared with the empty vector. Data shown are means ± SD of three independent experiments (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test).

we demonstrated that *GmCOL1a* overexpression in transgenic soybean plants improved salt tolerance and drought resistance, and knockout mutant *co-9* transgenic soybean plants showed opposite phenotypes (Figures 2E and 4A). The *GmCOL1a* gene contains two B-box domains and a CCT domain, which are typical B-box proteins (BBXs; Putterill et al., 1995; Gangappa and Botto, 2014). Previous studies have demonstrated that *GbBBX25* improves salt acclimation in transgenic *Populus* (Huang et al., 2021). The homolog of GmCOL1a was AtCOL4 (BBX5), with a sequence similarity of 35.62%, which also contained two B-box domains and a CCT domain. This enhances plant tolerance to abiotic stress by participating in an ABA-dependent signaling pathway (Min et al., 2015). *MdBBX7*/*MdCOL9* overexpression enhanced drought tolerance in transgenic apples and regulated the effect of the MdMIEL1-MdBBX7 module on the drought stress response (Chen et al., 2022). Therefore, we hypothesized that GmCOL1a was involved in the abiotic stress response in soybean plants, probably because of the roles of the B-Box domain and CCT domain.

Transgenic *GmCOL1a-ox-2* soybean had a reduced Na^+/K^+ ratio, improving salt tolerance

Reducing the Na^+/K^+ ratio was a key factor in improving salt tolerance in plants by regulating the ion dynamic balance. Salt induction affects the circulation and transport of K^+ in specific tissues, balancing excess Na^+ and thus conferring

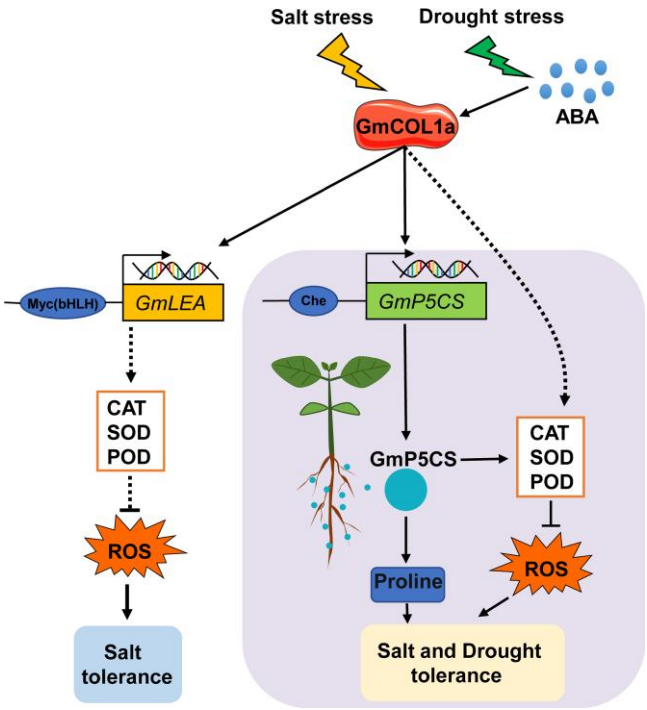

Figure 9 Proposed model depicting the function of GmCOL1a in the regulation of salt and drought stress tolerance. The solid arrows indicate activation of transcription, dotted-lines denote a relationship that has not been established as direct, and lines ending with a dash denote repression of transcription. GmCOL1a is induced by salt and drought tolerance. GmCOL1a is mediated by the abscisic acid (ABA), which enhances drought resistance. GmCOL1a promoted the transcriptional level of *GmLEA*, probably by decreasing the accumulation of ROS, thus improving salt tolerance in *GmCOL1a*-overexpressing transgenic plants. In addition, GmCOL1a not only increased the transcript level of *GmP5CS* but also promoted GmP5CS protein accumulation, which increased proline content and reduced ROS production, thereby improving stress tolerance in soybean plants.

salt tolerance to the plant (Álvarez-Aragón et al., 2016). In the present study, we initially investigated the effect of the *GmCOL1a* gene on the Na^+/K^+ ratio under salt stress conditions. We observed that the Na^+/K^+ ratio of *GmCOL1a-ox-2* transgenic soybean was significantly lower in stems and leaves but not in the roots compared to those in the WT and *co-9* after salt stress (Figure 3A). We speculated that *GmCOL1a* reduced Na^+ ion toxicity by reducing its transport from the roots to the stems and leaves and especially maintained the lower dynamic balance of Na^+/K^+ in leaves. Most *NHX* transgenic plants have improved salt stress adaptation by reducing the Na^+ content in the cytoplasm or by maintaining a higher K^+/Na^+ ratio (Wu et al., 2016; Sun et al., 2021). *PgTIP1*-trasgenic soybean reduced the Na^+/K^+ ratio in stems and leaves but not in roots (An et al., 2017), which was similar to the results of the present study. Although the K^+ content of *GmCOL1a-ox-2* transgenic soybean stems and leaves was reduced under salt stress conditions, the Na^+/K^+ ratio remained relatively low, improving plant salt tolerance.

GmP5CS increased the proline content

The GmP5CS protein regulated abiotic stress responses in the WT and *GmCOL1a*-overexpressing transgenic soybeans. Previous research has established that the gene encoding *AtP5CS* was induced by salt stress, drought stress, and ABA in *Arabidopsis* (Yoshiba et al., 1995). The two homologs of GmP5CS were *Glycine max* (*gmp5cs*), with a sequence similarity of 28.69%, and *Lactuca sativa* (*lsp5cs*), with a sequence similarity of 29.86%, which were reported to be up-regulated only under drought stress (Porcel et al., 2004). However, a functional analysis of the *P5CS* gene in soybean plants under salt stress has not been performed. Here, we reported the facilitation of abiotic stress responses by the *GmP5CS* gene in soybean because *GmP5CS* enhanced the ability of soybean to resist high salt and drought conditions (Figure 7, B and C). Furthermore, *P5CS* plays a key role in plant proline biosynthesis (Hu et al., 1992). This was consistent with the results that *GmP5CS* transgenic hairy roots were able to accumulate more proline content, further increasing the proline content under salt and drought treatments (Supplemental Figure S1, A). In addition, we described a previously uncharacterized mechanism whereby GmCOL1a not only increased the transcript level of *GmP5CS* but also promoted GmP5CS protein accumulation, resulting in an increased proline content and reduced ROS production, thereby improving stress resistance in soybean plants.

Materials and methods

Identification and phylogenetic relationship analysis of *GmCOL1a* genes

Multiple full-length GmCOL1a amino acid sequences of soybean (*Glycine max*) and other plants were aligned using the ClustalW program (http://www.clustal.org/). An NJ tree was constructed using MEGA7.0 and polished using EvolView with 1,000 bootstrap trials.

Plant materials and growth conditions

For the germination assay, three T_3 generation transgenic soybean lines *GmCOL1a-ox-2*, *GmCOL1a-ox-4*, and *GmCOL1a-ox-17*, T_3 generation *co-9* Crispr/Cas9 mutant, and WT (DN50) seeds were chlorine-sterilized and placed on 1/2 MSB medium (1/2 Murashige and Skoog basal nutrient salts, B5 vitamins, and 2% sucrose, pH 5.7) supplemented with 0 and 250 mM NaCl and germinated at 25°C under LDs (16 h/8 h light/dark). Images were taken, and germination rates and hypocotyl lengths were recorded at the end of the experiment.

Three *GmCOL1a-ox* transgenic lines, the *co-9* mutant, and WT soybeans were also used for phenotype analysis. Soybean seeds were germinated for 4 days in grass charcoal soil and vermiculite (1:1 ratio), and uniformly growing seedlings were transferred to LDs at 25°C and 250 μmol m^{-2} s^{-1} with white light and 60% relative humidity and grown in plastic root growth bags containing a half-strength

Hoagland solution. In the root growth phenotype assay, the movement of roots was minimized, and five parallel bags were used for each treatment, with all genotypes contained in the same number of bags. Survival rate and root length were photographed and recorded by adding 100 mM NaCl to the hydroponic solution and changing the solution every 3 days for 21 days of treatment.

N. benthamiana was grown in a greenhouse at 22°C under LDs.

RNA extraction and RT-qPCR analysis

To analyze the expression pattern of *GmCOL1a* (*Glyma.08G255200*), *GmLEA* (*Glyma.13G149000*), and *GmP5CS* (*Glyma.18G188000*) genes under salt and drought stress, the soybean cultivar "DN50" was grown under LDs at 25°C and 250 μmol m^{-2} s^{-1} with white light. When the first trifoliate leaves were fully expanded, soybean seedlings were treated with control (a half-strength Hoagland solution), 10% [w/v] PEG 6000, and 150 mM NaCl. Samples were collected after 0, 1, 3, and 6 h, immediately frozen in liquid nitrogen, and stored at −80°C.

Total RNA was extracted from soybean plants using Trizol reagent (Invitrogen), as described previously (Zhao et al., 2013). The resulting cDNA was used as a template using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). *GmActin4* (GenBank accession number AF049106) was used as an internal control. Three biological replicates and three technical replicates were applied to the assays. The primers used for RT-qPCR are listed in Supplemental Table S1.

Plasmid construction and generation of transgenic soybean

Total RNA was reverse transcribed into first-strand cDNA. A 1047 bp *GmCOL1a* gene fragment was cloned from the DN50 using the *GmCOL1a-3F6H*-F and *GmCOL1a-3F6H*-R primers (Supplemental Table S1). The *GmCOL1a* gene fragment was reassembled with the *pENTRY-3F6H* vector (named *35S:GmCOL1a-3F6H-pENTRY*) using the In-Fusion Cloning System (Clontech, USA). The recombinant plasmid *35S:GmCOL1a-3F6H-pENTRY* was synthesized into the *pB7WG2* vector by the LR reaction (named *35S:GmCOL1a-3F6H-pB7WG2*). The construct was then transferred into *Agrobacterium tumefaciens* (*EHA105*). According to the method described previously (Zhao et al., 2018), transgenic soybean DN50 expressing *35S:GmCOL1a-3F6H-pB7WG2* was obtained.

The design of the target sequence adapters for the *GmCOL1a* gene was performed using the online tool CRISPR-P (http://cbi.hzau.edu.cn/crispr/). The DNA oligonucleotide pairs (*GmCOL1a-cas9*-F and *GmCOL1a-cas9*-R) of the synthesized sgRNA were annealed and generated into a dimer bound to the *pGES-201* vector (Bai et al., 2020). The recombinant vector was introduced into the *Agrobacterium tumefaciens* strain, *EHA105*, and then transformed into soybean DN50 (Zhao et al., 2018). Transgenic soybean plants were screened using PCR. One representative homozygous line (*co-9*) was selected for further study.

For the cloning of *GmCOL1a* into the *pDEST17* vector to express the protein in *E. coli*, the full-length coding region of *GmCOL1a* was amplified by PCR using *GmCOL1a-TOPO-F* and *GmCOL1a-TOPO-R* primers (Supplemental Table S1), cloned into *pENTR/D-TOPO* (Thermo Fisher Scientific, USA), and transferred to the expression vector *pDEST17* through an LR reaction to generate the *HIS-GmCOL1a* fusion vector. The construct was transformed into the *E. coli* competent cell line *BL21* (*DE3*; Transgene, Beijing, China) to produce recombinant proteins. For subcellular localization, the *GmCOL1a-TOPO* plasmid was also transferred to the expression vector *pGWB506* through an LR reaction to generate the *35S:GFP-GmCOL1a* fusion vector.

To clone *GmP5CS* into the *pCAMBIA1302-no35S* plasmid, *pCAMBIA1302* was first digested with Hind III and Nco I to remove the 35S fragment, blunted by T_4 DNA polymerase, and ligated by T_4 DNA ligase to produce the circular plasmid *pCAMBIA1302-no35S*. The full-length sequences consisting of 1427-bp promoter regions were amplified from the genomic DNA of DN50 using *GmP5CS-P*-F and *GmP5CS-P*-R, and 2163-bp coding sequences (CDSs) of *GmP5CS* were amplified from the cDNA of DN50 by overlapping PCR with pairs of *GmP5CS-CDS*-F and *GmP5CS-CDS*-R. PCR was then performed using *GmP5CS-P*-F and *GmP5CS-CDS*-R primers to obtain the *GmP5CS:GmP5CS-GFP* PCR product (Supplemental Table S1; Yang et al., 2021). The PCR products were purified and cloned into the *pCAMBIA1302-no35S* vector containing the GFP sequence in the C-terminus of the cloning site linearized by Bgl II using the In-Fusion cloning system to construct the recombinant *GmP5CS:GmP5CS-GFP-pCAMBIA1302* fusion expression vector. The recombinant vector (*GmP5CS:GmP5CS-GFP-pCAMBIA1302*) was transferred into the *Agrobacterium rhizogene* (*A. rhizogenes*) strain *K599*.

Identification of phenotypes under salt and drought stress

Three transgenic *GmCOL1a-ox* lines, the *co-9* mutant, and WT soybeans were planted in 10 pots, each with three seedlings. For the salt stress tests, the plants were immersed in a 200 mM NaCl solution when the first trifoliate leaves were fully expanded. After 8 days of salt treatment, the RWC was determined in the first pair of unifoliolate leaves of soybean plants, as previously described (Bao et al., 2014). According to a previous report, measurements of Na^+ and K^+ concentration were performed using atomic absorption spectrophotometry (AAS; Hitachi Z-2000; Xu et al., 2006; Sun et al., 2021). The analysis was repeated in triplicate. For dehydration experiments, the water supply was stopped at the first untreated growth stage to ensure consistent watering in each pot, and the control group was watered with half-strength Hoagland solution every 3 days when the first trifoliate leaves of the plants were fully expanded.

Stomatal aperture measurement

Three-week-old soybean leaves were placed in stomatal opening solution (30 mM KCl and 10 mM MES-KOH, pH 6.15) for 2 h and then treated with 10 μM ABA for 3 h (Li et al., 2013). Stomatal observation was performed with a confocal microscope (TCS SP5; Leica Microsystems). The experimental setup followed by: bit depth was eight bit, depth of focus was 1.72 μm, digital offset was 0, digital gain was 1 (Martin et al., 2009).

Histochemical and physiological index analysis

For 3,3-DAB staining, root tips or leaf samples were immersed in 50 mM DAB solution (Solarbio, China) for 12 or 24 h and then decolorized in 95% [v/v] ethanol until the color turned white. For NBT staining, root tips or leaf samples were immersed in 50 mM NBT solution (Creek Huizhi, China) for 15 min or 16 h and then decolorized in 95% [v/v] ethanol until the color turned white (Wang et al., 2017). Images were taken using a Canon 50D (Canon, Japan) camera. After treatment, 100 mg of root or leaf samples was subjected to H_2O_2 content measurement (Solarbio, China).

For physiological parameter measurements, 2-week-old seedlings of three *GmCOL1a-ox* transgenic lines, the *co-9* mutant, and WT soybeans were exposed to 150 mM NaCl or 10% [w/v] PEG 6000 for 6, and 100 mg of leaf tissues was used to measure the free proline (Bates et al., 1973) and MDA content (Gao et al., 2011) and catalase (CAT; Abei, 1984), SOD (He et al., 2009), and POD activity (Kong et al., 2005). A half-strength Hoagland nutrient solution was used as a control.

Immunoblot analysis

To measure the protein expression of the *GmCOL1a* gene driven by the 35S promoter in transgenic soybean, the 35S:GmCOL1a-3F6H protein was extracted in extraction buffer for the extraction of whole proteins from soybean leaves (Wang et al., 2021). The HRP-coupled anti-FLAG antibody (A8592, Sigma) was used to detect the 35S:GmCOL1a-3F6H protein. A mouse β-actin monoclonal antibody (HRP-60008, Proteintech) was used as a control to detect actin proteins.

The GmP5CS:GmP5CS protein in transgenic hairy roots of in vitro-generated "composite" plants was extracted in the same extraction buffer. The protein extracts were detected with a GFP-Tag antibody (AE011, Abclonal), followed by an HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H + L) Secondary Antibody (SA00001–2, Proteintech). Anti-HPT (AbM59707–5-PU, BPI), followed by HRP-conjugated goat anti-mouse IgG (H + L) Secondary Antibody (G-21040, Thermo Fisher Scientific), was used as a control to detect HPT proteins.

Super Signal West Pico chemiluminescent substrate kits (Thermo Fisher Scientific, USA) were used to detect the signals by chemiluminescent imaging (Amersham Imager 600). All experiments were performed at least three times with independent biological replicates.

ChIP-qPCR

After 14 days of growth, *35S:GmCOL1a-3F6H* transgenic soybean seedlings and WT seedlings were transferred to 150 mM NaCl or 10% [w/v] PEG 6000 solution for 0 or 3 h at LDs. Half-strength Hoagland solution was used as a control, and the tissues were fixed with a 1% [v/v] formaldehyde crosslinking method for 15 min. ChIP-qPCR assays were performed as previously described (Wang et al., 2021). Immunoprecipitation reactions were performed using an anti-FLAG antibody (mouse-produced Monoclonal ANTI-FLAG M2 antibody, F1804, Sigma-Aldrich) and an anti-IgG antibody (Normal Rabbit IgG, 2729, Cell Signaling Technology) control. Complexes of chromatin antibodies were captured with protein G beads (Invitrogen), and DNA was purified using a QIAquick PCR purification kit (QIAGEN). Enriched DNA fragments were identified by RT-qPCR analysis, and three biological replicates and three technical replicates were performed for each sequence segment. The primers used in ChIP-qPCR are listed in Supplemental Table S1.

EMSA

Binding of the GmCOL1a protein to the Myc(bHLH) motif and Che motif in the promoter regions of *GmLEA* and *GmP5CS*, respectively, was examined by EMSA. Oligonucleotide probes containing the Myc(bHLH) motif (CACGTG) and the Che motif (GGATTCTC) were synthesized and labeled with biotin at the 5′ end. Probe sequences are shown in Supplemental Table S1. For competition with the unlabeled probe, an unlabeled probe was added to the reactions. *BL21(DE3)* containing the *HIS-GmCOL1a* fusion vector was induced using 0.5 mM isopropyl-b-ß-1-thiogalactopyranoside at 18°C for 12 h. The empty vector (*pET-28a*) was used as the negative control. The recombinant protein was purified using a 6×HIS-Tagged Protein Purification Kit (Soluble Protein; CWBIO, China) according to the manual provided by the manufacturer. The HIS-GmCOL1a fusion protein was incubated with the labeled probe and unlabeled DNA fragments (100-fold excess of the labeled probe). According to the manufacturer's protocol, the biotin-labeled probes were visualized using a Light Shift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, USA). The protein–DNA complexes were separated with 6% [w/v] native polyacrylamide gels and visualized on nylon membranes by chemiluminescence imaging (Amersham Imager 600).

Transient expression assays in *N. benthamiana* leaves

GmLEA and *GmP5CS* promoters were amplified from the genomic DNA of DN50 using the specific primers listed in Supplemental Table S1. The *pGreenII-0800-GmLEA* and *pGreenII-0800-GmP5CS* recombinant vectors were constructed using the In-Fusion Cloning System (Clontech, USA). The recombinant plasmid vectors were introduced into *Agrobacterium tumefacien GV3101* by electroporation and transformed into *N. benthamiana*, as previously described (Sheikh et al., 2014). Firefly and Renilla luciferase

activities were detected and analyzed, as described previously (Yang et al., 2021).

Transformation and detection of soybean hairy roots

Seven-day-old soybean DN50 seedlings were transformed by *A. rhizogenes* to form hairy roots using hypocotyl inoculation. Transgenic hairy roots were generated as described previously (Kereszt et al., 2007; Wang et al., 2015). The "composite" transgenic soybean plants were treated with 200 mM NaCl or subjected to drought for phenotypic observations, and positive "composite" transgenic soybean plants were used to determine the RWC, proline content, and CAT, SOD, and POD activities.

The use of *A. rhizogenes*-mediated cotyledonary transformation to obtain an in vitro-generated plant followed the inoculation method (Li et al., 2010; Cao et al., 2011). When hairy roots emerged, they were screened with a confocal microscope (TCS SP5; Leica Microsystems) to visualize GFP expression. The experimental setup followed by: excitation wavelength and acquisition bandwidth of laser at 488/490–540 nm for GFP, bit depth was eight bit, depth of focus was 1.72 µm, digital offset was 0, digital gain was 1(Martin et al., 2009). Expression of the *GmP5CS* gene in in vitro-generated plants with transgenic cotyledon hairy roots was tested using GFP fluorescence, RT-qPCR, and western blot.

Subcellular localization of GmCOL1a

The recombinant *35S:GFP-GmCOL1a* construct was introduced into *Agrobacterium GV3101* and subsequently transformed into *N. benthamiana* (Sheikh et al., 2014). A red nuclear marker plasmid (H2B-RFP) was used to confirm the location of the cell nucleus (Goodin et al., 2002). Two days after transfection, the leaves of *N. benthamiana* were imaged using a confocal microscope (TCS SP5; Leica Microsystems). The experimental setup followed by: excitation wavelength and acquisition bandwidth of laser at 561/580–630 nm for RFP, 488/490–540 nm for GFP, respectively, bit depth was eight bit, depth of focus was 1.72 µm, digital offset was 0, digital gain was 1(Martin et al., 2009).

Accession numbers

Sequence data from this article can be found in the Phytozome data libraries under accession numbers: *GmCOL1a* (*Glyma.08G255200*), *GmLEA* (*Glyma.13G149000*), *GmP5CS* (*Glyma.18G188000*), *GmACTIN* (*Glyma.12G063400*).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Physiological assays of *GmP5CS: GmP5CS* transgenic hairy root "composite" plants under salt and drought stress.

Supplemental Table S1. Primer sequences used in this study.

Supplemental Table S2. Peak sequences.

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