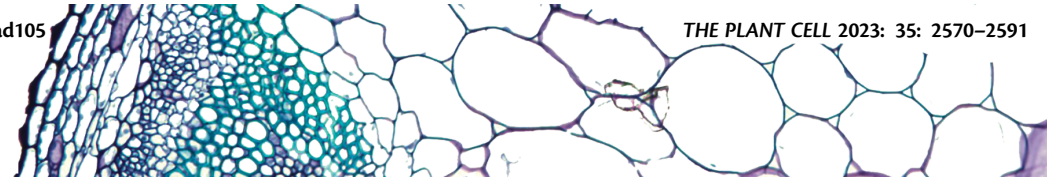
https://doi.org/10.1093/plcell/koad105 THE PLANT CELL 2023: 35: 2570–2591



Research Article

CycC1;1–WRKY75 complex-mediated transcriptional regulation of *SOS1* controls salt stress tolerance in *Arabidopsis*

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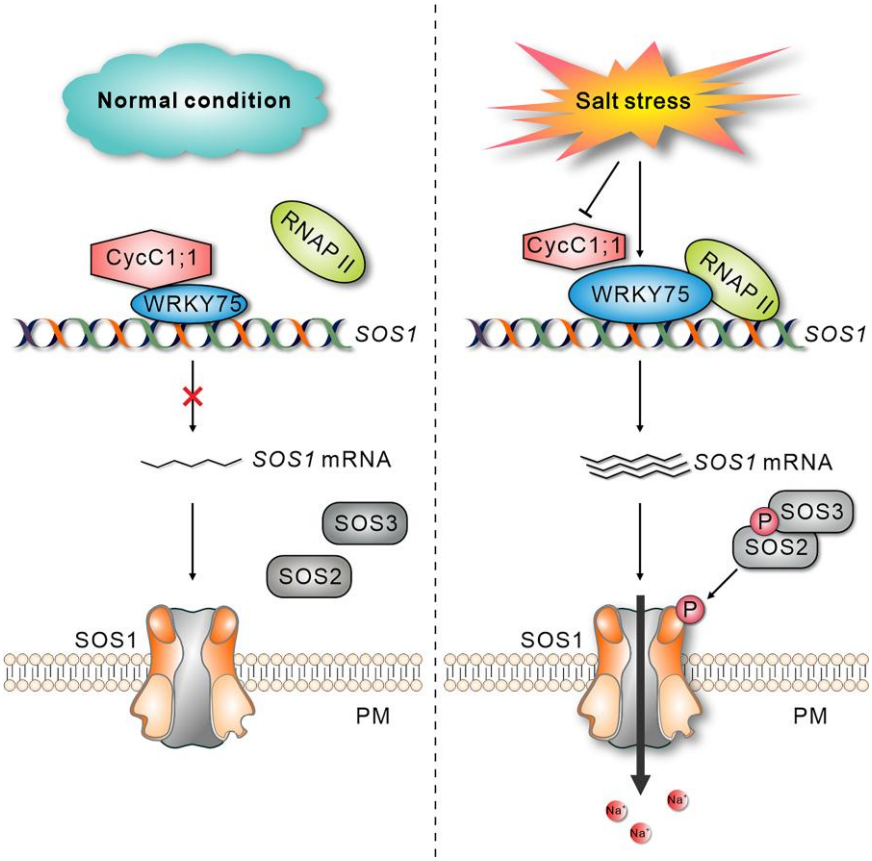
Abstract

SALT OVERLY SENSITIVE1 (SOS1) is a key component of plant salt tolerance. However, how *SOS1* transcription is dynamically regulated in plant response to different salinity conditions remains elusive. Here, we report that C-type Cyclin1;1 (CycC1;1) negatively regulates salt tolerance by interfering with WRKY75-mediated transcriptional activation of *SOS1* in *Arabidopsis* (*Arabidopsis thaliana*). Disruption of *CycC1;1* promotes *SOS1* expression and salt tolerance in *Arabidopsis* because CycC1;1 interferes with RNA polymerase II recruitment by occupying the *SOS1* promoter. Enhanced salt tolerance of the *cycc1;1* mutant was completely compromised by an *SOS1* mutation. Moreover, CycC1;1 physically interacts with the transcription factor WRKY75, which can bind to the *SOS1* promoter and activate *SOS1* expression. In contrast to the *cycc1;1* mutant, the *wrky75* mutant has attenuated *SOS1* expression and salt tolerance, whereas overexpression of *SOS1* rescues the salt sensitivity of *wrky75*. Intriguingly, CycC1;1 inhibits WRKY75-mediated transcriptional activation of *SOS1* via their interaction. Thus, increased *SOS1* expression and salt tolerance in *cycc1;1* were abolished by *WRKY75* mutation. Our findings demonstrate that CycC1;1 forms a complex with WRKY75 to inactivate *SOS1* transcription under low salinity conditions. By contrast, under high salinity conditions, *SOS1* transcription and plant salt tolerance are activated at least partially by increased *WRKY75* expression but decreased *CycC1;1* expression.

Received October 10, 2022. Accepted March 20, 2023. Advance access publication April 12, 2023

Graphical Abstract



IN A NUTSHELL

Background: Soil salinization is a major environmental hazard that severely affects plant growth and development. Plants have evolved sophisticated mechanisms that help them withstand elevated soil salinity, and Salt Overly Sensitive1 (SOS1) plays a crucial role in plant salt stress tolerance by facilitating the extrusion of excess Na^+ from the cells. Although previous reports have demonstrated the important role of posttranslational regulation of SOS1 in plant salt stress tolerance, how *SOS1* transcription is dynamically modulated in response to different salinity conditions remains unclear.

Question: What are the molecular mechanisms by which plants regulate *SOS1* expression at the transcriptional level in response to salinity stress?

Findings: Disruption of the CycC1;1 subunit of the plant Mediator complex promotes salt-induced *SOS1* expression and salt tolerance in *Arabidopsis* because CycC1;1 interferes with RNA polymerase II recruitment by occupying the *SOS1* promoter. *SOS1* mutation in the *cycc1;1* mutant completely compromised its enhanced salt tolerance. Moreover, CycC1;1 can physically interact with the transcription factor WRKY75, which can directly bind to the *SOS1* promoter and activate its expression. In contrast to the *cycc1;1* mutant, the *wrky75* mutant has attenuated *SOS1* expression and salt tolerance, whereas overexpression of *SOS1* can rescue the salt sensitivity of the mutant. Intriguingly, CycC1;1 inhibits WRKY75 transcriptional activation activity for *SOS1* through their interaction; thus, increased *SOS1* expression and salt tolerance in the *cycc1;1* mutant were abolished by the *WRKY75* mutation. In addition, *CycC1;1* expression is repressed, and *WRKY75* expression is stimulated in response to high salinity.

Next steps: In a future study, we will explore whether other components of the Mediator complex are coordinated with CycC1;1 to precisely control *SOS1* transcription, and investigate how salinity affects *CycC1;1* and *WRKY75* expression in the plant's response to different salinity conditions.

Introduction

Soil salinization is one of the major abiotic stresses that severely affect plant growth and development, causing huge losses in crop production worldwide (Zhu 2001; Yang and Guo 2018a; Van Zelm et al. 2020). During evolution, plants have developed various adaptive strategies to cope with high salinity stress (Zhu 2002, 2003; Van Zelm et al. 2020). The Salt Overly Sensitive (SOS) pathway plays a crucial role in plant salt stress tolerance (Zhu 2001; Yang and Guo 2018a). Three core proteins have been identified in the SOS pathway in *Arabidopsis* (*Arabidopsis thaliana*): SOS1, SOS2, and SOS3 (Yang and Guo 2018b; Zhu 2001). SOS1 is a Na^+/H^+ antiporter located at the plasma membrane that acts downstream in the SOS pathway by facilitating the efflux of excess Na^+ from cells and regulating long-distance transport of Na^+ in plants (Shi et al. 2002; Zhu 2002). It is well-known that a salt stress–induced cytosolic calcium signal is perceived by SOS3, an EF-hand calcium-binding protein, which then interacts with and activates SOS2, a Ser/Thr protein kinase (Ishitani et al. 2000). The activated SOS2 phosphorylates SOS1, promoting its activity and thus increasing salt tolerance (Liu et al. 2000; Zhu 2002). SOS1 activity is tightly regulated in plants: stimulated under high salinity conditions while inactivated under low salinity conditions through multiple different regulatory mechanisms, enabling intracellular sodium homeostasis.

Besides the three core proteins in the SOS pathway, other factors are implicated in plant salt response and tolerance. CALCIUM BINDING PROTEIN8 (SCaBP8)/Calcineurin B-like10 (CBL10) is a SOS3-like protein that recruits SOS2 to the plasma membrane in a calcium-dependent manner, thereby activating SOS1 and promoting plant salt tolerance (Liu et al. 2000; Quan et al. 2007; Lin et al. 2009). Mitogen-activated protein kinase (MPK) signaling cascades are also involved in plant salt stress tolerance through MPK6-mediated phosphorylation of SOS1 (Yu et al. 2010). In addition, VPS23A, the component of the endosomal sorting complex required for transport (ESCRT) enhances SOS2–SOS3 interaction and their localization at the plasma membrane, thus increasing SOS1 activity and salt tolerance (Lou et al. 2020). Interestingly, salt stress increases the conversion of phosphatidylinositol into phosphatidylinositol 4-phosphate, enhancing plant salt tolerance by activating SOS1 activity at the plasma membrane (Yang et al. 2021). A recent study reported that a small peptide PAMPINDUCED SECRETED PEPTIDE 3 (PIP3) could bind to and activate a leucine-rich repeat receptor-like kinase RECEPTOR-LIKE KINASE 7 (RLK7), leading to activation of MPK3 and MPK6 as well as salt tolerance in *Arabidopsis* (Zhou et al. 2022). In contrast, some negative regulators of the SOS pathway have also been identified and documented. For example, the photoperiodic and circadian clock oscillator protein Gigantea (GI) interacts with and inhibits SOS2 to inactivate the SOS pathway under normal conditions, whereas high salinity stress decreases GI protein accumulation, resulting in the activation of the pathway (Kim et al. 2013). The 14-3-3 proteins λ and κ can also interact with and inhibit SOS2 activity in the absence of salt stress, while salt stress reduces the interaction between the 14-3-3 proteins and SOS2, leading to activation of the SOS pathway for higher salt tolerance in *Arabidopsis* (Zhou et al. 2014). In addition to posttranslational modulation, SOS1 activity is also significantly modulated at the transcriptional level. It has long been known that, upon salt stress treatment, the *SOS1* transcripts are rapidly induced and accumulate, and their stability is promoted in *Arabidopsis* (Shi et al. 2000; Chung et al. 2008). Although a few transcription factors, including WRKY1 and MYB73, have been identified as positive or negative factors affecting *SOS1* transcription in *Arabidopsis* (Kim et al. 2013; Wu et al. 2022), how *SOS1* transcription is dynamically regulated to adjust plant responses to different salinity conditions remains to be further elucidated.

Mediator is a conserved protein complex in eukaryotes that significantly affects gene transcription by acting as a bridge between transcription factors and RNA polymerase II (RNAP II) (Asturias et al. 1999; Harper and Taatjes 2018; Agrawal et al. 2021). The Mediator complex is composed of 4 major components, including head, middle, tail, and kinase modules. The head, middle, and tail modules form the core part of the Mediator complex, providing interfaces for physical interaction between RNAP II and sequence-specific transcription factors (Jeronimo and Robert 2017). The kinase module is a separate part of the Mediator complex consisting of Cyclin-Dependent Kinase 8 (CDK8), C-type Cyclin (CycC), Mediator Complex Subunit12 (MED12), and MED13 (Wang and Chen 2004; Mathur et al. 2011; Maji et al. 2019) that significantly alters gene transcription by binding to the core part of Mediator (Poss et al. 2013). The Mediator complex functions in a variety of versatile roles in plant growth, development, and environmental responses (Agrawal et al. 2021; Chen et al. 2012; Chen et al. 2019; Guo et al. 2021; Maji et al. 2019), but whether and how it is involved in plant salt stress responses and tolerance remain unknown.

In this study, we identified C-type Cyclin1;1 (CycC1;1) as a negative regulator of salt stress tolerance in *Arabidopsis* by repressing *SOS1* expression via disrupting the recruitment of RNAP II on *SOS1* promoter. On the contrary, we found that WRKY75 positively regulated salt-induced *SOS1* expression by binding to the *SOS1* promoter. Furthermore, CycC1;1 interacted with WRKY75 and inhibited its transcriptional activation of *SOS1* expression, and this inhibition was relieved both by high salinity-mediated repression of *CycC1;1* expression and by induction of *WRKY75* expression. Our results demonstrate an important role for the CycC1;1–WRKY75 complex in transcriptional regulation of *SOS1*, allowing plants to respond to different salinity conditions.

Results

CycC1;1 negatively modulates salt tolerance

As a component of the Mediator complex, CycC is known to affect plant immunity by modulating the expression of

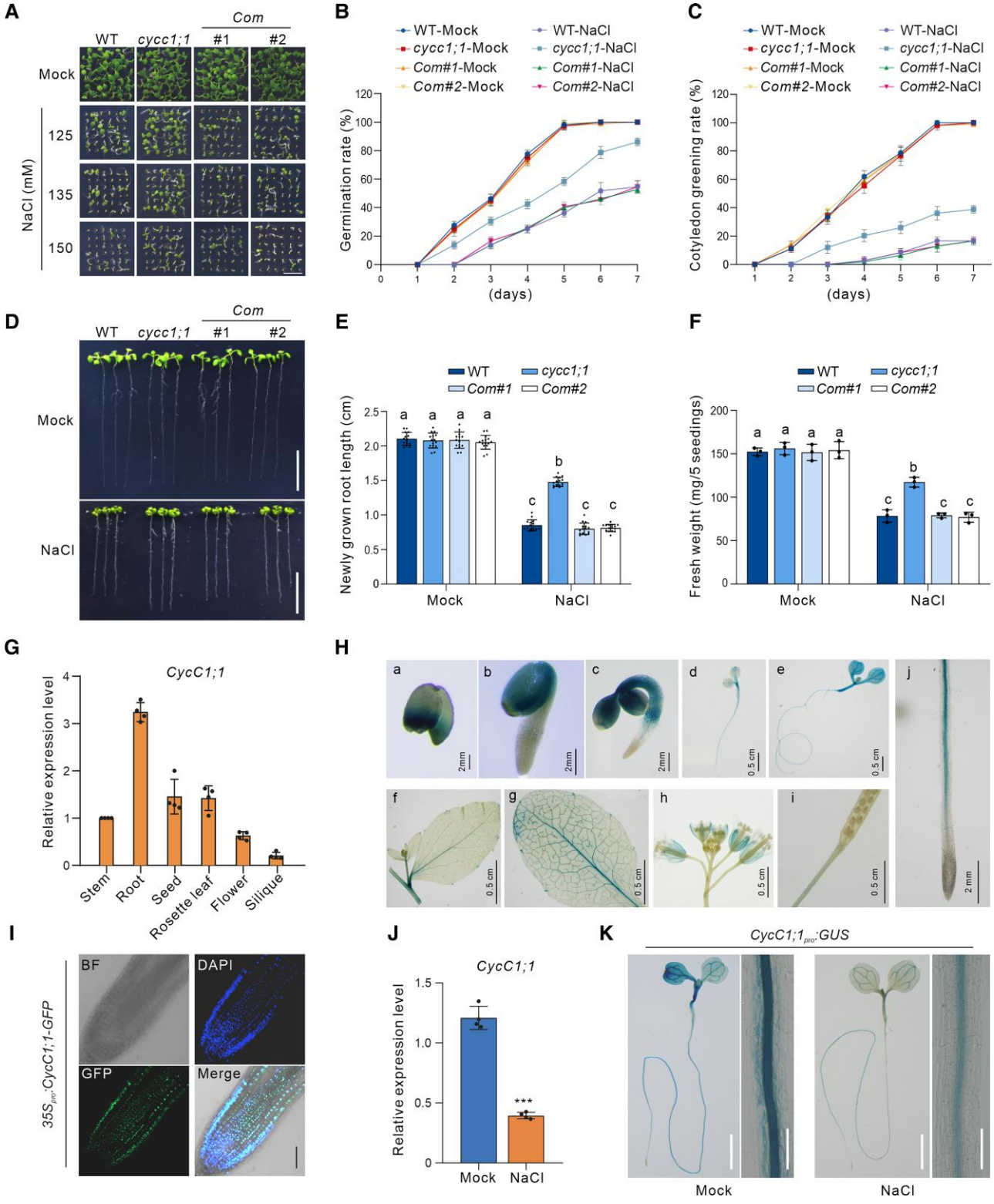


Figure 1. CycC1;1 negatively regulates plant salt tolerance. **A** to **C)** Phenotypes **A)** of the wild-type, *cycc1;1*, and complementation (*Com*) plants grown on 1/2× MS medium supplemented with 0 mM, 125 mM, 135 mM, or 150 mM NaCl for 5 d. Bar = 0.5 cm. Quantitative analysis of seed germination **B)** and cotyledon greening rates **C)** of plants grown on 1/2× MS medium supplemented with 0 mM or 125 mM NaCl for 7 d. Data are means ± SD of 3 independent biological repeats. **D** to **F)** Root elongation and fresh weight analysis. Five-day-old wild-type, *cycc1;1*, and *Com* plants were transferred to 1/2× MS medium supplemented with 0 mM or 125 mM NaCl for additional growth. Photographs were taken 5 d after transfer **D)**. Bar = 1 cm. The lengths of newly grown roots **E)** and the fresh weights **F)** of the seedlings were also analyzed. Data are means ± SD ($n = 15$ for root

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defense-related genes (Zhu et al. 2014), but it has not been characterized in the context of abiotic stress responses in plants. We had previously identified *cycc1;1* (SALK_053291), a T-DNA insertion mutant allele of *CycC1;1*, that showed decreased expression of *CycC1;1* (Guo et al. 2022). We recently found that this mutant also showed lower sensitivity to high salinity compared with the wild-type (WT) as evidenced by increased seed germination and cotyledon greening rates after 5 d in high-salt (125 to 150 mM) conditions (Fig. 1, A to C), suggesting the involvement of *CycC1;1* in the plant salt stress response. To test its salt tolerance, 5-d-old wild-type and the *cycc1;1* mutant seedlings grown on 1/2× Murashige–Skoog (1/2× MS) medium were subjected to high salinity for 2 additional days. Our results showed that the *cycc1;1* mutant had primary root length and fresh weight comparable to the wild-type under nonstress conditions, while the mutant displayed longer primary roots and a higher fresh weight than the wild-type under high salinity conditions (Fig. 1, D to F), revealing that the *cycc1;1* mutant was more tolerant to the salt stress than the wild-type plant. To confirm whether the salt-tolerant phenotype of the *cycc1;1* mutant was caused by the disruption of *CycC1;1*, transgenic complementary lines in which the expression of *CycC1;1* was driven by its native promoter (Guo et al. 2022) were analyzed for salt sensitivity. As expected, the complementary lines had similar salt tolerance to the wild-type regarding seed germination, primary root elongation, and fresh weight (Fig. 1, A to F). Furthermore, we previously assayed the salt tolerance of the *CycC1;1*-overexpression transgenic lines, CaMV $35S_{pro}$:*CycC1;1-GFP*, and found that the expression of *CycC1;1* was significantly higher than that in the wild-type plants (Guo et al. 2022). In contrast to the *cycc1;1* mutant, when exposed to high salinity, the *CycC1;1*-overexpression plants had lower germination rates, shorter primary roots, and lower fresh weights than the wild-type plants (Supplemental Fig. S1). These results indicate that CycC1;1 is a negative regulator of plant salt stress tolerance.

To better understand the *CycC1;1* expression pattern in *Arabidopsis*, we first measured the expression levels of *CycC1;1* in different tissues and organs of the wild-type plants, including stem, root, seed, rosette leaf, flower, and silique, and found that *CycC1;1* was highly expressed in root (Fig. 1G). To confirm this, we generated stable transgenic β-glucuronidase (GUS) reporter lines, $CycC1;1_{pro}$:*GUS*, in which *GUS* expression was under the control of the *CycC1;1* native promoter, enabling us to observe *CycC1;1* expression levels by GUS staining. GUS activity was higher in the germinating seeds and seedlings than in the mature leaves, cauline leaves, flower, and siliques (Fig. 1H), suggesting that *CycC1;1* is indeed highly expressed during the vegetative growth period, especially in the early stages. In addition to its expression pattern, we also examined the subcellular localization of CycC1;1 by observing the green fluorescence in the roots of $35S_{pro}$:*CycC1;1-GFP* transgenic plant seedlings (Fig. 1I). The location of CycC1;1-GFP fluorescence coincided well with the blue fluorescence resulting from staining with 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent dye specific for nuclei. This indicated that CycC1;1 is a nuclear protein in *Arabidopsis*.

To determine how *CycC1;1* responds to high salinity, we analyzed its expression levels in wild-type seedlings treated with or without high salinity. Our reverse transcription quantitative PCR (RT-qPCR) results showed that *CycC1;1* expression was lower in the salt-treated plants than in the untreated control plants (Fig. 1J), implying that salt stress represses *CycC1;1* expression. In agreement with this, when the $CycC1;1_{pro}$:*GUS* seedlings were subjected to high salinity, both the GUS staining and activity were obviously reduced compared with the unstressed plants (Figs. 1K and S2), further supporting that salt stress suppresses *CycC1;1* expression in *Arabidopsis*. Notably, *CycC1;1* was highly expressed in the stele of the root elongation and mature zones based on the GUS staining results, while such staining was very much repressed by salt stress (Fig. 1K).

CycC1;1 negatively regulates salt-induced *SOS1* expression

These results demonstrated that CycC1;1 negatively modulates salt tolerance, prompting us to analyze the Na^+ content in roots of salt-treated *cycc1;1* mutant plants. Using enhanced NaTrium Green-2 AM (ENG-2 AM), a sodium-specific fluorescent dye, our results showed that Na^+ accumulation was higher in both the salt-treated wild-type and *cycc1;1* mutant seedlings than in the untreated plants; nevertheless, the accumulation of Na^+ was significantly lower in the mutant than in the wild-type when treated with high salinity (Fig. 2, A and B). Because the SOS pathway plays a

(**Figure 1.** Continued)
length and $n = 3$ for fresh weight). Bars with different letters indicate significant differences at $P < 0.05$, determined using ANOVA with a Tukey's multiple comparison test (Supplemental Data Set 1). **G)** RT-qPCR results showing the expression of *CycC1;1* in different plant tissues, including stem, root, seed, rosette leaf, flower, and silique. The experiment was repeated 3 times. Data are means ± SD ($n = 4$). *ACTIN2* was used as a reference gene. **H)** Glucuronidase (GUS) staining images of the $CycC1;1_{pro}$:*GUS* transgenic reporter plants. a, seed; b, 1-d-old germinating seed; c, 3-d-old seedling; d, 5-d-old seedling; e, 7-d-old seedling; f, cauline leaf; g, rosette leaf; h, flower; i, silique; j, 5-d-old seedling root tip. a to c, j, bar = 2 mm; d to i, bar = 0.5 cm. **I)** Nuclear localization of CycC1;1-GFP in the 5-d-old $35S_{pro}$:*CycC1;1-GFP* transgenic plant root. DAPI was used to stain the nucleus. Bar = 100 μm. **J)** The expression of *CycC1;1* in 5-d-old wild-type plant seedlings treated with or without 100 mM for 12 h. The experiment was repeated 3 times. Error bars indicate mean ± SD ($n = 4$). Asterisks indicate significant differences determined by Student's *t* test (***$P < 0.001$). **K)** GUS staining images of the 5-d-old $CycC1;1_{pro}$:*GUS* transgenic seedlings treated without or with 100 mM for 12 h. Bar = 0.5 cm.

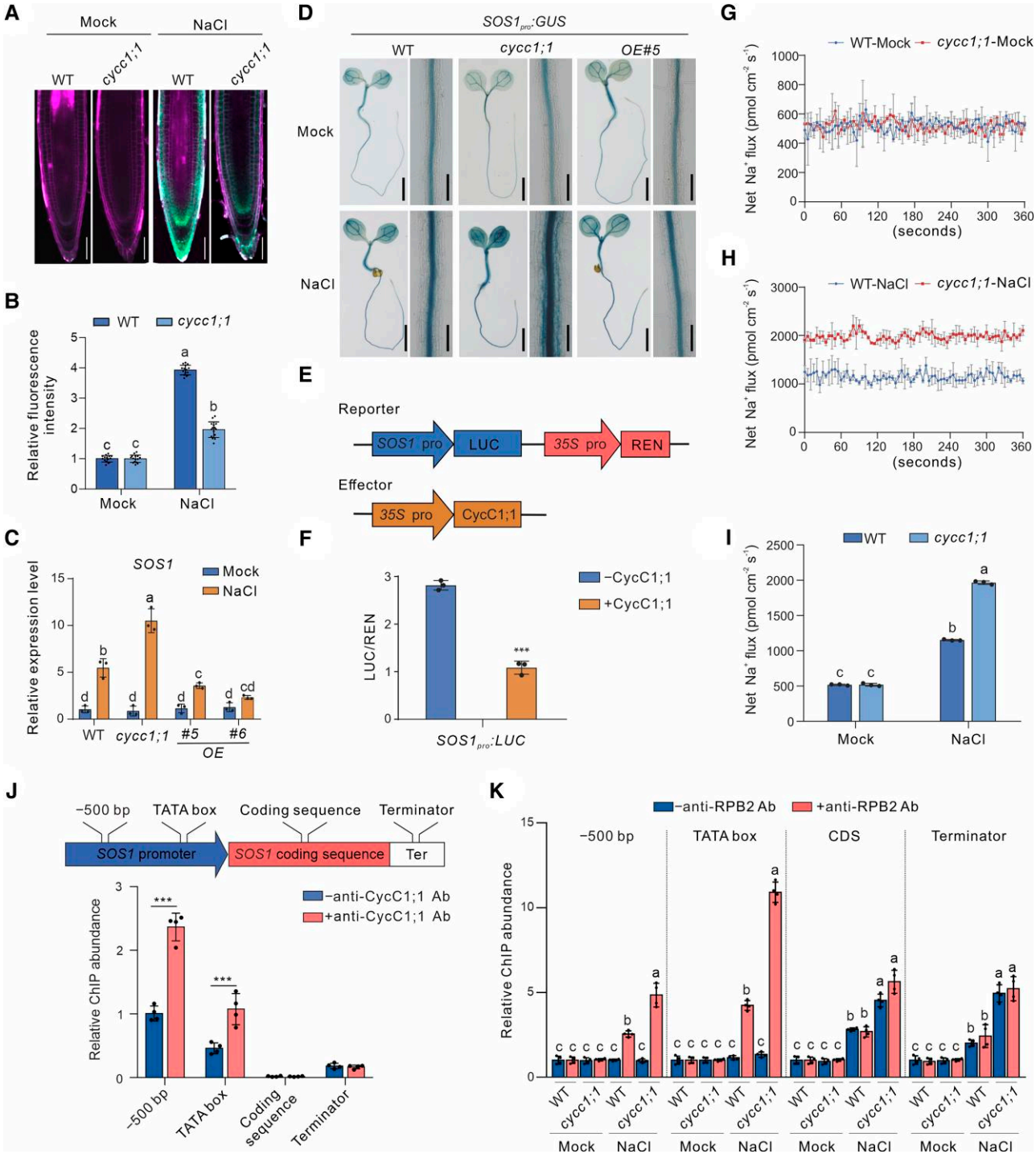

Figure 2. CycC1;1 negatively regulates salt-induced *SOS1* expression in plants. **A, B)** Sodium accumulation in wild-type and *cycc1;1* mutant seedling roots. Five-day-old wild-type and *cycc1;1* seedlings were treated with 100 mM NaCl for 3 h and then stained in a 10 μM ENG-2 AM solution containing 0.05% Pluronic F-127 for 3 h. Fluorescence images **A)** were taken, and the ENG-2 AM fluorescence intensity **B)** was analyzed. Bar = 50 μm. Data are means ± SD of 3 independent repeats ($n = 15$). Bars with different letters indicate significant differences at $P < 0.05$, revealed using ANOVA with a Tukey's multiple comparison test (Supplemental Data Set 1). **C)** The expression of *SOS1* in the wild-type and *cycc1;1* mutant seedlings subjected to salt stress. Seven-day-old wild-type and *cycc1;1* mutant seedlings treated with or without 100 mM NaCl for 12 h. Data are means ± SD ($n = 3$). Bars with different letters indicate significant differences at $P < 0.05$, revealed using ANOVA with a Tukey's multiple comparison test (Supplemental Data Set 1). **D)** GUS staining images of 5-d-old transgenic $SOS1_{pro}$:*GUS* in the wild-type, *cycc1;1*, and OE backgrounds treated with 0 mM or 100 mM NaCl for 12 h. Bar = 0.5 cm. **E, F)** LUC reporter gene assay to examine the effect of CycC1;1 on *SOS1* expression. The schematic diagram **E)** shows the reporters and effectors used in the assay. The relative LUC intensity **F)** represents the $SOS1_{pro}$:*LUC* activity relative to the internal control (REN driven

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predominant role in controlling plant salt stress tolerance by reducing Na^+ overaccumulation, we then assayed the expression of *SOS1*, *SOS2*, and *SOS3*, three genes encoding core components of the SOS pathway in *Arabidopsis*, in the wild-type and *cycc1;1* mutants. Our RT-qPCR results showed that the expression of these *SOS* genes was obviously induced by high salinity treatment in the wild-type, while such induction of the expression of *SOS1* but not *SOS2* or *SOS3* was further enhanced in the *cycc1;1* mutant (Figs. 2C and S3), suggesting that CycC1;1 greatly modulates the expression of *SOS1*, but not *SOS2* or *SOS3* expression. This finding was confirmed by results showing that salt-induced expression of *SOS1*, but not *SOS2* or *SOS3*, was compromised in the *CycC1;1*-overexpression lines compared with the wild-type plants (Figs. 2C and S3). In addition, we generated stable $SOS1_{pro}$:*GUS* transgenic reporter lines in the wild-type and *cycc1;1* mutant backgrounds in which the expression of *GUS* was driven by the *SOS1* promoter, enabling us to evaluate in fine detail the effect of CycC1;1 on salt-induced *SOS1* expression. Consistent with the RT-qPCR results, GUS staining and activity were increased by the treatment of salt stress in $SOS1_{pro}$:*GUS* plants, while such staining and activity were further enhanced in the *cycc1;1* mutant but suppressed in the *CycC1;1*-overexpression line (Figs. 2D and S4). This further indicates that CycC1;1 negatively regulates salt-induced *SOS1* expression in *Arabidopsis*. To further determine the role of CycC1;1 in the modulation of *SOS1* expression, we performed dual-luciferase (LUC) reporter gene assays. With $35S_{pro}$:*Renilla luciferase* (*REN*) as an internal control, *LUC* driven by the *SOS1* promoter as a reporter was coexpressed with $35S_{pro}$:*CycC1;1-GFP* as an effector in *Nicotiana benthamiana* leaves (Fig. 2E). Our results showed that the LUC activity was significantly repressed in the presence of CycC1;1 (Fig. 2F), revealing that CycC1;1 indeed plays a negative role in the regulation of *SOS1* expression in *Arabidopsis*.

SOS1, a Na^+/H^+ antiporter localized at the plasma membrane, protects plants under high salinity conditions by facilitating Na^+ efflux from the cells and regulating Na^+ transport from roots to shoots (Shi et al. 2002; Munns and Tester 2008; Deinlein et al. 2014; Van Zelm et al. 2020). Because the mutant had higher *SOS1* expression than the wild-type, we speculated that Na^+ efflux and/or long distance of Na^+ transport was affected in the *cycc1;1* mutant. To test this hypothesis, we first measured the Na^+ efflux at the root apex using noninvasive microtest technology (NMT). Our results showed that in the absence of a high salinity treatment, both the wild-type and mutant roots had similar low net Na^+ efflux, but following salt stress, the net Na^+ efflux in both plants markedly increased and was much higher in the mutant than in the wild-type (Fig. 2, G to I). This result reveals that Na^+ excretion is higher in the *cycc1;1* mutant than in the wild-type under salt stress. In addition, we measured the Na^+ content in the xylem sap from the wild-type and *cycc1;1* mutant plants. We found that both the wild-type and *cycc1;1* mutants had a similar low level of Na^+ in xylem sap under normal conditions, whereas the Na^+ content was significantly higher in the salt-treated wild-type than in the treated mutant plants (Supplemental Fig. S5), implying a role of CycC1;1 in the regulation of Na^+ accumulation in the xylem transpirational stream under high salinity conditions. These results further support the role of CycC1;1 in salt tolerance via changes of *SOS1* expression in plants.

Previous reports documented that CycC1;1 affects transcription through the action of Mediator associating with the promoter of target genes (Agrawal et al. 2021; Guo et al. 2022). We thus asked whether CycC1;1 could be associated with the *SOS1* promoter. To this end, the wild-type plant seedlings and an anti-CycC1;1 antibody (Guo et al. 2022) were used for a chromatin immunoprecipitation (ChIP) experiment. We used the immunoprecipitated DNA as templates for qPCR with the primers designed to bind at different positions of *SOS1* genomic DNA, including −500 bp from the translation start site (ATG), the TATA box (the RNAP II binding site), the coding region, and the terminator (Fig. 2J). The DNA fragments of the −500 bp upstream and TATA box but not the coding region or terminator were significantly enriched in CycC1;1 antibody-treated samples vs. antibody-lacking controls (Fig. 2J), implying that CycC1;1 is associated with the *SOS1* promoter in vivo in *Arabidopsis*.

The Mediator complex acts as a bridge connecting RNAPII and gene transcription factors, thus affecting the expression of associated genes (Agrawal et al. 2021). Whether CycC1;1 association with the *SOS1* promoter affects RNAPII binding

(**Figure 2.** Continued)
by $35S_{pro}$). The activity of $SOS1_{pro}$:*LUC* without CycC1;1 expression was set to 1. Data are means ± SD ($n = 3$). Asterisks indicate significant differences determined by Student's *t* test (***$P < 0.001$). **G** to **I)** NMT showing Na^+ fluxes. Ten-day-old wild-type and *cycc1;1* mutant seedlings cultured in 1/2× MS liquid medium were treated with 0 mM **G)** or 150 mM NaCl **H)** for 5 h, and then the continuous transient Na^+ fluxes were recorded for about 6 min. Each point is the mean of 4 individual plants. Quantitative analysis of the means of net Na^+ fluxes within a continuous period of 0 to 6 min **I)**. Data are means ± SD ($n = 3$). Bars with different letters indicate significant differences at $P < 0.05$, revealed using ANOVA with a Tukey's multiple comparison test (Supplemental Data Set S1). **J)** A diagram showing the positions of *SOS1* gene primers used for ChIP-qPCR is shown at the top. The ChIP-qPCR results showing the association of CycC1;1 with *SOS1* is shown at the bottom. Chromatin was extracted from 7-d-old wild-type seedlings and then precipitated with either an anti-CycC1;1 antibody (+Ab) or only IgG (−Ab). Data are means ± SD ($n = 3$). Asterisks indicate significant differences determined by Student's *t* test (***$P < 0.001$). **K)** Occupancy of RNAP II at the *SOS1* promoter in the wild-type and *cycc1;1* mutants. Chromatin was extracted from 7-d-old wild-type and *cycc1;1* mutant seedlings and precipitated with an anti-RPB2 antibody (+RPB2) or only IgG (−RPB2). Data are means ± SD ($n = 4$). Bars with different letters indicate significant differences at $P < 0.05$, determined using ANOVA with a Tukey's multiple comparison test.

to the *SOS1* genomic DNA was unknown. To address this, wild-type and *cycc1;1* mutant seedlings were used for ChIP-qPCR analysis using a specific antibody raised against the RNAP II subunit B2 (anti-RPB2). Our results showed that the amounts of RNAP II-associated *SOS1* genomic DNA fragments, including the −500 bp upstream, TATA box, coding region, and terminator, were similar in the untreated wild-type and *cycc1;1* mutant seedlings, whereas after salt treatment, increased DNA fragments were detected in seedlings of both genotypes, but much more in the mutant than in the wild-type (Fig. 2K). These results reveal that salt-induced RNAP II association with *SOS1* is promoted in the *cycc1;1* mutant, in agreement with the increased *SOS1* expression and salt tolerance of the mutant.

Disruption of *SOS1* abolishes salt stress tolerance of the *cycc1;1* mutant

To analyze the genetic role of CycC1;1 in *SOS1* expression for salt tolerance, we obtained a *cycc1;1 sos1* double mutant by crossing *cycc1;1* with the Col-0 background T-DNA insertion mutant of *SOS1* (SALK_046400) and tested its salt stress tolerance. Similar to the above findings and a previous report (Shi et al. 2000), the *cycc1;1* and *sos1* mutants exhibited much lower and higher salt stress sensitivity than the wild-type, respectively, in terms of the germination and cotyledon greening rate (Fig. 3, A to C). By contrast, sensitivity of the *cycc1;1 sos1* double mutant when subjected to high salinity was comparable to the *sos1* mutant (Fig. 3, A to C), revealing that *CycC1;1* acts upstream of *SOS1* in plant salt sensitivity. Moreover, we also evaluated the salt tolerance of these plants by treating seedlings with high salinity and then assessing their primary root length and fresh weight. Our analysis results showed that the increased salt tolerance of the *cycc1;1* mutant was completely compromised by the *SOS1* mutation, as evidenced by similar primary short root lengths and low fresh weights in both the *cycc1;1 sos1* double mutant and the *sos1* mutant (Fig. 3, D to F). This further supports the idea that *CycC1;1* acts upstream of *SOS1* in plant salt stress tolerance.

To verify the genetic relationship between *CycC1;1* and *SOS1*, lower concentrations of NaCl were used to test the salt sensitivity of their single and double mutants. Consistent with the previous reports, the *sos1* mutant was indeed very sensitive to salt stress, even though only 25 mM or 50 mM NaCl was used, while the *cycc1;1* mutant was less sensitive to these salinity conditions than the wild-type in terms of seed germination, root length, and fresh weight (Supplemental Fig. S6). This clearly showed that the *cycc1;1 sos1* double mutant displayed salt hypersensitive phenotypes similar to the *sos1* single mutant under lower salt stress conditions (Supplemental Fig. S6). Consistent with this genetic evidence, the NMT analysis revealed that, upon salt stress treatment, the *cycc1;1* and *sos1* mutants had higher and lower net Na^+ efflux than the wild-type, respectively, whereas the net Na^+ efflux in salt-treated *cycc1;1 sos1* double mutant was comparable to that in the treated *sos1* mutant (Fig. 3, G to I). Moreover, the Na^+ content in the xylem sap of the *cycc1;1* mutant was similar to that in the *cycc1;1 sos1* plant under either normal conditions or high salinity conditions (Supplemental Fig. S7). These data further show that the negative role of CycC1;1 in the regulation of salt tolerance is mainly dependent on *SOS1* in *Arabidopsis*.

CycC1;1 interacts with WRKY75 for binding to the *SOS1* promoter

Together, the above data demonstrated that CycC1;1 suppresses salt-induced *SOS1* expression by interfering with RNAP II recruitment to the *SOS1* promoter; however, the gene-specific transcription factors involved in CycC1;1-mediated repression of *SOS1* expression remained unknown. To address the relationship between CycC1;1 and *SOS1* transcription, we sought to identify transcription factors that not only can target the *SOS1* promoter but also can interact with CycC1;1, thus providing a physical link between CycC1;1 and the *SOS1* promoter. As a recent study reported that the transcription factor WRKY1 can bind to *SOS1* promoter and activate its expression (Wu et al. 2022), we first examined whether CycC1;1 could interact with WRKY1 in a yeast 2-hybrid (Y2H) experiment. Regrettably, no interaction between CycC1;1 and WRKY1 was observed in yeast cells (Supplemental Fig. S8), suggesting that WRKY1 may not be involved in CycC1;1-mediated transcriptional regulation of *SOS1*.

Therefore, we screened CycC1;1-interacting proteins by Y2H from a yeast library, and unexpectedly, another WRKY family member WRKY75 was identified as a possible CycC1;1-interacting partner (Fig. 4A). To confirm their interaction, we performed a bimolecular fluorescence complementation (BiFC) assay. When we coexpressed WRKY75 fused to the N-terminal half of yellow fluorescent protein (YFP) and CycC1;1 fused to the C-terminal half of YFP in *N. benthamiana* leaves, with H2B-RFP as a nuclear marker (Rosa et al. 2014), we observed reconstituted YFP fluorescence in the nucleus but not in the negative controls (Fig. 4B), indicating that CycC1;1 interacts with WRKY75 in planta. The interaction between CycC1;1 and WRKY75 was verified by a co-immunoprecipitation (Co-IP) experiment in which HA-tagged CycC1;1 and GFP-tagged WRKY75 were coexpressed in *N. benthamiana* leaves and immunoprecipitated using an anti-GFP antibody. Our results showed that HA-CycC1;1 could be specifically detected by an anti-HA antibody in the protein precipitants immunoprecipitated by the anti-GFP antibody (Fig. 4C), further supporting the previous result that CycC1;1 interacts with WRKY75 in vivo. To test their interaction in vitro, we purified glutathione S-transferase (GST)-tagged CycC1;1 and 6×His-tagged WRKY75 proteins that were expressed in *Escherichia coli* and performed a GST pull-down assay using Glutathione Sepharose beads. Our results showed that His-WRKY75 was specifically pulled down by GST-CycC1;1, but not by a construct containing the GST tag alone

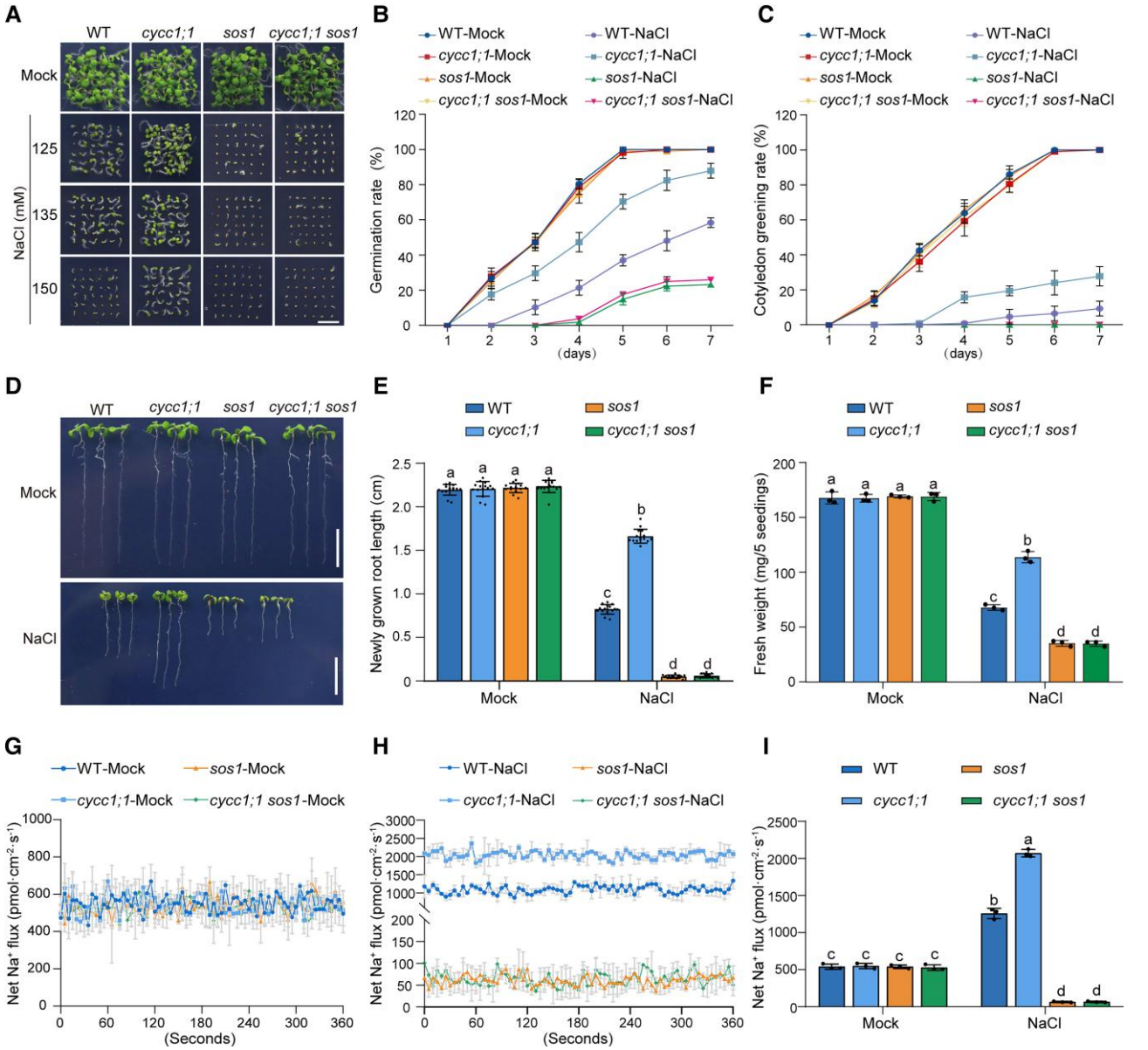

Figure 3. CycC1;1 affects plant salt tolerance through SOS1. **A** to **C)** Phenotypes **A)** of the wild-type, *cycc1;1*, *sos1*, and *cycc1;1 sos1* plants grown on 1/2× MS medium supplemented with 0 mM, 125 mM, 135 mM, or 150 mM NaCl for 5 d. Quantitative analysis of seed germination **B)** and cotyledon greening rates **C)** of plants grown on 1/2× MS medium supplemented with 0 mM or 125 mM NaCl for 7 d. Data are means ± SD of 3 independent experiments ($n = 3$). **D** to **F)** Root elongation and fresh weight analysis. Five-day-old wild-type, *cycc1;1*, *sos1*, and *cycc1;1 sos1* plants were transferred to 1/2× MS medium supplemented with 0 mM or 125 mM NaCl for additional growth. The photographs were taken 5 d after transfer **D)**. Bar = 1 cm. The lengths of newly grown roots **E)** and the fresh weights **F)** of the seedlings were also analyzed. Data are means ± SD ($n = 15$ for root length and $n = 3$ for fresh weight). Bars with different letters indicate significant differences at $P < 0.05$, revealed using ANOVA with a Tukey's multiple comparison test (Supplemental Data Set 1). **G** to **I)** Net Na^+ fluxes in root tips using NMT. Ten-day-old wild-type, *cycc1;1*, *sos1*, and *cycc1;1 sos1* mutant seedlings cultured in 1/2× MS liquid medium were treated with 0 mM **G)** or 150 mM NaCl **H)** for 5 h, and then continuous transient Na^+ fluxes were recorded for about 6 min. Each point is the mean of data from 4 individual plants. Quantitative analysis of the means of net Na^+ fluxes within a continuous period of 0 to 6 min **I)**. Data are means ± SD ($n = 3$). Bars with different letters indicate significant differences at $P < 0.05$, revealed using ANOVA with a Tukey's multiple comparison test.

(Fig. 4D), clearly indicating that CycC1;1 has physical interaction with WRKY75 in vitro. These results reveal that CycC1;1 interacts with WRKY75 both in vivo and in vitro.

To investigate whether WRKY75 can target the *SOS1* promoter, we analyzed the *cis*-elements of the *SOS1* promoter and identified a typical W-box motif in the *SOS1* promoter (Fig. 4E), raising the possibility that WRKY75 binds to the *SOS1* promoter. As expected, results from an electrophoresis mobility shift assay (EMSA) revealed that 6×His-SUMO-tagged WRKY75 purified form *E. coli* bound to a biotin-labeled

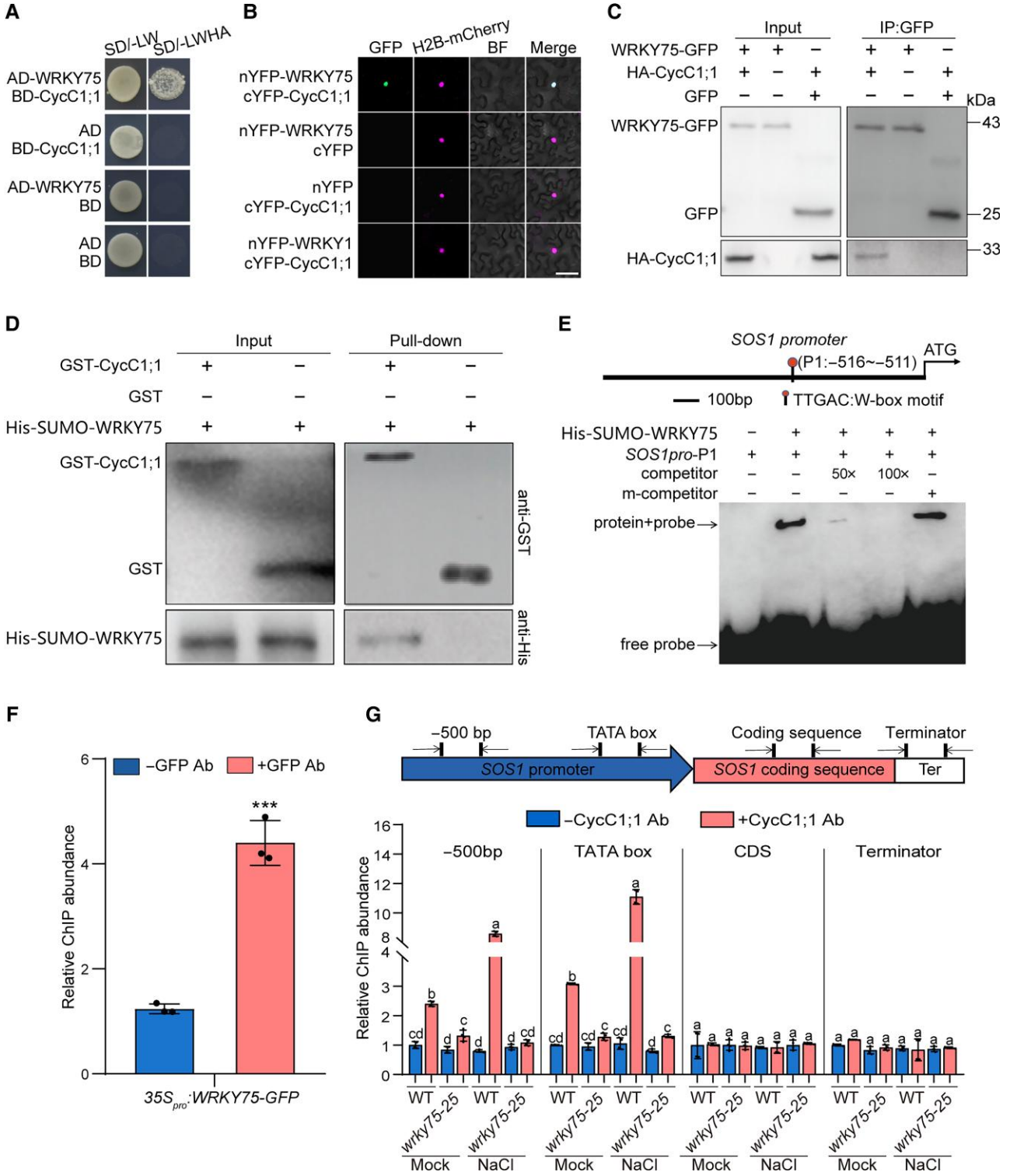

Figure 4. CycC1;1 associates with *SOS1* promoter by interacting with WRKY75. **A)** Interaction between WRKY75 and CycC1;1 examined by Y2H assay. Protein interactions were examined based on the growth of yeast cells on selective media. SD indicates synthetic dropout medium. −L/W indicates Leu and Trp dropout plates. −L/W/H/A indicates Trp, Leu, His, and Ade dropout plates. **B)** BiFC showing CycC1;1 interaction with WRKY75. nYFP-WRKY75 and cYFP-CycC1;1 were transiently coexpressed in *N. benthamiana* leaves, and YFP fluorescence was observed under confocal microscopy. H2B-mCherry (Rosa et al. 2014) was used as a nuclear marker. Bar = 20 μm. **C)** Interaction between WRKY75 and CycC1;1 assayed by Co-IP. GFP or GFP-tagged WRKY75 and HA-tagged CycC1;1 were transiently coexpressed in *N. benthamiana* leaves, and proteins were immunoprecipitated using anti-GFP antibody-conjugated agarose beads. The resulted precipitates were detected using anti-GFP and anti-HA antibodies,

(continued)

P1 probe containing the W-box of the *SOS1* promoter (Fig. 4E). This binding was blocked by an unlabeled P1 probe but not by an unlabeled P1 probe with mutations in the W-box motif (Fig. 4E), indicating the specific binding of WRKY75 to *SOS1* promoter. In addition, a $35S_{pro}$:*WRKY75-GFP* transgenic plant (Guo et al. 2017) and an anti-GFP antibody were used for ChIP-qPCR, and our results showed that the amounts of DNA fragments enriched by the anti-GFP antibody were much higher than those in the antibody-lacking control (Fig. 4F), further supporting that WRKY75 can bind to the *SOS1* promoter in *Arabidopsis*.

To test whether WRKY75 links CycC1;1 and the *SOS1* promoter in plants, we performed ChIP-qPCR by isolating CycC1;1-associated *SOS1* DNA fragments from the wild-type and *wrky75-25* mutant using an anti-CycC1;1 antibody. Our results showed that *SOS1* promoter DNA fragments including −500 bp upstream and the TATA box were enriched in samples from the untreated wild-type plant, and high salinity treatment significantly increased such enrichment. However, this enrichment was greatly repressed in the *wrky75-25* mutant (Fig. 4G), suggesting that CycC1;1 associates with *SOS1* promoter in a WRKY75-dependent manner. Together, these results reveal CycC1;1 association with *SOS1* promoter through its physical interaction with WRKY75.

WRKY75 confers plant salt tolerance by increasing *SOS1* expression

Our results so far have revealed that WRKY75 can bind to the *SOS1* promoter, leading us to further study its role in *SOS1* transcription. Two T-DNA insertion mutant alleles of *WRKY75*, *wrky75-1* and *wrky75-25* (Fig. 5A; Chen et al. 2021; Guo et al. 2017), were identified and used to measure the expression of *SOS1*. In contrast to the *cycc1;1* mutant, salt-induced *SOS1* expression was greatly inhibited in both *wrky75-1* and *wrky75-25* mutants compared with that in the wild-type (Fig. 5B), revealing the involvement of WRKY75 in salt-induced *SOS1* expression. To confirm this, we obtained $SOS1_{pro}$:*GUS wrky75-1* plants by crossing the $SOS1_{pro}$:*GUS* line with the *wrky75-1* mutant. We found that GUS staining and activity in the $SOS1_{pro}$:*GUS* seedlings were highly induced by salt stress, whereas this induction was dampened in the $SOS1_{pro}$:*GUS wrky75-1* plant (Figs. 5C and S9), indicating that WRKY75 plays an important role in salt-induced *SOS1* expression. In addition, using a dual LUC reporter gene assay in which both $SOS1_{pro}$:*LUC* as a reporter and $35S_{pro}$:*REN* as an internal control were coexpressed with GFP or $35S_{pro}$:*WRKY75-GFP* as an effector in *N. benthamiana* leaves (Fig. 5D), we found that WRKY75-GFP, but not GFP alone, could activate LUC activity (Fig. 5D). These results demonstrate that WRKY75 can bind to the *SOS1* promoter and activate its expression in response to high salinity. Consistent with the above findings, the *wrky75* mutants and *WRKY75*-overexpression transgenic plants (Guo et al. 2017) displayed lower and higher tolerance to salt stress than the wild-type, respectively, in terms of germination and cotyledon greening rates, primary root length, and fresh weight (Fig. 5, E to J), indicating that WRKY75 is a positive regulator of salt stress tolerance in *Arabidopsis*. Consistently, Na^+ accumulation in the *wrk75-25* mutant root and xylem sap was significantly higher than in the wild-type plants when subjected to high salinity (Figs. 5, K and L, and S10).

To provide genetic evidence supporting that WRKY75 confers salt tolerance by acting upstream of *SOS1*, we first obtained the *SOS1*-overexpression (*SOS1-OE*) transgenic line and then crossed it with the *wrky75-25* mutant, producing *wrky75-25 SOS1-OE* plants. Our results showed that the *SOS1-OE* plants indeed had stronger tolerance to the salt stress than the wild-type, while the *wrky75-25 SOS1-OE* plant exhibited similar salt tolerance to the *SOS1-OE* line (Fig. 6, A to F), implying that the salt-sensitive phenotype of the *wrky75-25* mutant could be rescued by the overexpression of *SOS1*.

To assess how *WRKY75* responds to salt stress, we first assayed its transcription levels in the wild-type seedlings treated with or without high salinity and showed that *WRKY75* expression was significantly higher in salt-treated plants than in the untreated plant (Fig. 6G). We also obtained a previously reported $WRKY75_{pro}$:*GUS* line (Guo et al. 2017) and subjected its seedlings to high salinity treatment. We found that the GUS was highly active in seedling roots, while salt treatment further increased such activity in both root and shoot (Figs. 6H and S11). These results clearly indicate that salt stress induces *WRKY75* expression in *Arabidopsis*. We

(**Figure 4.** Continued)
respectively. **D)** GST pull-down for the analysis of in vitro interaction between WRKY75 and CycC1;1. 6×His-SUMO-WRKY75 were mixed with GST-CycC1;1 or GST and immobilized on Glutathione Sepharose beads. After washing, the eluted proteins were subjected to immunoblot analysis with anti-GST or anti-His antibodies, respectively. **E)** The EMSA experiment showing that WRKY75 can bind to the *SOS1* promoter in vitro. A diagram showing the *SOS1* promoter has a typical W-box motif (−516∼−511 bp) recognized by WRKY75 transcription factor is shown at the top. Purified 6×His-SUMO-WRKY75 was incubated with biotin-labeled *SOS1* promoter probes, and unlabeled probes (mutated or not mutated) were used as competitors. **F)** ChIP-qPCR showing that WRKY75 can bind to the *SOS1* promoter in vivo. Chromatin was extracted from 7-d-old $35S_{pro}$:*WRKY75-GFP* transgenic seedlings and then precipitated with either an anti-GFP antibody (+Ab) or only IgG (−Ab). Data are means ± SD ($n = 3$). Asterisks indicate significant differences determined by Student's *t* test (***$P < 0.001$). **G)** A diagram showing the positions of *SOS1* gene primers used for ChIP-qPCR is shown at the top. The ChIP-qPCR results showing the changes of CycC1;1 association with *SOS1* in the wild-type and *wrky75-25* mutant are shown at the bottom. Chromatin was extracted from 7-d-old wild-type and *wrky75-25* mutant seedlings and precipitated with anti-CycC1;1 antibody (+Ab) or only IgG (−Ab). Data are means ± SD ($n = 4$). Bars with different letters indicate significant differences at $P < 0.05$, determined using ANOVA with a Tukey's multiple comparison test.

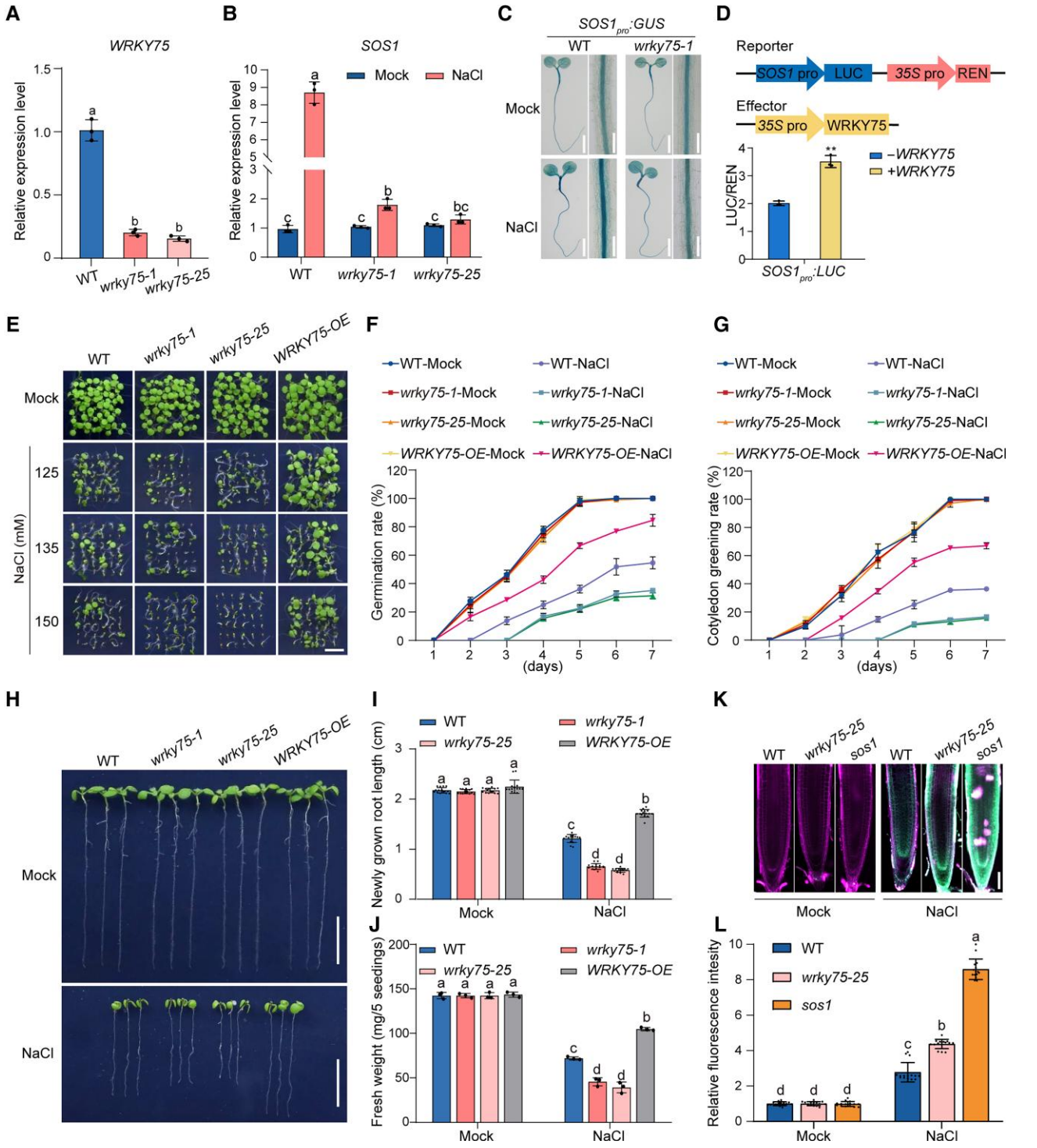

Figure 5. WRKY75 is required for salt-induced *SOS1* expression. **A)** Expression of *WRKY75* in 5-d-old wild-type, *wrky75-1*, and *wrky75-25* mutant plants. Data are means ± SD ($n = 3$). Bars with different letters indicate significant differences at $P < 0.05$, determined using ANOVA with a Tukey's multiple comparison test. **B)** Expression of *SOS1* in 5-d-old wild-type, *wrky75-1*, and *wrky75-25* mutant plants treated with 0 mM (Mock) or 100 mM NaCl for 12 h. Data are means ± SD ($n = 3$). Bars with different letters indicate significant differences at $P < 0.05$, revealed using ANOVA with a Tukey's multiple comparison test. **C)** GUS staining images of 5-d-old $SOS1_{pro}$*:GUS* and $SOS1_{pro}$*:GUS wrky75-1* seedlings treated with 0 mM or 100 mM NaCl for 12 h. Bar = 0.5 cm. **D)** LUC reporter gene assay showing WRKY75-mediated activation of *SOS1* expression. The schematic diagrams at the top show the reporters and effectors used in the assay. The relative LUC intensity represents the $SOS1_{pro}$*:LUC* activity relative to the internal control (REN driven by the *35S* promoter). The activity of $SOS1_{pro}$*:LUC* without *WRKY75* expression was set to 1. Data are means ± SD ($n = 3$). Asterisks indicate significant differences determined by Student's *t* test (**$P < 0.01$). **E** to **G)** Phenotypes **E)** of the wild-type, *wrky75-1*, *wrky75-25*, and

(continued)

also noticed that *WRKY75* was highly expressed in the stele of root elongation and mature zones, based on the GUS staining experiment (Fig. 6H), and that this pattern likely overlapped with the staining pattern of $CycC1;1_{pro}$:*GUS* and $SOS1_{pro}$:*GUS* seedling roots (Figs. 1K and 2D). To exactly study their expression pattern in roots, transverse sections of the GUS-stained roots from the three GUS reporter lines were analyzed under a microscope, revealing that *CycC1;1*, *WRKY75*, and *SOS1* were highly expressed in the root stele parenchyma cells (Fig. 6I).

CycC1;1 interferes with WRKY75 transcriptional activation activity for *SOS1*

We further asked whether CycC1;1 involves WRKY75-mediated transcriptional regulation of *SOS1* through their interaction. We performed dual LUC reporter gene assays, in which $SOS1_{pro}$:*LUC* as a reporter and $35S_{pro}$:*WRKY75-GFP* as an effector were coexpressed with or without CycC1;1 in *N. benthamiana* leaves. Our results showed that LUC activity was activated when WRKY75-GFP was expressed, whereas WRKY75-promoted LUC activity was significantly suppressed in the presence of CycC1;1 (Fig. 7A). This revealed that CycC1;1 interferes with WRKY75-mediated transcriptional activation of *SOS1*. Therefore, mutation of *WRKY75* should dampen both higher *SOS1* expression and salt tolerance of the *cycc1;1* mutant. To test this, we crossed the *cycc1;1* mutant with the *wrky75-25* mutant, producing a *cycc1;1 wrky75-25* double mutant. The *cycc1;1 wrky75-25* double mutant had lower *SOS1* expression in the presence of high salinity, a level similar to that in the *wrky75-25* mutant (Fig. 7B). In addition, we analyzed the expression levels of *WRKY75* in the wild-type and *cycc1;1* mutant seedlings and found that *WRKY75* expression in the wild-type was similar to that in the *cycc1;1* mutant, either in the presence or absence of high salinity (Fig. 7C), revealing that CycC1;1 does not affect *WRKY75* expression in response to salt stress. Furthermore, the *cycc1;1 wrky75-25* double mutant displayed a salt-sensitive phenotype similar to that of the *wrky75-25* mutant regarding seed germination, cotyledon greening, primary root length, and fresh weight (Fig. 7, D to I), results consistent with measurements of the Na^+ content in the xylem sap of the wild-type and mutant plants treated with high salinity (Supplemental Fig. S12). These results indicate that CycC1;1 interferes WRKY75-mediated transcriptional activation of *SOS1* in plant response to high salinity.

Discussion

Due to their sessile lifestyle, plants cannot avoid adverse environments such as high salinity by changing location; thus, they must precisely perceive and respond to environmental stresses to survive. SOS1 plays a critical role in salt tolerance in *Arabidopsis* by pumping excess Na^+ out of the cell (Zhou et al. 2018; Chai et al. 2020). Numerous reports have documented that *SOS1* expression is induced by salt stress (Shi et al. 2000, 2002; Wu et al. 2022), but the underlying mechanism remains unclear. Here, we report that CycC1;1 negatively regulates plant salt tolerance by interfering with WRKY75-mediated transcriptional activation of *SOS1*. Biochemical and genetic analyses revealed that CycC1;1 negatively regulates *SOS1* expression and salt tolerance by interacting with WRKY75 to interfere with the association of RNAP II with the *SOS1* promoter. Thus, our study reveals the critical role of CycC1;1 as a component of the Mediator complex in linking gene-specific transcription factor WRKY75 and RNAP II during the dynamic regulation of *SOS1* transcription in plants. We propose a model illustrating this in Fig. 8.

It has been widely accepted that activation of SOS1 at the posttranslational level is important for the plant salt stress response, and several components key to this response have been identified, especially the SOS2–SOS3 kinase complex (Qiu et al. 2002; Quintero et al. 2002, 2011). In fact, a potential role of *SOS1* transcription in plant salt tolerance was proposed when this gene was initially cloned and analyzed, as salt stress could highly induce *SOS1* expression in both plant roots and shoots (Shi et al. 2000, 2002). This finding was later confirmed by numerous reports, and further work supports the key role of *SOS1* transcription in plant salt tolerance based on the fact that overexpression of *Arabidopsis SOS1* or its homologous genes from other plant species, such as *Crossostephium chinense*, *Artemisia japonica*, and *Chrysanthemum*, can significantly enhance salt tolerance in plants (Shi et al. 2002; Yang et al. 2009; Gao et al. 2016).

(**Figure 5.** Continued)
$35S_{pro}$:*WRKY75-GFP* (*WRKY75-OE*) plants grown on 1/2× MS medium supplemented with 0 mM, 125 mM, 135 mM, or 150 mM NaCl for 5 d. Quantitative analysis of seed germination **F)** and cotyledon greening **G)** of plants grown on 1/2× MS medium supplemented with 0 mM or 125 mM NaCl for 7 d. Data are means ± SD of 3 independent repeats. **H** to **J)** Root elongation and fresh weight analysis. Five-day-old wild-type, *wrky75-1*, *wrky75-25*, and *WRKY75-OE* plants were transferred to 1/2× MS medium supplemented with 0 mM or 125 mM NaCl for additional growth. The photographs in **H)** were taken 5 d after transfer. Bar = 1 cm. The length of newly grown roots **I)** and the fresh weight **J)** of the seedlings were also analyzed. Data are means ± SD of 3 independent repeats ($n = 15$ for root length and $n = 3$ for fresh weight). Bars with different letters indicate significant differences at $P < 0.05$, determined using ANOVA with a Tukey's multiple comparison test. **K, L)** Sodium accumulation in wild-type, *wrky75-25*, and *sos1* mutant seedling roots. Five-day-old plant seedlings were treated with 100 mM NaCl for 12 h and then stained by 10 μM ENG-2 AM solution containing 0.05% Pluronic F-127 for 3 h. Fluorescence images **K)** were taken, and relative ENG-2 AM fluorescence intensity **L)** was analyzed. Data are means ± SD of 3 independent repeats ($n = 15$). Bars with different letters indicate significant differences at $P < 0.05$, revealed using ANOVA with a Tukey's multiple comparison test.

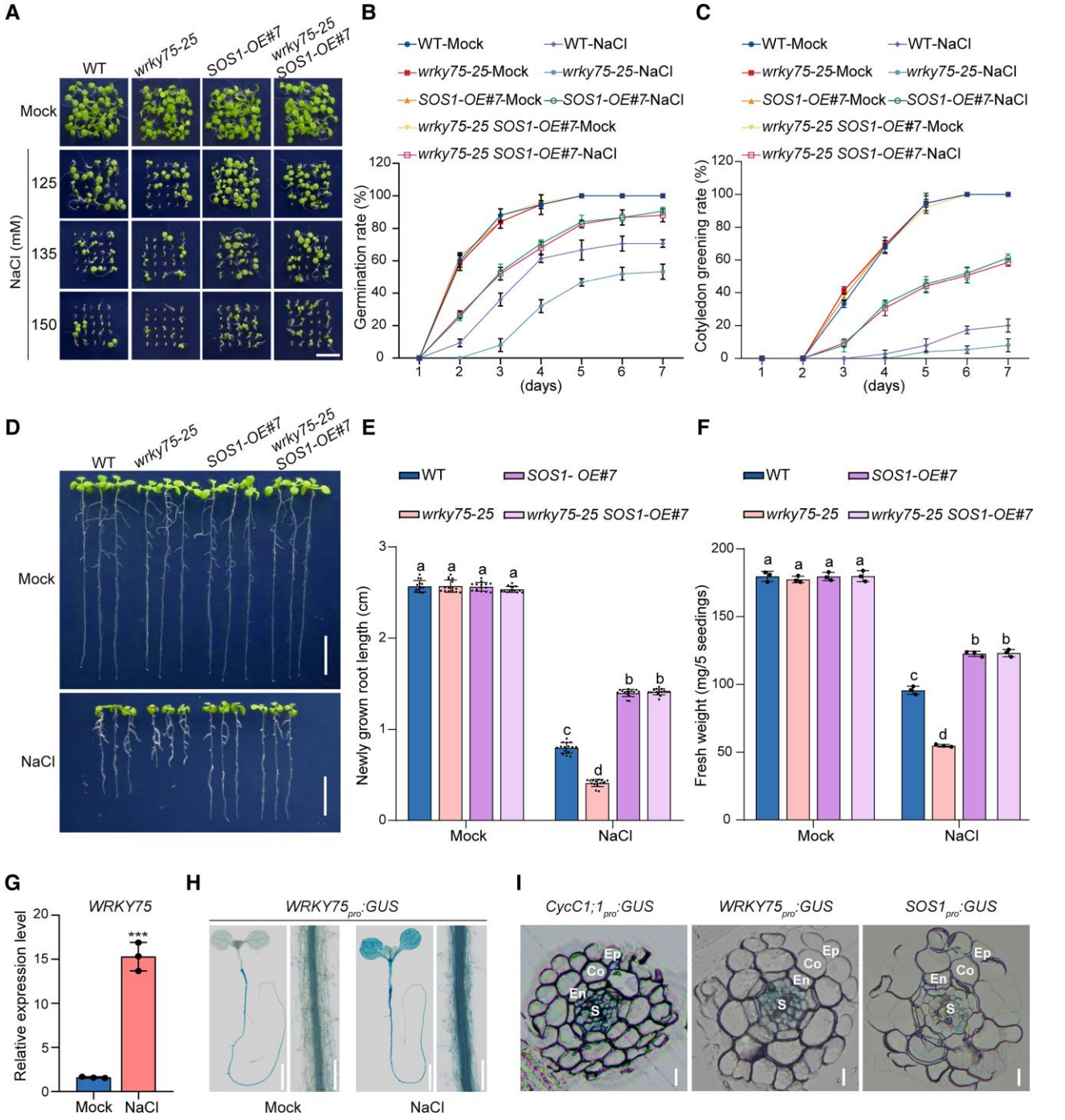

Figure 6. WRKY75 acts upstream of *SOS1* in the regulation of plant salt stress tolerance. **A** to **C)** Phenotypes **A)** of the wild-type, *wrky75-25*, $35S_{pro}$:*SOS1-GFP#7* (*SOS1-OE#7*), and *wrky75-25 SOS1-OE#7* plants grown on 1/2× MS medium supplemented with 0 mM, 125 mM, 135 mM, or 150 mM NaCl for 5 d. Quantitative analysis of seed germination **B)** and cotyledon greening rates **C)** of plants grown on 1/2× MS medium supplemented with 0 mM or 125 mM NaCl for 7 d. Data are means ± SD ($n = 3$). **D** to **F)** Root elongation and fresh weight analysis. Five-day-old wild-type, *wrky75-25*, $35S_{pro}$:*SOS1-GFP#7* (*SOS1-OE#7*), and *wrky75-25 SOS1-OE#7* plants were transferred to 1/2× MS medium supplemented with 0 mM or 125 mM NaCl for additional growth. The photographs were taken 5 d after transfer **D)**. Bar = 1 cm. The lengths of newly grown roots **E)** and the fresh weights **F)** of the seedlings were also analyzed. Data are means ± SD ($n = 15$ for root length and $n = 3$ for fresh weight). Bars with different letters indicate significant differences at $P < 0.05$, determined using ANOVA with a Tukey's multiple comparison test. **G)** The expression of *WRKY75* in 5-d-old wild-type plants treated with 0 mM or 100 mM NaCl for 12 h. Data are means ± SD ($n = 3$). Asterisks indicate significant differences determined by Student's *t* test (***$P < 0.001$). **H)** GUS staining images of 5-d-old $WRKY75_{pro}$:*GUS* transgenic plant seedlings and longitudinal sections of roots of plants treated with 0 mM or 100 mM NaCl for 12 h. Bar = 0.5 cm. **I)** GUS staining images showing transverse sections of 7-d-old $CycC1;1_{pro}$:*GUS*, $WRKY75_{pro}$:*GUS*, and $SOS1_{pro}$:*GUS* seedling roots. Ep, epidermis; Co, cortex; En, endodermis; S, stele. Bar = 10 μm.

These previous reports, as well as this study, show that dynamic regulation of SOS1 activity is likely achieved by precise transcriptional as well as posttranslational regulation of SOS1 during the response of plants to different salinity conditions.

Our study showed that CycC1;1 forms a transcriptional repression complex with WRKY75 via their physical interaction and that this complex hinders the transcription of *SOS1* by interfering with RNAP II recruitment to the *SOS1* promoter when plants are grown under normal conditions. The relief of this transcriptional repression of *SOS1* is at least partially caused by decreased *CycC1;1* expression, but increased *WRKY75* expression, under high salinity conditions, thereby leading to increased *SOS1* transcription and salt tolerance in plants. Thus, we infer that the protein complex formed by CycC1;1 and WRKY75 and its transcriptional regulation of *SOS1* is dynamically affected by the different expression levels of *CycC1;1* and *WRKY75* in plant response to different salinity conditions.

Nevertheless, many more biochemical experiments must be performed in the future to further elucidate whether and how the CycC1;1–WRKY75 complex is associated with other components of the Mediator complex. It will also be important to determine their distinct status in plant responses to different salinity conditions. In addition to the impacts of differential *CycC1;1* and *WRKY75* expression on the CycC1;1–WRKY75 complex in plants when challenged by salinity, we cannot rule out another possibility that salt stress may abolish formation of the CycC1;1–WRKY75 complex to upregulate *SOS1* transcription by dampening their interaction. Although it is difficult to directly investigate the changes of CycC1;1–WRKY75 interaction in planta, it is certainly worth exploring the role of salinity on the interaction between CycC1;1 and WRKY75 and further investigating these effects on the transcriptional activation of *SOS1* and plant salt stress tolerance.

It is also worth noting that CycC1;1 association with the *SOS1* promoter was nearly completely abolished when *WRKY75* expression was largely disrupted in the *wrky75* mutant, implying that CycC1;1 associates with *SOS1* in a WRKY75-dependent manner in planta. This is consistent with the current model that the Mediator complex functions in gene transcription as a bridge connecting transcription factors with RNAP II. Whether CycC1;1 can repress *SOS1* expression by inhibiting WRKY75 binding to the *SOS1* promoter in addition to interfering WRKY75 transcriptional activation activity remains to be further elucidated and is worthy of further experimental exploration.

SOS1 is specifically expressed in the xylem parenchyma cells and the epidermis of plant roots (Shi et al. 2002; Chung et al. 2008). In our study, we generated a GUS reporter line where the GUS activity is under the control of the *SOS1* native promoter. This enabled us to observe the changes of *SOS1* expression patterns in roots of plants of in the wild-type, *cycc1;1*, and *wrky75-25* mutant backgrounds. Our results showed that *SOS1* is indeed specifically expressed in epidermal and xylem parenchyma cells (Fig. 6I), consistent with the previous report (Shi et al. 2002). Our biochemical and genetic analyses revealed that WRKY75 functions in salt-induced *SOS1* expression by directly binding to the W-box in the *SOS1* promoter. Indeed, the GUS staining and activity of $SOS1_{pro}$:*GUS* were lower in the *wrky75-25* mutant roots than in the wild-type roots (Figs. 6I and S11), especially in the stele of the seedling roots. This further supports the positive role of WRKY75 in the regulation of *SOS1* expression in xylem parenchyma cells. However, observations using the $WRKY75_{pro}$:*GUS* reporter line showed obvious GUS activity only in the stele, but not in the epidermis in roots (Guo et al. 2017), demonstrating that *WRKY75* is specifically expressed in the stele. Since a previous report confirmed that WRKY75 RNA or protein can move into the epidermal cells from the cells where it is transcribed (Rishmawi et al. 2014), we infer that salt stress increases *WRKY75* transcription in stele and then *WRKY75* moves into the epidermis to promote *SOS1* expression, thus conferring plant enhanced salt tolerance. By contrast, under normal conditions, WRKY75-regulated *SOS1* expression is inhibited by CycC1;1 through its interaction, as CycC1;1 is widely distributed in the whole root tissues, including epidermal and xylem parenchyma cells (Fig. 6I). Investigation of these complex and dynamic expression patterns of CycC1;1–WRKY75 signaling should contribute to our understanding of the precise regulation of *SOS1* expression in a proper plant response to low or high salt conditions.

In addition to the expression in roots, our GUS staining experiments using the $SOS1_{pro}$:*GUS* reporter line revealed that *SOS1* is also expressed in shoots (Fig. 2D), consistent with previous reports and the prediction that SOS1 functions to expel Na^+ from the xylem parenchyma cells into the apoplastic space of mesophyll cells in leaves (Shi et al. 2002; Zhu et al. 2016). Interestingly, salt-induced GUS staining in shoots was also increased in the *cycc1;1* mutant but decreased in the *wrky75-25* mutant (Figs. 2D and 5C), implying a similar regulatory role of CycC1;1 and WRKY75 in *SOS1* expression in both shoots and roots. How shoot-expressed *SOS1* functions in plant salt tolerance remains unclear and is worthy of experimental exploration, which will provide more insights into the role of SOS1 in long-distance transport of Na^+ in plants.

Emerging evidence has revealed that many subunits of the Mediator complex participate in plant growth, development, and stress responses (Zhu et al. 2014; Guo et al. 2021). It is reported that the Mediator complex tail module can interact with different transcription factors to affect gene expression. For example, MED25 interacts with MYC2 and ABSCISIC ACID INSENSITIVE5 (ABI5), 2 master transcription factors in JA and ABA signaling pathways, respectively, thus differentially regulating plant responses to JA and ABA (Chen et al. 2012). In addition to the tail module, the kinase module can also interact with transcription factors to modulate the expression of associated genes. CDK8, a subunit of the kinase module, regulates plant immunity and drought stress tolerance by interacting with the transcription factor WAX INDUCER1 and ERF/AP2 transcription factor RAP2.6, respectively (Zhu et al. 2014; Zhu et al. 2020). Unlike the well-

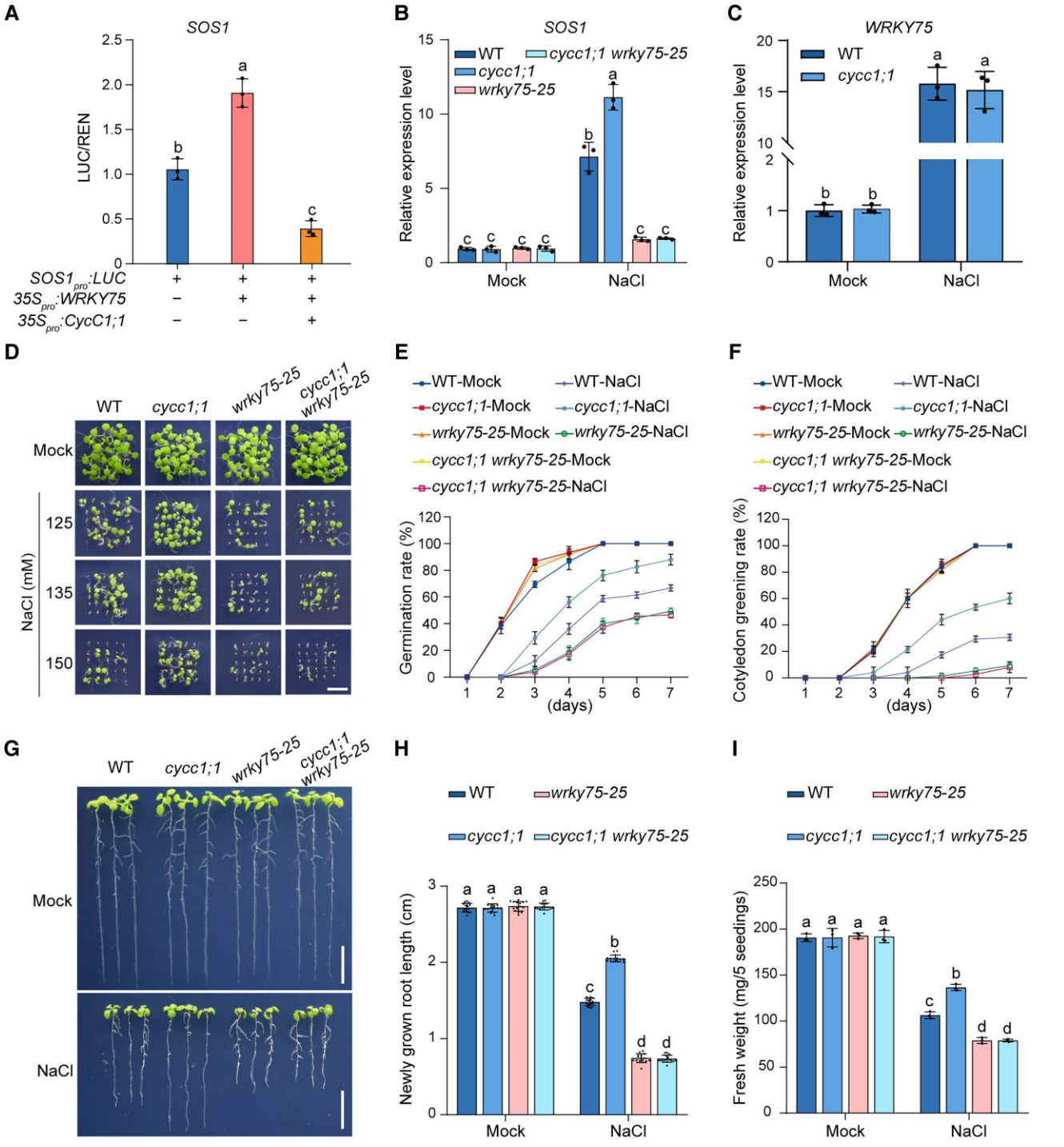

Figure 7. CycC1;1 interferes WRKY75-mediated transcriptional activation for *SOS1*. **A)** LUC reporter gene assay showing the effect of CycC1;1 on WRKY75-mediated activation of *SOS1* expression. The relative LUC intensity represents the *SOS1*$_{pro}$:*LUC* activity relative to the internal control (REN driven by the 35S promoter). The activity of SOS1$_{pro}$:LUC without *WRKY75* expression was set to 1. Data are means ± SD ($n = 3$). **B)** The expression of *SOS1* in 5-d-old wild-type, *cycc1;1*, *wrky75-25*, and *cycc1;1 wrky75-25* mutant seedlings treated with 0 mM or 100 mM NaCl for 6 h. Data are means ± SD ($n = 3$). Bars with different letters indicate significant differences at $P < 0.05$, determined using ANOVA with a Tukey's multiple comparison test. **C)** The expression of *WRKY75* in 5-d-old wild-type and *cycc1;1* mutant seedlings treated without or with 100 mM NaCl for 12 h. Data are means ± SD ($n = 3$). ns, no significant differences. **D** to **F)** Phenotypes **D)** of the wild-type, *cycc1;1*, *wrky75-25*, and *cycc1;1 wrky75-25* mutant plants grown on 1/2× MS medium supplemented with 0 mM, 125 mM, 135 mM, or 150 mM NaCl for 5 d. Quantitative analysis of seed germination **E)** and cotyledon greening rates **F)** of plants grown on 1/2× MS medium supplemented with 0 mM or 125 mM NaCl for 7 d. Data are means ± SD ($n = 3$). **G** to **I)** Root elongation and fresh weight analysis. Five-day-old wild-type, *cycc1;1*, *wrky75-25*, and *cycc1;1 wrky75-25* mutant plants were transferred to 1/2× MS medium supplemented with 0 mM or 125 mM NaCl for additional growth. The photographs were taken 5 d after transfer **G)**. Bar = 1 cm. The lengths of newly grown roots **H)** and the fresh weights **I)** of the seedlings were also analyzed. Data are means ± SD ($n = 15$ for root length and $n = 3$ for fresh weight). Bars with different letters indicate significant differences at $P < 0.05$, determined using ANOVA with a Tukey's multiple comparison test.

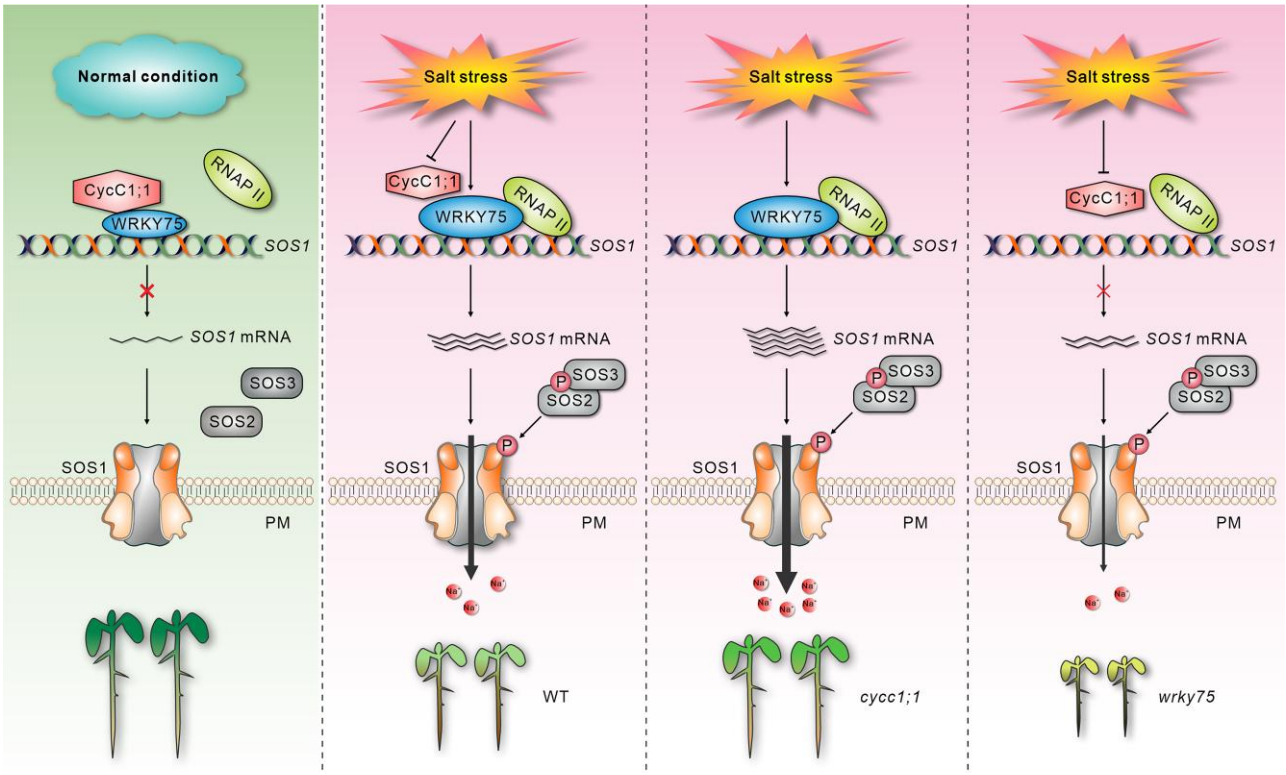

Figure 8. A proposed model showing the mechanism of CycC1;1–WRKY75 complex-mediated transcriptional regulation of *SOS1* in response to different salinity conditions in *Arabidopsis*. Under the normal condition, CycC1;1 interacts with WRKY75 to form a transcriptional repression complex that inactivates *SOS1* expression by interfering RNAP II occupancy on the promoter of *SOS1* in WT seedlings. When plants are subjected to high salinity stress, the expression of *CycC1;1* is suppressed while *WRKY75* expression is stimulated, leading to increased recruitment of RNAP II to the *SOS1* promoter, thereby activating *SOS1* expression and enhancing salt tolerance in WT seedlings. When *CycC1;1* is disrupted in the *cycc1;1* mutant, WRKY75 transcriptional activation of *SOS1* is further enhanced under high salinity conditions, thus leading to higher salt stress tolerance in the mutant than in the WT. In contrast to the *cycc1;1* mutant, the *wrky75* mutant has impaired salt-induced *SOS1* transcription and thus attenuated salt stress tolerance. WT, wild-type; PM, plasma membrane; *cycc1;1*, CycC1;1 loss-of-function mutant; *wrky75*, WRKY75 loss-of-function mutant.

studied CDK8 subunit in the kinase module, our knowledge of CycC regulation of plant stress responses is poor as is its role in resistance to necrotrophic pathogens in *Arabidopsis* (Zhu et al. 2020). This study showed that CycC1;1 plays a critical role in plant salt tolerance by interacting with and inhibiting WRKY75, a key transcription factor positively regulating *SOS1*, shedding light on how CycC1;1 is involved in the plant salt stress response and tolerance. However, we have noticed that although CDK8 promotes drought tolerance by increasing the expression of RAP2.6-targeted genes such as *RD29A* and *COR15A*, as documented in a previous study (Zhu et al. 2020), CycC1;1 suppresses ABA signaling and the expression of ABA-responsive genes as shown in our recent report (Guo et al. 2022). Also, the *cdk8* and *cycc1* mutants exhibit similar susceptibility to the necrotrophic pathogen *Alternaria brassicicola* but show different resistance against *Botrytis cinerea*, another necrotrophic pathogen (Zhu et al. 2014). However, flowering and a prolonged reproductive phase are similarly delayed in both mutants of *CDK8* and *CycC* in pea (*Pisum sativum* L.) (Hasan et al. 2020). Based on these findings that different subunits of Mediator complex exert similar or different effects on plant growth and stress responses, we hypothesize that CycC elicits its role in these processes in both CDK8-dependent and CDK8-independent manners. However, it is still important to perform additional experiments to investigate the role of CDK8 in the plant response to salt stress and possible relationships with CycC1;1.

In summary, our study reveals how the CycC1;1–WRKY75 signaling module is a key component of the precise and dynamic regulation of *SOS1* expression and elucidates the underlying mechanism by which CycC1;1 interferes WRKY75-mediated transcriptional activation of *SOS1* under normal conditions. It also reveals how *CycC1;1* expression is suppressed but *WRKY75* expression is upregulated to promote *SOS1* expression in *Arabidopsis*, leading to enhanced salt tolerance under salt stress conditions (Fig. 8).

Materials and methods

Plant materials and growth conditions

Arabidopsis (*A. thaliana*) ecotype Columbia-0 was used as the WT. The *cycc1;1* (SALK_053291) and *sos1*

(SALK_046400) mutants were obtained from the Arabidopsis Biological Resource Center. The complementation (Com) lines of the *cycc1;1* mutant and *CycC1;1*-overexpressing transgenic plants (OE) were generated in our previous report (Guo et al. 2022). The *wrky75-1*, *wrky75-25* (Guo et al. 2017; Zhang et al. 2018), and *WRKY75*$_{pro}$*:GUS* (Guo et al. 2017) seeds were previously reported and verified by genomic DNA PCR. The *cycc1;1 sos1* and *cycc1;1 wrky75-25* double mutants were obtained by genetic crossing. The transgenic plants *CycC1;1*$_{pro}$*:GUS*, *35S*$_{pro}$*:SOS1* (*SOS1-OE*), and *SOS1*$_{pro}$*:GUS* were generated as described below in methods. Subsequently, the *SOS1*$_{pro}$*:GUS cycc1;1*, *SOS1*$_{pro}$*:GUS CycC1;1-OE*, *SOS1*$_{pro}$*:GUS wrky75-1*, and *wrky75-25 SOS1-OE* plants were obtained by genetic crossing.

Seeds were surface sterilized using 10% (*w*/*v*) sodium hypochlorite (NaClO) for 5 min, washed 5 times with sterile water, and sown on a 1/2× MS medium (pH 5.8, containing 1% [*w*/*v*] sucrose and 0.8% [*w*/*v*] agar). Plates were kept at 4 °C for 3 d, and then seeds were germinated and grown under a 16-/8-h light/dark photoperiod at 100 μmol m^{-2} s^{-1} at 22 °C.

Construction of transgenic plants

To generate the *CycC1;1*$_{pro}$*:GUS* transgenic reporter line, the genomic sequence containing 1,030 bp upstream of the *CycC1;1* translation start codon (ATG) was amplified and cloned into pCambia1381 at the *Eco*RI site according to our previous report (Wang et al. 2022). The resulting plasmid was introduced into the wild-type plant via *Agrobacterium tumefaciens*-mediated floral transformation using the floral dip method (Clough and Bent 1998). Com T4 transgenic plants were used for GUS staining analysis.

To generate *SOS1*-overexpression lines, the full-length coding sequence (CDS) of *SOS1* was cloned into pCambia1300 at the *Sal*I site under the control of the cauliflower mosaic virus *35S* promoter, and the resulting plasmid was used to transform the wild-type *Arabidopsis* plants by *A. tumefaciens*-mediated transformation as described above. At least 8 T4 homozygous *35S*$_{pro}$*:SOS1-GFP* transgenic lines were selected, and one of them (*SOS1-OE #7*) with higher *SOS1* transcription and salt tolerance than the wild-type was used for our study. The *wrky75-25 SOS1-OE* line was obtained by genetic crossing *wrky75-25* with the selected *SOS1-OE #7* transgenic plant.

To generate the *SOS1*$_{pro}$*:GUS* transgenic reporter line, the genomic sequence containing 1,386 bp upstream of the *SOS1* translation start codon (ATG) was amplified and cloned into pCambia1381 at the *Bam*HI site. The resulting plasmid was introduced into the wild-type plant via *A. tumefaciens*-mediated floral transformation. T4 transgenic plants were used for GUS staining analysis. The *SOS1*$_{pro}$*:GUS cycc1;1*, *SOS1*$_{pro}$*:GUS CycC1;1-OE*, and *SOS1*$_{pro}$*:GUS wrky75-25* plants were obtained by genetic crossing the *SOS1*$_{pro}$*:GUS* line with the *cycc1;1*, *CycC1;1-OE#5*, and *wrky75-25* plants, respectively. The primers used for genotyping, plasmid construction, and RT-qPCR are listed in Supplemental Table S1.

Analysis of seed germination and cotyledon greening rates

Mature *Arabidopsis* seeds were harvested and dried at room temperature for 3 wk and then used for the germination assays. Sterilized seeds were plated on 1/2× MS medium without or with the indicated concentrations of NaCl. Following stratification at 4 °C for 3 d in the dark, the plates were transferred to a growth chamber with a 16-h light/8-h dark cycle at 100 μmol m^{-2} s^{-1} at 23 °C. The germination and cotyledon greening rates were analyzed from the first day to the seventh day after the plates were transferred to the light.

Analysis of root elongation and fresh weight

Five-day-old wild-type, mutant, or overexpression transgenic seedlings grown on 1/2× MS medium were transferred to 1/2× MS containing 0 mM or 125 mM NaCl for another 5 d, and then lengths of the newly grown roots were measured using ImageJ (https://imagej.nih.gov/ij/). At least 15 seedling roots were analyzed per group for each experiment. For the fresh weight analysis, a group of 5 seedlings was used as 1 biological sample, and at least 3 biological replicates were used for the fresh weight analysis.

RT-qPCR analysis

Tissues from treated or untreated wild-type, mutant, or overexpression transgenic seedlings were collected for total RNA isolation, first-strand cDNA synthesis, and RT-qPCR as we described previously (Wang et al. 2022). The constitutively expressed *ACTIN2* gene was used as an internal control. The primers used for RT-qPCR are listed in Supplemental Table S1.

Measurement of net Na^+ flux using NMT

The wild-type and mutant seedlings were grown on 1/2× MS medium for 10 d, incubated in liquid medium or 150 mM NaCl for 5 h, and then subjected to measurement of Na^+ fluxes using NMT (NMT150; YoungerUSA, Amherst, MA, USA; Xuyue [Beijing] Sci. & Tech., Beijing, China) according to the previous reported method (Lou et al. 2020). The continuous transient Na^+ fluxes were recorded for about 6 min. Each point is the mean of 4 individual plants.

Determination of sodium content by ENG-2 AM staining

The sodium-specific fluorescent dye, enhanced NaTrium Green-2 AM (ENG-2 AM, MX4514, Shanghai Maokang Biotechnology, Shanghai, China), was used to determine sodium accumulation in roots. Five-day-old plant seedlings grown on 1/2× MS medium were treated with or without 100 mM NaCl for 12 h, and then the seedlings were stained with a 10 μM ENG-2 AM solution containing 0.05% Pluronic F-127 (MS4301, Shanghai Maokang Biotechnology, Shanghai, China) for 3 h. The Pluronic F-127 is a nonionic surfactant that can promote the dissolution capacity of the fluorescent dye ENG-2 AM. The seedlings were stained

with 20 μg/mL propidium iodide (PI) solution for 3 min, after which the green fluorescence of ENG-2 AM (488 nm excitation, 505 to 530 nm emission, and 5% laser intensity) and the red fluorescence of PI (514 nm excitation, 575 to 615 nm emission, and 2% laser intensity) were detected using a confocal laser scanning microscope equipped with Plan-Apochromat 20×/0.8 objective (ZEISS LSM 980, Zeiss, https://www.zeiss.com). The fluorescence intensities were analyzed using ImageJ. At least 15 seedling roots were analyzed per group for each experiment.

Determination of Na^+ content in xylem sap

Determination of Na^+ accumulation in xylem sap was performed according to a previously reported method (Shi et al. 2002). Briefly, plants were grown in soil for 3 wk in a growth chamber under a 16-/8-h light/dark photoperiod at 100 μmol m^{-2} s^{-1} at 22 °C and then irrigated with water or 100 mM NaCl solution for 1 d. The plants were then kept in a chamber with 100% humidity, and their rosette leaves and inflorescence stems were cut at the base. The water droplet on the cut surface of the inflorescence stem was collected, and the Na^+ content of this xylem sap was analyzed using inductively coupled plasma-optical emission spectrometer (ICP-OES, Agilent 5110).

Y2H assays

pGBKT7-CycC1;1 was constructed in our previous report (Guo et al. 2022). The full-length CDSs of *WRKY1* and *WRKY75* were cloned into pGADT7 (Clontech) at the *Bam*HI site, respectively. Yeast transformation and growth were carried out using the Matchmaker system (Clontech) according to the manufacturer's protocols. Yeast transformants were selected on double dropout medium lacking Leu and Trp (−LW), and protein interactions were analyzed on quadruple dropout medium lacking Leu, Trp, His, and Ade (−LWHA). Primer sequences are listed in Supplemental Table S1.

BiFC assay

The CDSs of *CycC1;1*, *WRKY75*, and *WRKY1* were cloned into the pSPYCE or pSPYNE vector (Walter et al. 2004) containing the C-terminal of YFP (cYFP) and or the N-terminal of YFP (nYFP), respectively. The nuclear marker H2B-mCherry (Rosa et al. 2014), nYFP-WRKY75 (N-terminal tag), and cYFP-CycC1;1 (C-terminal tag) were coexpressed in *N. benthamiana* leaves for 3 d, after which YFP (excitation 488 nm and emission 520 to 560 nm) and mCherry (excitation 561 nm and emission 600 to 630 nm) fluorescence signals were detected using a laser scanning confocal microscope (Zeiss LSM980, Zeiss, Germany). Primer sequences are listed in Supplemental Table S1.

Co-IP assays

To perform Co-IP assays, the CDS of *WRKY75* was cloned into pCambia1300 at the *Sal*I site, including sequences encoding a GFP tag fused to its C-terminus and under the control of the 35S promoter. The Co-IP experiment was performed according to the previously reported methods with some modifications (Nie et al. 2022; Liu et al. 2022a). Briefly, GFP-tagged WRKY75 and HA-tagged CycC1;1 were transformed into *Agrobacterium* GV3101 and infiltrated into 3-wk-old *N. benthamiana* leaves. After 3 d, total proteins were extracted from the infected leaves by homogenization in IP buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail [Roche]) and then immunoprecipitated by an anti-GFP antibody (ABclonal, AE012, diluted 1:1,000). The resulted precipitates were resuspended and detected using an anti-GFP (ABclonal, AE012) and anti-HA (Sungene Biotech, KM8004) antibodies, respectively.

EMSA assay

The CDS of *WRKY75* was cloned into pPSUMO (pET28a-6×His-SUMO, Wang et al. 2022) at the *Bam*HI site, where sequences encoding a 6×His-SUMO tag allowed fusion to its N-terminus. The resulted pPSUMO-His-WRKY75 vector was transformed and induced in the *E. coli* BL21 (DE3) strain, and then the 6×His-SUMO-tagged WRKY75 protein was purified using NTA-Ni beads (Ni-NTA Purose 6 Fast Flow, A41002-06, Qianchun Bio, Jiaxing, China) according to our previous report (Wang et al. 2022). The EMSA assay was performed using a Light Shift Chemiluminescent EMSA kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, biotin-labeled probes were incubated with or without purified 6×His-WRKY75 in binding buffer (2.5% glycerol, 50 mM KCl, 5 mM $MgCl_2$, and 10 mM EDTA) for 30 min at room temperature. For the EMSA competition experiments, unlabeled unmutated and mutated probes used as competitors were added to the binding reactions, respectively. The probe sequences are listed in Supplemental Table S1.

GST pull-down assay

The CDSs of full-length *WRKY75* and *CycC1;1* were cloned into the pPSUMO (6×His-SUMO) and pGEX4T-1 vectors, respectively. The GST pull-down assay was performed according to our previous reports (Guo et al. 2022; Liu et al. 2022a). Briefly, GST-CycC1;1 and 6×His-SUMO-WRKY75 were expressed and purified from *E. coli*. 6×His-SUMO-WRKY75 was mixed with GST-CycC1;1 or GST alone on ice for 1 h and then incubated with GST-Sefinose Resin 4FF (Settled Resin) (Sangon Biotech, C600031) at 4 °C for 3 h. After washing, the eluted protein with Elution Buffer (10 mM GSH in 50 mM Tris-HCl, pH 8.0) was detected with anti-His (EnoGene, #E12-004-3) and anti-GST (ABclonal, AE006) antibodies, respectively. Primer sequences are listed in Supplemental Table S1.

LUC reporter gene assay

The LUC reporter gene assay was performed according to previous reports (Liu et al. 2022b). The *SOS1* promoter was cloned into pGreen0800 (Zhang et al. 2020) at the *Bam*HI

site, and LUC expression was controlled by *SOS1* promoter activity. The resulted reporter and the internal control (*35S$_{pro}$:REN* in pGreen0800 vector) were transiently coexpressed with or without the effector pEGAD-CycC1;1 (Guo et al. 2022) in *N. benthamiana* leaves for 3 d, and then the activities of LUC and REN were detected using a Multimode Reader Platform (Tecan Spark, Tecan Group Ltd., Zurich, Switzerland) according to our previous reports (Guo et al. 2022; Liu et al. 2022b). Primer sequences are listed in Supplemental Table S1.

ChIP-qPCR analysis

To test the association of CycC1;1 with *SOS1* genomic DNA, 7-d-old wild-type and *wrky75-25* mutant seedlings were used for ChIP assays according to a previously reported method (Guo et al. 2022). The chromatin was immunoprecipitated from the wild-type plants with or without the anti-CycC1;1 antibody (Guo et al. 2022), and then both the input and the immunoprecipitated DNA fragments were quantified by qPCR with specific primers. *ACTIN7* was used as a reference gene. The primers used for ChIP-qPCR are listed in Supplemental Table S1.

To assay the binding of WRKY75 to the *SOS1* promoter, chromatin was extracted from 7-d-old *35S$_{pro}$:WRKY75-GFP* (Guo et al. 2017) seedlings and precipitated using GFP antibody-conjugated agarose beads (ABclonal, AE074). DNA fragments in both input and immunoprecipitated samples were quantified by qPCR. *ACTIN7* was used as a reference gene. The primers used for ChIP-qPCR are listed in Supplemental Table S1.

To examine the effect of CycC1;1 on RNAP II recruitment to the *SOS1* promoter, chromatin was extracted from 7-d-old wild-type and *cycc1;1* mutant seedlings and precipitated with an anti-RPB2 antibody (ABclonal, A5928, diluted 1:1,000). DNA fragments in both input and immunoprecipitated samples were quantified by qPCR. *ACTIN7* was used as a reference gene. The primers used for ChIP-qPCR are listed in Supplemental Table S1.

GUS staining and GUS activity

Untreated and salt-treated *CycC1;1$_{pro}$:GUS*, *SOS1$_{pro}$:GUS*, and *WRKY75$_{pro}$:GUS* transgenic plant seedlings or tissues were stained in GUS staining solution (100 mM sodium phosphate buffer, pH 7.5, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1 mM 5-bromochloro-3 -indolyl-b-D-glucuronide, and 0.1% Triton X-100). The samples were rinsed with 70% ethanol several times to remove the chlorophyll, and images were then taken under a microscope (Leica DMI8, Leica Microsystems, Germany). The GUS activities of the treated and untreated promoter-GUS transgenic seedlings were assayed according to a method described previously (Li et al. 2015).

Statistical analysis

Data are means ± SD of 3 biological replicates, and the asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, Student's *t* test). Bars with different letters indicate significant differences at $P < 0.05$ by ANOVA with Tukey's multiple comparison test. Detailed statistical analysis results can be found in Supplemental Data Set 1.

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: CycC1;1 (AT5G48640), SOS1 (AT2G01980), SOS2 (AT5G35410), SOS3 (AT5G24270), ACTIN2 (AT3G18780), ACTIN7 (AT5G09810), WRKY1 (AT2G04880), and WRKY75 (AT5G13080).

Acknowledgments

We thank the anonymous reviewers for their thoughtful reviews and constructive suggestions which have improved this paper. We thank Prof. Hongwei Guo (Southern University of Science and Technology, China) and Prof. Diqiu Yu (Yunnan University, China) for kindly providing the *WRKY75$_{pro}$:GUS*, *35S$_{pro}$:WRKY75-GFP*, *wrky75-1*, and *wrky75-25* seeds. We thank Man-cang Zhang (Electrophysiology platform, Henan University) for performing the NMT experiment.

Author contributions

W.-C.L. and Y.-T.L. designed and directed the project. K.-K.L. performed the experiments with assistance of R.-F.S., J.-X.G., Y.Z., H.-H.C., J.-X.Z., C.-Y.L., and X.-Y.H. K.-K.L., W.-C.L., and F.R. analyzed the data. W.-C.L. wrote the manuscript.

Supplemental data

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

Supplemental Figure S1. *CycC1;1*-overexpressing plants are sensitive to salt stress.

Supplemental Figure S2. Relative GUS activity of the *CycC1;1$_{pro}$:GUS* transgenic plants subjected to salt stress.

Supplemental Figure S3. *SOS2* and *SOS3* expression in the *cycc1;1* mutant.

Supplemental Figure S4. Relative GUS activity of the *SOS1$_{pro}$:GUS* transgenic plants subjected to salt stress.

Supplemental Figure S5. Na^+ accumulation in the xylem sap of the wild-type and *cycc1;1* mutant plants.

Supplemental Figure S6. Sensitivity of single and double mutants of *cycc1;1* and *sos1* to mild salt stress.

Supplemental Figure S7. Na^+ accumulation in the xylem sap of single and double mutants of *cycc1;1* and *sos1*.

Supplemental Figure S8. CycC1;1 does not interact with WRKY1.

Supplemental Figure S9. Relative GUS activity of the *SOS1$_{pro}$:GUS* and *SOS1$_{pro}$:GUS wrky75-1* transgenic plants subjected to salt stress.

Supplemental Figure S10. Na^+ accumulation in the xylem sap of the *wrky75* mutant.

Supplemental Figure S11. Relative GUS activity of *WRKY75$_{pro}$:GUS* transgenic plants subjected to salt stress.

Supplemental Figure S12. Na^+ accumulation in the xylem sap of single and double mutants of *cycc1;1* and *wrky75*.

Supplemental Table S1. Primers used in this study.

Supplemental Data Set 1. Statistical analysis results.

Funding

This work was supported by the National Natural Science Foundation of China (#32000150 and #31971814) and the Natural Science Foundation of Henan Province (#222300420401).

Conflict of interest statement. None declared.

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