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Research Article

CycC1;1 negatively modulates ABA signaling by interacting with and inhibiting ABI5 during seed germination

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Abstract

Regulation of seed germination is important for plant survival and propagation. ABSCISIC ACID (ABA) INSENSITIVE5 (ABI5), the central transcription factor in the ABA signaling pathway, plays a fundamental role in the regulation of ABA-responsive gene expression during seed germination; however, how ABI5 transcriptional activation activity is regulated remains to be elucidated. Here, we report that C-type Cyclin1;1 (CycC1;1) is an ABI5-interacting partner affecting the ABA response and seed germination in Arabidopsis (*Arabidopsis thaliana*). The *CycC1;1* loss-of-function mutant is hypersensitive to ABA, and this phenotype was rescued by mutation of *ABI5*. Moreover, CycC1;1 suppresses ABI5 transcriptional activation activity for ABI5-targeted genes including *ABI5* itself by occupying their promoters and disrupting RNA polymerase II recruitment; thus the *cycc1;1* mutant shows increased expression of *ABI5* and genes downstream of ABI5. Furthermore, ABA reduces the interaction between CycC1;1 and ABI5, while phospho-mimic but not phospho-dead mutation of serine-42 in ABI5 abolishes CycC1;1 interaction with ABI5 and relieves CycC1;1 inhibition of ABI5-mediated transcriptional activation of downstream target genes. Together, our study illustrates that CycC1;1 negatively modulates the ABA response by interacting with and inhibiting ABI5, while ABA relieves the CycC1;1 interaction with and inhibition of ABI5 to activate ABI5 activity for the ABA response, thereby inhibiting seed germination.

Introduction

Seed dormancy is a crucial process for angiosperms to adapt to changing environments especially stressful conditions (Finch-Savage and Leubner-Metzger, 2006). When sensing the favorable circumstance for growth, plants start with the release of seeds dormancy and launch of germination for seedling establishment (Liu et al., 2016; Nonogaki, 2017). Thus, the balance between seed dormancy and germination is precisely controlled in plants, which plays a vital role in

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plant survival and propagation as well as agricultural production (Shu et al., 2018). Various endogenous phytohormones affecting seed germination have been widely studied, and among them, abscisic acid (ABA) plays a pivotal role in promoting seed dormancy, inhibiting seed germination, and arresting seedling growth (Zhao et al., 2018). During the seed maturation and dormancy induction processes, the endogenous ABA level is substantially increased and ABA signaling pathway is strongly activated; on the contrary, ABA accumulation is decreased and its signaling is suppressed during seed germination and postgerminative seedling growth (Shu et al., 2017). Under adverse conditions or ABA treatment, increased ABA level in seeds is perceived by its receptors PYRABACTIN RESISTANCE PROTEINS/PYR-LIKE PROTEINS/REGULATORY COMPONENTS OF ABA RECEPTOR, relieving PHOSPHATASE2C-mediated repression of SNF1-RELATED PROTEIN KINASE2 (SnRK2) kinases (Ma et al., 2009; Park et al.,2009; Zhao et al., 2018). Many ABA-responsive element-binding factors (ABFs) implicated in plant ABA response are phosphorylated by the activated SnRK2 kinases, resulting in changes of gene expression (Fujii et al., 2009; Yoshida et al., 2014; Vishwakarma et al., 2017).

The basic leucine zipper (bZIP) transcription factor, ABA INSENSITIVE5 (ABI5) is a central player in ABA-mediated inhibition of seed germination (Zhao et al., 2020). ABI5 recognizes and binds to the ABA response elements (ABREs), a G-box of numerous ABA-responsive genes, such as *EARLY METHIONINE-LABELED1* (*EM1*), *EM6*, and *RESPONSIVE TO DESICCATION29A* (*RD29A*), which encode products important for plant ABA response during seed germination, and transcriptionally activates the expression of these genes (Yamaguchi-Shinozaki et al., 1992; Nakashima et al., 2009). Both EM1 and EM6 are belonging to the late embryogenesis abundant proteins, and have been used as molecular markers of late embryogenesis (Gaubier et al., 1993; Yang et al., 2021). Although *EM1* and *EM6* have different expression pattern, but both of them are strongly activated by ABA and thus involved in the acquisition of desiccation response and establishment of embryo dormancy (Wise, 2003; del Viso et al., 2007). ABI5 expression and activity are tightly regulated in plants to ensure optimal responses to internal and external cues. SnRK2-mediated phosphorylation of ABI5 results in its stabilization and activation in plant response to ABA. Serine (Ser)-42, Ser-145, and Threonine (Thr)-201 are the major phosphorylation sites of ABI5, and the phospho-dead forms of ABI5 at these sites severely block its transactivation of the targeted genes while the phospho-mimic forms promote such activity (Wang et al., 2013; Zhou et al., 2015). On the contrary, ABI5 activity can be inactivated by phytochrome-associated Ser/Thr protein phosphatase/Ser/Thr-specific phosphoprotein phosphatase 6-mediated de-phosphorylation (Dai et al., 2013). In addition, ABI5 is also posttranslationally regulated by DWD HYPERSENSITIVE TO ABA1-mediated ubiquitination (Lee et al., 2010), nitric oxide-mediated *S*-nitrosylation (Albertos et al., 2015), and SAP AND MIZ1 DOMAIN-CONTAINING LIGASE1-mediated sumoylation (Zheng et al., 2012), which disrupts plant ABA response through promoting ABI5 degradation.

In addition to the posttranslational regulation, ABI5 is also regulated at the transcriptional level. Several transcription factors that positively or negatively regulate *ABI5* expression by binding to *ABI5* promoter have been identified, including ANAC060, ABI3, WRKY40, HY5, a B box-containing protein BBX19, MCM1/AGAMOUS/DEFICIENS/SRF-box transcription factors AGL21 and RELATED TO ABI3/VP1 (Xu et al., 2014; Yu et al., 2017, 2020; Bai et al., 2019; Wang et al., 2020). In addition, ABI5 can upregulate *ABI5* transcription by binding to its own promoter (Xu et al., 2014; Bai et al., 2019). Notably, a REGULATOR OF CHROMATIN CONDENSATION1 (RCC1) family protein SENSITIVE TO ABA1 (SAB1) and the transcription factor BRINSENSITIVE1-EMS-SUPPRESSOR1 suppress the expression of *ABI5* and ABI5-targed genes through physical interaction, thereby interfering plant responses to ABA (Ji et al., 2019; Zhao et al., 2019).

The regulatory information of most targeted gene transcription conveyed by transcription factors generally needs to be delivered to RNA polymerase II (RNAP II), and the Mediator complex as a signal processor plays a necessary role in this process by establishing the linkage (Asturias et al., 1999; Agrawal et al., 2021). Mediator complex is conserved in eukaryotes, and comprises four modules, including head, middle, tail, and kinase modules. The head, middle, and tail modules form the core part of Mediator complex, while the kinase module is a separable part of the complex consisting of CYCLIN-DEPENDENT KINASE8 (CDK8), C-type cyclin (CycC), MEDIATOR COMPLEX SUBUNIT12 (MED12), and MED13 (Wang and Chen, 2004; Mathur et al., 2011; MaJi et al., 2019). The head and the tail modules serve as the physical interaction interfaces for RNAP II and sequence-specific transcription factors, respectively, and the kinase module is able to alter the function of Mediator through association or de-association with the core part, thereby negatively or positively regulating gene transcription (Poss et al., 2013). Emerging evidence has revealed the involvement of the Mediator complex in plant ABA responses. For example, MED25, a subunit of Mediator tail module, physically interacts with ABI5 and exerts a negative effect on the expression of ABI5-targeted genes (Chen et al., 2012). MED16 can compete with MED25 to interact with ABI5, thus positively regulating plant ABA signaling (Guo et al., 2021). The biological role of CDK8, a subunit of the kinase module, has also been studied in plant ABA response, where it is associated with ERF/AP2 transcription factor RAP2.6 and SnRK2.6 to positively modulate ABA signaling and drought response in Arabidopsis (*Arabidopsis thaliana*) (Zhu et al., 2020). In contrast, CycC in Arabidopsis is a small family consisting with two members, CycC1;1 and CycC1;2, and in addition to their role in resistance to necrotrophic pathogens in Arabidopsis (Zhu et al., 2020), functions of CycC in plant stress responses remains elusive.

In this study, we report that CycC1;1 is an ABI5-interacting protein, and negatively modulates ABA-inhibited

seed germination by inhibiting the transcriptional activation activity of ABI5 for the downstream targeted genes including *ABI5* itself. Mutation of *CycC1;1* resulted in increased sensitivity in plant response to ABA whereas its overexpression led to decreased sensitivity. CycC1;1 physically interacts with ABI5 to occupy the promoters of ABI5-targeted genes and *ABI5* itself, and thus interfering its transcriptional activation activity for its target genes as well as *ABI5*; while ABA-induced ABI5 phosphorylation at Ser-42 relieves its repression of ABI5 by impairing their physical interaction. Thus, our study unravels the role of CycC1;1 in ABA signaling transduction by interacting with and inhibiting ABI5 during seed germination.

Results

CycC1;1 physically interacts with ABI5

ABI5, as a central transcription factor in ABA signaling pathway, plays a fundamental role in seed dormancy and germination by regulating ABA-triggered gene expression. We assumed that identification of ABI5-interacting proteins would gain more insights into our understanding for the precise regulation of ABI5 in seed germination and ABA response. Using ABI5 as a bait, a C-type Cyclin family member CycC1;1 (AT5G48630) was identified as a putative partner that could interact with ABI5 in yeast two-hybrid (Y2H) system. To verify this interaction, we cloned full-length coding sequence (CDS) of *CycC1;1* and re-examined its interaction between ABI5 in yeast cells, our results showed that CycC1;1 and ABI5 indeed had interaction in yeast cells (Figure 1A). The interaction was also confirmed by bimolecular fluorescence complementation (BiFC) assay, in which ABI5 fused to the N-terminal half of yellow fluorescent protein (YFP), CycC1;1 fused to the C-terminal half of YFP and nuclear marker H2B-mCherry (Rosa et al., 2014) were co-expressed in *Nicotiana benthamiana* leaves. Reconstituted fluorescence was observed in the nucleus while no fluorescence was observed in negative controls (Figure 1B), indicating that CycC1;1 interacts with ABI5 in planta. Similarly, our luciferase (LUC) complementation imaging (LCI) experiments further confirmed the interaction between CycC1;1 and ABI5, as LUC activity was only detected in *N. benthamiana* leaves where CycC1;1 fused with the N-terminal of LUC and ABI5 fused with the C-terminal of LUC were co-expressed (Figure 1C). To examine whether CycC1;1 has physical interaction with ABI5 in vitro, we purified 6 × histidine (His)-tagged CycC1;1 and glutathione *S*-transferase (GST)-tagged ABI5 proteins expressed in *Escherichia coli*, and performed GST pull-down assay using glutathione-sepharose beads. Our results showed that His-CycC1;1 could be specifically pulled down by GST-ABI5 but not by GST tag alone (Figure 1D), indicating that CycC1;1 physically interacts with ABI5 in vitro. These results reveal that CycC1;1 has interaction with ABI5 in vitro and in vivo.

The C-type Cyclin family in Arabidopsis (*A. thaliana*) has another member, CycC1;2 (AT5G48630) in addition to CycC1;1, thus we further investigated whether CycC1;2 has interaction with ABI5 in plants. For this end, we performed Y2H experiment, and found that the yeast cells co-expressing both CycC1;2 and ABI5 could grow on triple dropout medium but not quadruple dropout medium (Figure 1A and Supplemental Figure S1A), revealing weaker interaction between CycC1;2 and ABI5 than that between CycC1;1 and ABI5 in yeast cells. We also carried out BiFC and LCI assays, showing that CycC1;2 could interact with ABI5 in *N. benthamiana* leaves (Figure 1, B and C). These results revealed that similar to CycC1;1, CycC1;2 also has interaction with ABI5 in planta. Considering that C-type cyclin and CDK8 are members of the kinase module in the Mediator complex, we wondered whether CDK8 could also interact with ABI5. CDK8 could indeed interact with CycC1;2; however, no interaction between CDK8 and ABI5 was observed in yeast cells (Supplemental Figure S1B).

CycC1;1 is epistatic to ABI5 to negatively regulate ABA-inhibited seed germination

The interaction of CycC1;1/CycC1;2 with ABI5 prompted us to test whether they functions in ABA-regulated seed germination. We first obtained a previously reported T-DNA insertion line of *CycC1;1*, SALK_053291 (Zhu et al., 2014), where the T-DNA is inserted in the second intron of *CycC1;1*, resulting in reduced expression of *CycC1;1* but not *CycC1;2* (Supplemental Figure S2, A–C). Our results showed that both the wild-type (WT) and *cycc1;1* mutant exhibited similar germination and cotyledon greening rates in the absence of ABA, whereas when subjected to ABA treatment, the mutant displayed significantly higher sensitivity to ABA than the WT as evidenced by less germination and cotyledon greening rates (Figure 2, A–C), revealing that *CycC1;1* negatively regulates ABA-mediated seed germination. To confirm that the increased ABA sensitivity of the *cycc1;1* mutant is due to defective *CycC1;1* expression caused by T-DNA insertion in the mutant, a binary vector harboring *CycC1;1::CycC1;1* was transformed into *cycc1;1* mutant for the complementation analysis. Decreased *CycC1;1* expression level in the *cycc1;1* mutant was fully restored in the transgenic complementation lines (Supplemental Figure S2B). Consistently, increased ABA sensitivity of the mutant in terms of the seed germination and cotyledon greening rates was also rescued by the expression of *CycC1;1* under the control of its native promoter (Figure 2, A–C), further supporting the negative effects of *CycC1;1* on ABA-inhibited seed germination. We also tested ABA sensitivity of the *CycC1;1*-overexpressing transgenic lines where the expression of *CycC1;1-GFP* was driven by the *35S* promoter (Figure 2B). In contrast to the *cycc1;1* mutant, *CycC1;1*-overexpressing lines exhibited remarkably insensitive phenotype to ABA as they had higher seed germination and cotyledon greening rates than the WT in the presence of ABA (Figure 2, A–C), further validating the negative regulation of *CycC1;1* in ABA-inhibited seed germination.

The genes of *CycC1;1* and *CycC1;2* are linked in the Arabidopsis genome (Supplemental Figure S2A). We could

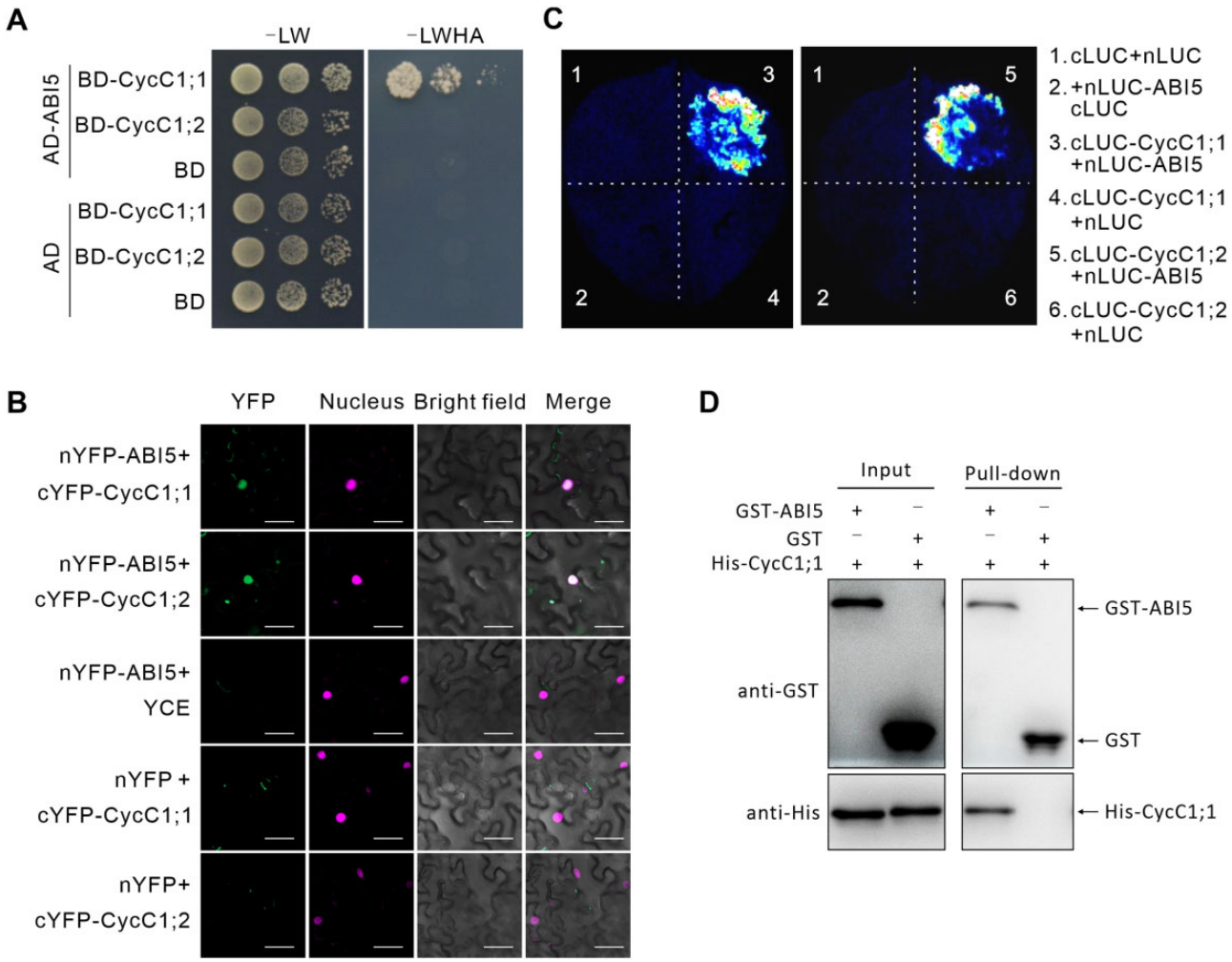

Figure 1 CycC1;1 and CycC1;2 interact with ABI5. A, Y2H assay shows the interaction between ABI5 and CycC1;1 or CycC1;2. CycC1;1 or CycC1;2 and ABI5 were fused to the transcriptional activation domain (AD) or DNA-BD of GAL4. Protein interactions were examined based on the growth of yeast cells on selective media. -L/W indicates Leu and Trp, -L/W/H/A indicates Trp, Leu, His, and Ade drop-out plates. B, BiFC analysis. ABI5-nYFP and cYFP-CycC1;1 or cYFP-CycC1;2 were transiently co-expressed in *N. benthamiana* leaves. H2B-mCherry (Rosa et al., 2014) was used as a nuclear marker. Scale bars = 20 μm. C, Split LUC assay. nLUC-ABI5 and cLUC-CycC1;1 or cLUC-CycC1;2 constructs were transiently expressed in *N. benthamiana* leaves. cLUC, C-terminus of LUC; nLUC, N-terminus of LUC. D, GST pull-down analysis. 6 × His-CycC1;1 were mixed with GST-ABI5 or GST and immobilized on glutathione sepharose beads. After washing, the eluted proteins were subjected to immunoblot analysis with anti-GST and anti-His antibodies, respectively.

not obtain the single mutant of *CycC1;2*, but another T-DNA insertion line SALK_039400, where the T-DNA is inserted in the intergenic region between *CycC1;1* and *CycC1;2*, was identified (Supplemental Figure S2A). Consistent with the previously reports (Zhu et al., 2014; Chen et al., 2019), the SALK_039400 line indeed had reduced expression of both *CycC1;1* and *CycC1;2*, thus it was named *cycc1;1/1;2* (Supplemental Figure S3A). Similar to *cycc1;1* mutant, the *cycc1;1/1;2* mutant also showed higher ABA sensitivity than the WT in terms of seed germination and cotyledon greening rate (Supplemental Figure S3, B–D), suggesting a similar negative role of *CycC1;2* to *CycC1;1* in regulating ABA-inhibited seed germination. To further confirm the role of *CycC1;2* in ABA response, we generated *CycC1;2*-overexpressing lines and tested their ABA sensitivity (Supplemental Figure S3A). As expected, the *CycC1;2*-overexpressing lines had higher seed germination and cotyledon greening rate than the WT in the presence of ABA (Supplemental Figure S3, B–D). These results further support that both CycC1;1 and CycC1;2 negatively regulate ABA-inhibited seed germination.

Considering that both CycC1;1 and CycC1;2 could interact with ABI5, we hypothesized that CycC1;1 and CycC1;2 may affect ABA-mediated seed germination through ABI5. If this was the case, mutation of *ABI5* in the *cycc1;1* mutant should revert its ABA hypersensitivity as the *ABI5* loss-of-function mutants are insensitive to ABA (Zhao et al., 2020). Therefore, to genetically analyze the relationship between *CycC1;1* and *ABI5*, we crossed *cycc1;1* with *abi5-7* (Zhao et al., 2020), resulting in *cycc1;1 abi5-7* double mutant. The *abi5-7* mutant was indeed very insensitive to ABA (Figure 2, D–F), which is consistent with previous reports (Zhao et al., 2020), the *cycc1;1*

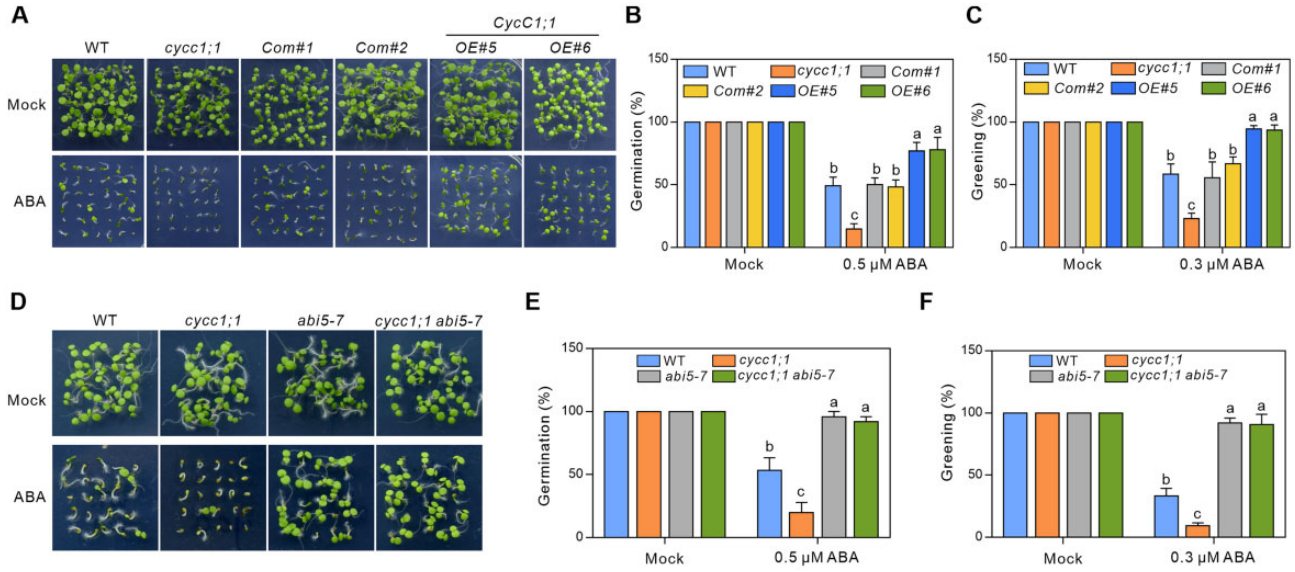

Figure 2 CycC1;1 suppresses ABA-inhibited seed germination through ABI5. A, Seedlings of 7-day-old various genotypes germinated on MS medium supplemented without or with 0.3 μM ABA. B, Germination percentages of the various genotypes in response to ABA. Seed germination on MS medium without or with 0.5 μM ABA was recorded after 3 days of stratification. C, Percentages of greening cotyledon of various genotypes. Cotyledon greening was recorded 7 days after stratification on MS medium supplemented without or with 0.3 μM ABA. D, Seedlings of 7-day-old WT, *cycc1;1*, *abi5-7*, and *cycc1;1 abi5-7* germinated on MS medium supplemented without or with 0.3 μM ABA. E, Germination percentages of the WT, *cycc1;1*, *abi5-7*, and *cycc1;1 abi5-7* in response to ABA. Seed germination on MS medium without or with 0.5 μM ABA was recorded after 3 days of stratification. F, Percentages of greening cotyledon of the WT, *cycc1;1*, *abi5-7*, and *cycc1;1 abi5-7*. Cotyledon greening was recorded 7 days after stratification on MS medium supplemented without or with 0.3 μM ABA. Data indicate mean ± SD ($n = 3$). Bars with different letters indicate significant differences at $P < 0.05$, revealed using ANOVA analysis.

mutant was hypersensitive to ABA while the *cycc1;1 abi5-7* double mutant showed identical ABA sensitivity to *abi5-7* in terms of seed germination and cotyledon greening rates upon ABA treatment (Figure 2, D–F). These results demonstrate that CycC1;1 acts epistatically to ABI5 in the negative regulation of ABA-mediated seed germination.

CycC1;1 interferes ABI5 transcriptional activation of the downstream targets

Our above results reveal that CycC1;1 negatively regulates ABA-inhibited seed germination, while ABI5 is a positive factor favoring plant ABA responses including inhibiting seed germination. We speculated that CycC1;1 may affect the transcriptional activation activity of ABI5 through their physical interaction. Because *EM1* is reported as a direct target gene of ABI5 during seed germination, we performed dual-LUC reporter gene assays in *N. benthamiana* leaves, where LUC was used as a reporter under the control of *EM1* promoter and *Renilla* LUC (REN) under the control of the constitutive *35S* promoter was used an internal control (Figure 3A). Our results showed that LUC activity was extensively stimulated when *35S::ABI5* as an effector was expressed but remained unchanged when *35S::CycC1;1* alone was expressed, while this ABI5-induced LUC activity was significantly compromised by the co-expression of *ABI5* and *CycC1;1* (Figure 3B), revealing that CycC1;1 interferes the activation of *EM1* expression by ABI5. Similarly, ABI5-induced LUC activity could also be suppressed by the expression of CycC1;2 (Figure 3B). Consistent with this, our reverse-transcription quantitative PCR (RT-qPCR) data showed that ABA-induced *EM1* transcripts in the WT were significantly enhanced in the *cycc1;1* mutant but repressed in the *CycC1;1*-overexpressing plants (Figure 3C), further supporting the negative effects of CycC1;1 on ABI5-induced *EM1* expression in plant response to ABA. In addition to *EM1*, we also examined the expression levels of other ABI5 target genes that are implicated in seed germination, including *EM6*, *RD29A*, and *ABF3*. The RT-qPCR results showed that the expression levels of these ABI5-targeted genes in the WT were greatly induced by ABA, while such changes were further promoted in the *cycc1;1* mutant and suppressed in the *CycC1;1*-overexpressing plants, respectively (Figure 3, D–F). Together, these data demonstrated that CycC1;1 interferes ABI5-mediated transcriptional activation of the downstream target genes during ABA-inhibited seed germination.

CycC1;1 represses *ABI5* expression by associating with its genomic DNA

Considering that ABI5 can bind to the promoter of its own gene and activate gene expression, we therefore further explored whether CycC1;1 affects *ABI5* expression at the transcriptional level. To achieve this, we first assayed whether CycC1;1 is associated with *ABI5* genomic DNA by chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR). *35S::GFP-CycC1;1* transgenic plants and anti-GFP antibody were used for the ChIP, and quantitative PCR primers designed at

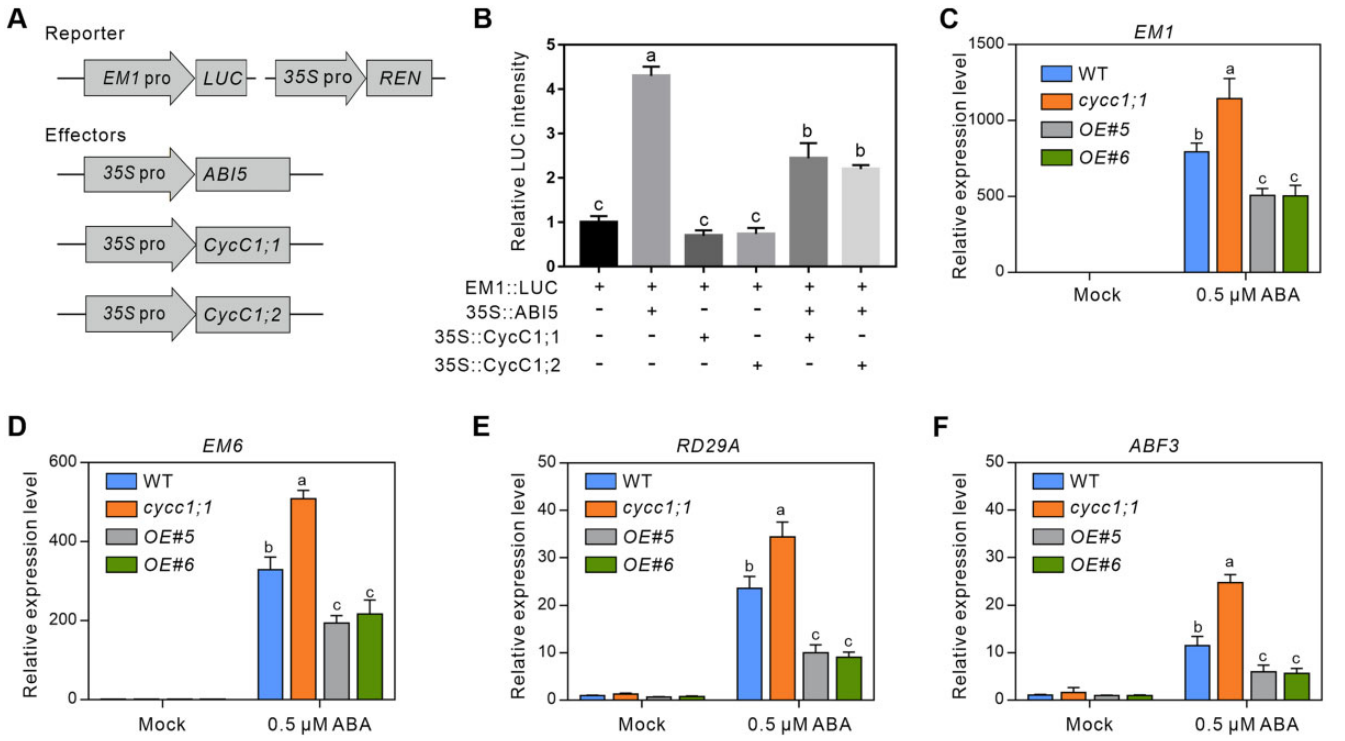

Figure 3 CycC1;1 and CycC1;2 suppress the transcriptional activation activity of ABI5 for the downstream genes. A and B, Dual-LUC reporter gene assay to examine the role of CycC1;1 and CycC1;2 in ABI5-activated *EM1* expression. The schematic diagram (A) shows the reporters and effectors used in the assay. The effector and reporter genes were co-expressed in *N. benthamiana* leaves, and the activities of LUC and REN were detected. The relative LUC intensity (B) represents the EM1::LUC activity relative to the internal control (REN driven by the 35S promoter). The activity of EM1::LUC alone was set to 1. Error bars indicate mean ± SD (n = 3). C–F, Expression of *EM1*, *EM6*, *RD29A*, and *ABF3* in germinating seeds of the WT, *cycc1;1* and *CycC1;1*-overexpression lines. Seeds were treated with MS medium or MS medium containing 10 μM ABA for 24 h. Error bars indicate mean ± SD (n = 3). Different letters indicate significant differences as determined using one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

different positions, including 500-bp upstream of translation start site, TATA box (the RNAP II binding site), coding and terminator regions of *ABI5* were used for the subsequent qPCR (Figure 4A). Our results showed that DNA fragments in −500 bp upstream, TATA box, and coding region but not terminator were significantly enriched by anti-GFP antibody compared with the control without antibody (Figure 4B), revealing that CycC1;1 is associated with *ABI5* promoter DNA. The core part of Mediator regulates gene transcription by acting as a bridge that connects RNAP II and transcription factors, and this connection can be affected positively or negatively by the kinase module (Agrawal et al., 2021). We then performed ChIP-qPCR to investigate the recruitment of RNAP II to *ABI5* in the WT and *cycc1;1* mutant plant using a specific antibody raised against the C-terminal domain of RNAP II. Our results showed that RNAP II was associated with the *ABI5* genomic DNA in the WT while this association was significantly enhanced in the *cycc1;1* mutant (Figure 4C), revealing that CycC1;1 disrupted RNAP II recruitment to *ABI5* in planta. We speculated that this enhanced association of RNAP II with *ABI5* promoter in *cycc1;1* mutant likely leads to increased expression of *ABI5*. To test this hypothesis, we carried out dual-reporter gene assay in *N. benthamiana* leaves by expressing *LUC* driven by *ABI5* promoter in the presence or absence of CycC1;1 or CycC1;2 (Figure 4D). The result showed that LUC expression under the control of *ABI5* promoter was significantly repressed by the co-expression of either CycC1;1 or CycC1;2 (Figure 4E). These data indicate that CycC1;1 and CycC1;2 can associate with *ABI5* promoter to reduce RNAP II recruitment, thereby repressing *ABI5* transcription.

Consistent with the above results, the RT-qPCR data showed that *ABI5* transcript levels in ABA-treated *cycc1;1* mutant and *CycC1;1*-overexpressing plants were significantly higher and lower than that in the treated WT, respectively (Figure 5A). In addition, GUS reporter activity under the control of *ABI5* promoter was also monitored in the WT, *cycc1;1* and *CycC1;1*-overexpressing background plants. We found that ABA-induced GUS activity in the germinating seeds and seedlings of the WT was strongly increased in the *cycc1;1* mutant but extensively decreased in the *CycC1;1*-overexpressing plants as evidenced by the GUS staining experiment (Figure 5, B and C). Furthermore, ABI5 protein abundance was increased in the *cycc1;1* mutant but decreased in the *CycC1;1*-overexpressing plants, respectively, compared with the WT when treated with ABA (Figure 5D).

To examine the effect of CycC1;1-repressed *ABI5* transcription on the activation of ABI5-targeted genes, intact *ABI5* genomic DNA *ABI5::ABI5g-Ter* including promoter, coding

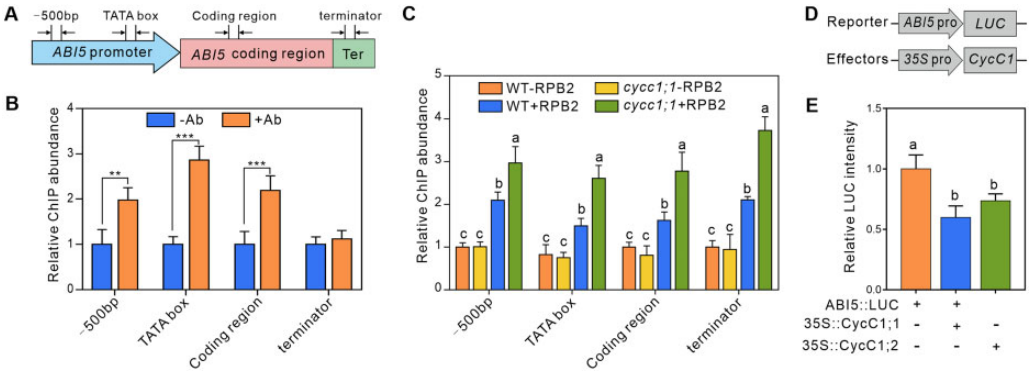

Figure 4 CycC1;1 decreases *ABI5* transcription by interfering RNAP II association with *ABI5* promoter. A, A diagram showing the positions of *ABI5* gene primers used for ChIP-qPCR. B, CycC1;1 associates with the *ABI5* promoter. Chromatin was extracted from 7-day-old *CycC1;1*-overexpression (*35S::GFP-CycC1;1*) lines and then precipitated with anti-GFP antibody (+ Ab) or only IgG (−Ab). Error bars indicate mean ± SD ($n = 3$). Asterisks indicate significant differences determined by Student's t test (**$P < 0.01$ and ***$P < 0.001$). C, CycC1;1 reduces RNAP II association to the *ABI5* promoter. Chromatin was extracted from 7-day-old WT and *cycc1;1* mutant seedlings and precipitated with anti-RPB2 antibody raised against the C-terminal domain of RNAP II (+ RPB2) or only IgG (−RPB2). Error bars indicate mean ± SD ($n = 3$). D and E, Dual-LUC reporter gene assay to examine the effects of CycC1;1 and CycC1;2 on *ABI5* expression. LUC under the control of *ABI5* promoter was co-expressed with CycC1;1 or CycC1;2 in *N. benthamiana* leaves. The activity of *ABI5::LUC* alone was set to 1. Error bars indicate mean ± SD ($n = 3$). Different letters indicate significant differences as determined using one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

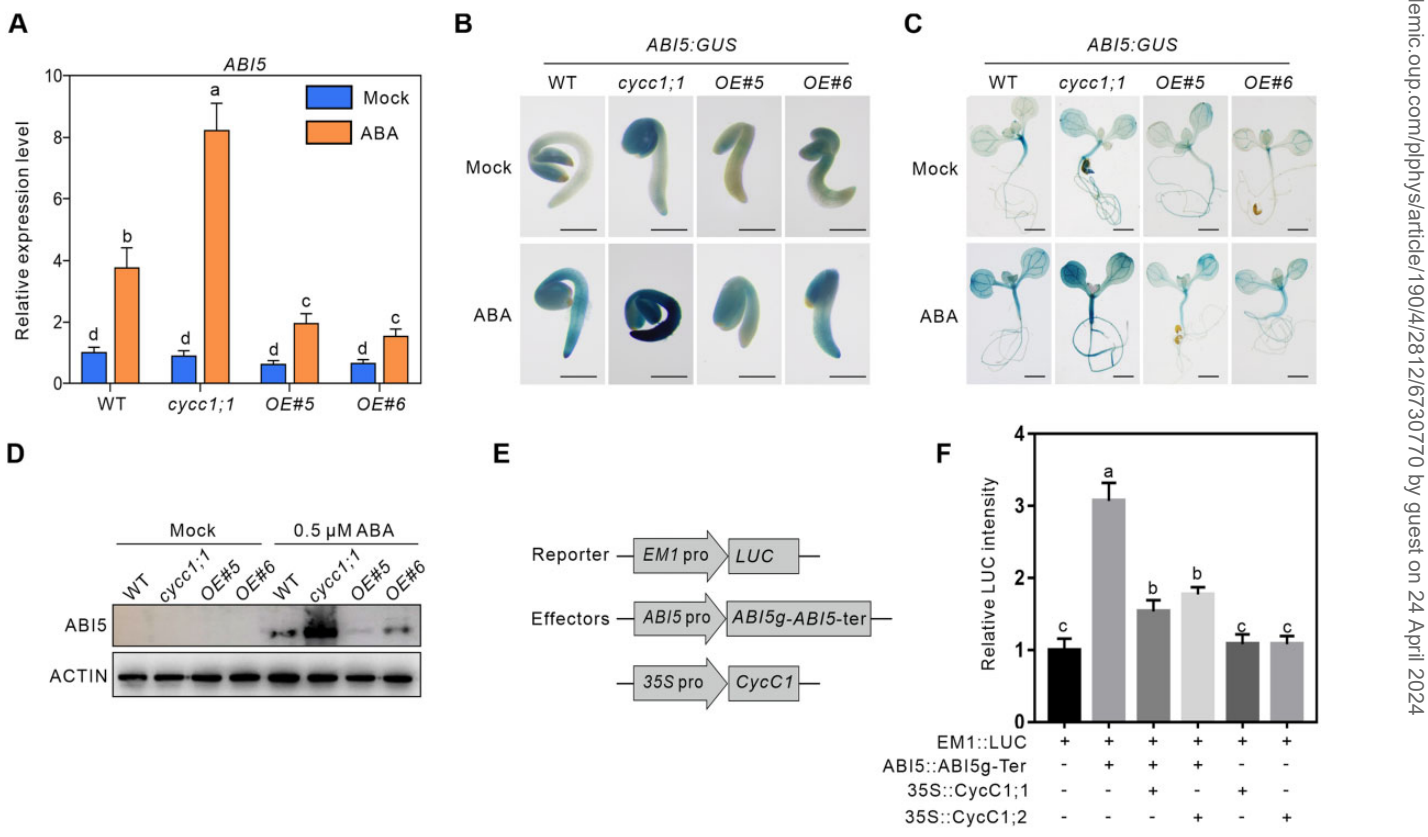

Figure 5 CycC1;1 represses ABI5 expression during seed germination. A, The expression of *ABI5* in germinating seeds of the WT, *cycc1;1*, and *CycC1;1-OE* lines. Seeds were treated with MS medium or MS medium containing 10 μM ABA for 24 h. Error bars indicate mean ± SD ($n = 3$). B and C, GUS staining of germinating seeds (B) and seedlings (C) of *ABI5::GUS* transgenic line in the WT, *cycc1;1* and *CycC1;1-OE* background plants. One-day-old and 7-day-old seedlings grown on MS medium were transferred to MS medium containing 10 μM ABA for 24 h, and then subjected to GUS staining. Bar, 0.2 cm. D, ABI5 protein abundance in the seeds of WT, *cycc1;1* and *CycC1;1-OE* lines. Seeds were treated with MS medium without or with 10 μM ABA for 24 h. The anti-ABI5 antibody was used in the immune analysis. E and F, Dual-LUC reporter gene assay to examine the effects of ABI5 and CycC1;1 on *EM1* expression. The intact *ABI5* genomic DNA containing its promoter, coding region, and terminator was cloned into a binary vector to transiently express ABI5 protein in *N. benthamiana* leaves. The activity of *EM1::LUC* alone was set to 1. Error bars indicate mean ± SD ($n = 3$). Different letters indicate significant differences as determined using one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

region, and terminator was constructed and employed as an effector, and *EM1::LUC* was used as a reporter (Figure 5, E and F). The dual-reporter gene assay results showed that expression of *ABI5* but not *CycC1;1* alone could significantly stimulate LUC activity while when *ABI5* and *CycC1;1* are co-expressed, ABI5-increased LUC activity was remarkably suppressed (Figure 5F). Such effects of *CycC1;1* on inhibiting ABI5-promoted *EM1* expression were also observed when *CycC1;2* was co-expressed (Figure 5F). These results indicate that CycC1;1 and CycC1;2 negatively regulate ABI5 activity at the transcription level, thus down-regulating the expression of ABI5-targeted genes.

ABA-induced phosphorylation of ABI5 at Ser42 dampens its interaction with CycC1;1

We have demonstrated that CycC1;1 negatively regulates ABI5 activity through physical interaction, whereas whether and how ABA affects their interaction remains elusive. We carried out split-LUC experiments by expressing both cLUC-CycC1;1 and nLUC-ABI5 in *N. benthamiana* leaves. Following treatment with different concentrations of ABA, LUC activity was monitored, and we found that the LUC signal intensity was extensively suppressed by the ABA treatments compared with the untreated control in the *N. benthamiana* leaves (Figure 6A), revealing ABA reduced the interaction between CycC1;1 and ABI5. This was confirmed by the co-immunoprecipitation (Co-IP) assay where untreated and ABA-treated *35S::ABI5-Myc* seedlings were employed to the isolate the CycC1;1-interacting proteins using anti-CycC1;1 antibody (Supplemental Figure S2D) and then Myc-tagged ABI5 protein was analyzed by anti-Myc antibody in the precipitants, and our result showed that the precipitated ABI5-Myc protein was less in the ABA-treated seedlings than the untreated control (Figure 6B). These results imply that ABA could reduce CycC1;1 interaction with ABI5 in planta. If this is really the case in plants, association of CycC1;1 on ABI5-targeted genes could be also suppressed by ABA. Previous reports documented that ABI5 binds to the ABRE motif containing *ACGTG* in the *EM1* or *EM6* promoter. To test this hypothesis, we performed ChIP-qPCR by analyzing the enrichment of CycC1;1 on these ABRE sites in *EM1* and *EM6* promoters. Without ABA treatment, CycC1;1 was indeed significantly associated with *EM1* or *EM6* promoter DNA, while ABA treatment remarkably inhibited such association (Figure 6, C–F), further supporting that ABA could dampen CycC1;1 interaction with ABI5 in plants.

To explore how ABA affects CycC1;1 interaction with ABI5, we mapped CycC1;1-interacting domain of ABI5 protein by Y2H. Based on the previous functional analysis of ABI5 protein structure, ABI5 has four conserved regions (C1, C2, C3, and C4) and a bZIP DNA binding domain (BD) (Bensmihen et al., 2002; Chen et al., 2012), thus we generated several truncated forms of ABI5 (Figure 7A). The Y2H results showed that only the C1 domain or the truncated C1C2C3 fragment covering the C1 region had interaction with CycC1;1, while the truncated fragments without C1 region including bZIPC4 and C2C3bZIPC4 did not (Figure 7B), suggesting that the C1 region of ABI5 is the major site for CycC1;1 interaction. To verify this, we purified GST-tagged full-length and truncated ABI5 proteins expressed in *E. coli*, and incubated them with 6 × His-tagged CycC1;1 protein for in vitro GST pull-down assay. Our result showed that His-CycC1;1 could be specifically pulled down by intact or truncated ABI5 proteins that contains the C1 region including C1 and C1C2C3, but not by the truncated forms without C1 region including bZIPC4 or C2C3bZIPC4 (Figure 7C), further supporting that CycC1;1 interacts with the C1 region of ABI5.

Previous reports demonstrate that phosphorylation of ABI5 plays a crucial role in plant response to ABA, and Ser-42, Ser-145, and Thr-201 are the major phosphorylation sites substantially affecting ABI5 protein activity (Wang et al., 2013; Zhou et al., 2015). Considering that ABI5 C1 region that interacts with CycC1;1 contains the Ser-42, we herein asked if phosphorylation of Ser-42 influences its interaction with CycC1;1. For this end, we mutated Ser-42 of ABI5 as alanine (Ala, A) or aspartic acid (Asp, D) to mimic the dephosphorylated or phosphorylated form, respectively, and tested their interaction with CycC1;1. Our Y2H experiment showed that, similarly to ABI5, ABI5-S42A, the phospho-dead form of ABI5 at Ser-42, could still interact with CycC1;1 (Figure 7D). Strikingly, ABI5-S42D, the phospho-mimic form of ABI5 at Ser-42, lost its interaction with CycC1;1 (Figure 7D), suggesting that phosphorylation of ABI5 at Ser-42 deprives its interaction with CycC1;1. This note was reinforced by the BiFC experiment, where ABI5 and ABI5-S42A but not ABI5-S42D could interact with CycC1;1 in *N. benthamiana* leaves (Figure 7E). In addition, phospho-mimic and phospho-dead forms of ABI5 at Ser-145 and Thr-201 were also produced to test the changes of their interaction with CycC1;1. Consistent with the interaction analysis, mutations of Ser-145 and Thr-201 in ABI5 as Ala or Asp did not affect ABI5 interaction with CycC1;1 (Figure 7D) as the two sites are localized in the C2 and C3 domains, respectively.

Further, we performed dual-reporter gene assay in *N. benthamiana* leaves to examine the effect of phospho-dead ABI5-S42A and phospho-mimic ABI5-S42D on the transactivation of *EM1* in the presence or absence of CycC1;1. Our results showed that when *EM1::LUC* was used as a reporter, CycC1;1 significantly suppressed ABI5- and ABI5-S42A-promoted LUC activity, but ABI5-S42D-activated LUC was not significantly impacted by CycC1;1 (Figure 7F), suggesting that CycC1;1 interferes ABI5 transcriptional activity through interacting with its un-phosphorylated form, whereas phosphorylation of Ser-42 blocks CycC1;1 interaction with and inhibition of ABI5. These results imply that ABI5 activity is interfered by CycC1;1 through their physical interaction while ABA-induced Ser-42 phosphorylation relieves their interaction, thereby promoting ABI5 activity and the

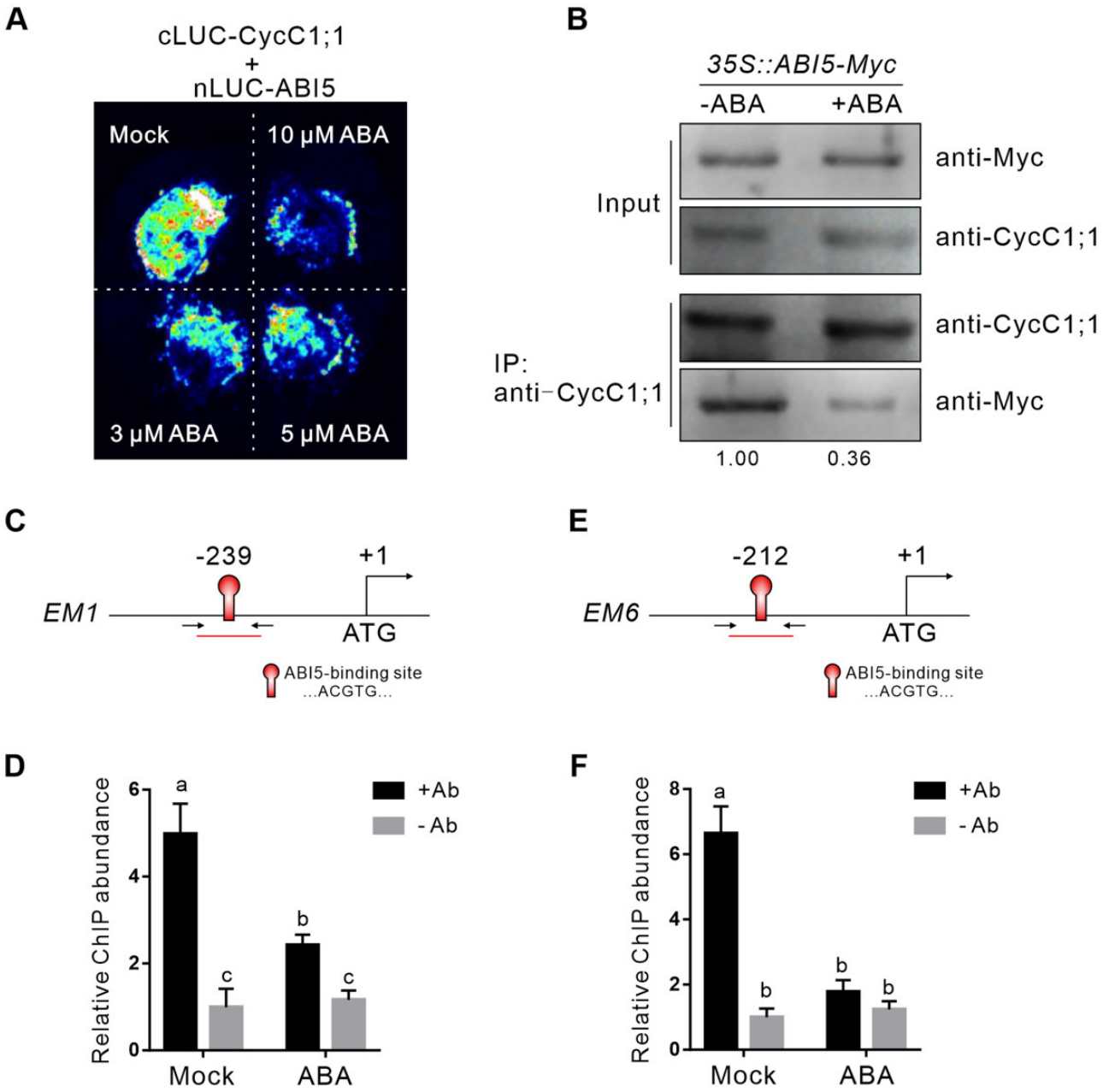

Figure 6 ABA reduces CycC1;1 interaction with ABI5 and association with the promoters of *EM1* and *EM6*. A, Split LUC assay to examine the interaction between CycC1;1 and ABI5 in the absence or presence of different concentrations of ABA. nLUC-ABI5 and cLUC-CycC1;1 constructs were transiently expressed in *N. benthamiana* leaves for 2 days, and then different concentrations of ABA solution were injected into the leaves. Following the injection for 1 day, the LUC images were captured. B, Co-IP to assay the effect of ABA on the interaction between CycC1;1 and ABI5 in Arabidopsis. Seven-day-old *35S::ABI5-Myc* transgenic seedlings treated with or without 10 μM ABA for 12 h were employed to isolate the total proteins, and then proteins were immunoprecipitated by the anti-CycC1;1 antibody. After immunoprecipitation, anti-Myc antibody was used to examine the ABI5-Myc protein in the precipitate. C–F, ChIP-qPCR to assay the association of CycC1;1 with the ABI5-binding sites on the *EM1* or *EM6* promoters. Diagrams showing the positions of ABI5-binding sites on the *EM1* and *EM6* promoters and the primers used for the ChIP-qPCR (C and E). Chromatin was extracted from 7-day-old *35S::GFP-CycC1;1* seedlings treated with or without 10 μM ABA for 12 h, and then precipitated with anti-GFP antibody (+ Ab) or only IgG (−Ab). Error bars indicate mean ± SD (n = 3). Different letters indicate significant differences as determined using two-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

expression of ABI5-targeted genes. In addition to the changes of CycC1;1 interaction with ABI5 caused by ABA-mediated ABI5 phosphorylation, whether ABA modulates CycC1;1 expression remains unclear. Therefore, we performed RT-qPCR experiment to determine the expression of *CycC1;1* and *CycC1;2* in ABA-treated WT plant seedlings,

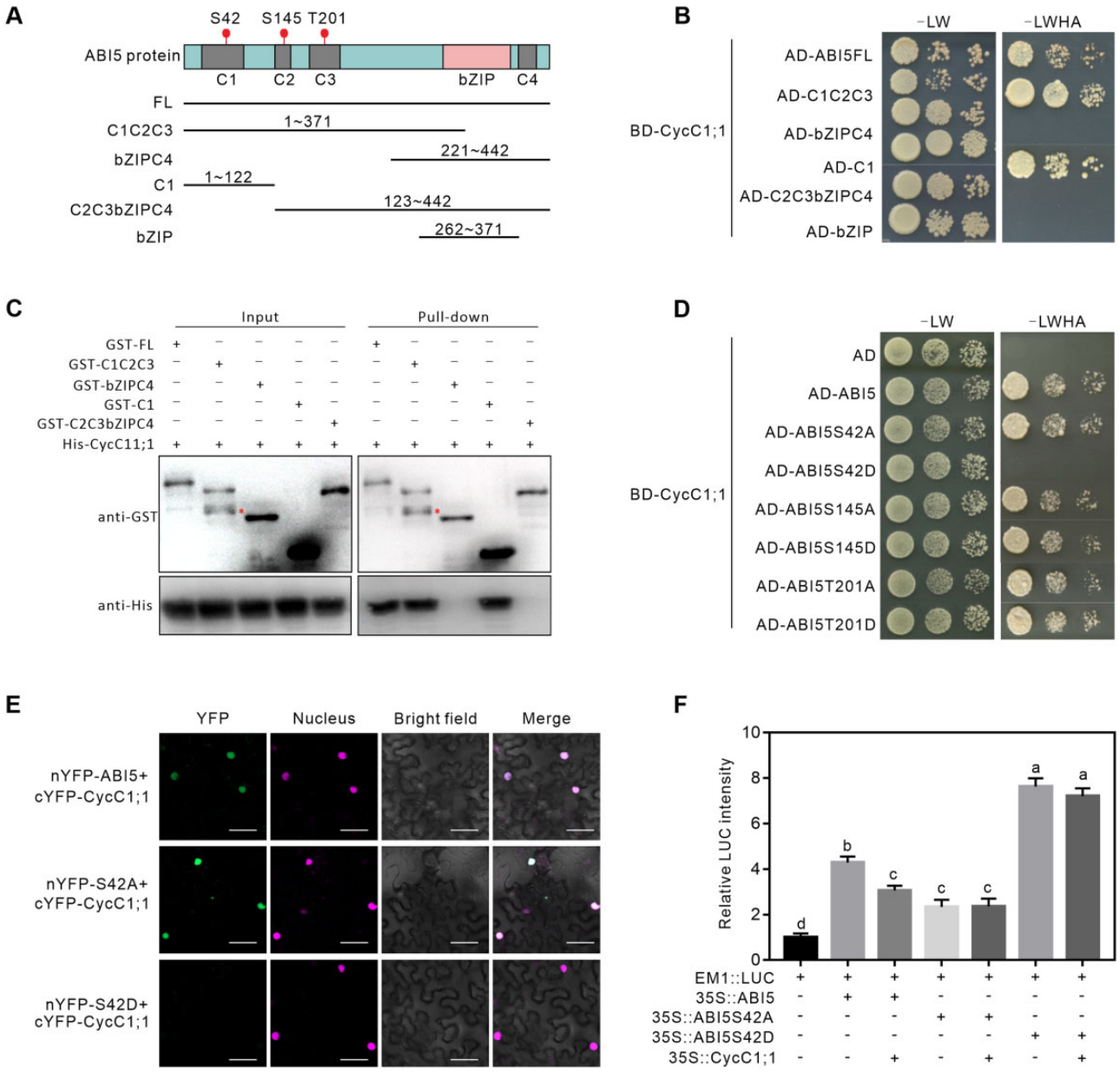

Figure 7 ABI5 Ser-42 phosphoamino acid affects ABI5 interaction with CycC1;1. A and B, Identification of the CycC1;1-interacting domain of ABI5 via Y2H assay. The diagram in the box (A) indicates the conserved domains in ABI5 and the truncated fragments tested in the assays. The full-length and truncated ABI5 proteins were fused to the AD of GAL4, and CycC1;1 was fused to the BD of GAL4. Protein interactions were examined based on the growth of yeast cells on selective media (B). C, GST pull-down assay to analyze the CycC1;1-interacting domain of ABI5. 6 × His-CycC1;1 were mixed with GST-tagged full-length or truncated ABI5 proteins and then immobilized on glutathione sepharose beads. After washing, the eluted proteins were subjected to immunoblot analysis with anti-GST and anti-His antibodies, respectively. D, Y2H assay to analyze the interaction between CycC1;1 and phospho-mimic or phospho-dead forms of ABI5 at the Ser42, Ser145, and Thr201 sites. E, BiFC analysis to examine the interaction between CycC1;1 and ABI5, ABI5S42A or ABI5S42D in *N. benthamiana* leaves. Scale bars = 20 μm. F, Dual-LUC reporter gene assay to examine the effects of ABI5, ABI5S42A, or ABI5S42D on *EM1* expression in the presence or absence of CycC1;1. The activity of EM1::LUC alone was set to 1. Error bars indicate mean ± SD (n = 3). Different letters indicate significant differences as determined using one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

and our result showed that the transcripts of *CycC1;1* and *CycC1;2* in ABA-treated plants were less than that in the untreated control (Supplemental Figure S4), indicating that ABA suppresses the expression of both *CycC1;1* and *CycC1;2* in plants for the further activation of ABI5 activity.

Discussion

Seed dormancy and germination are important physiological processes for angiosperms species, and crucial to plant survival and propagation. Long-term evolution enables plants to precisely determine whether seed germination starts or

not by sensing the changes of external environments. ABA is one of the most important phytohormones inhibiting seed germination. ABI5 functions as a central transcription factor in ABA signaling, facilitates plant versatile ABA responses, including inhibition of seed germination and repression of postgerminative seedling growth. It has now been widely accepted that ABI5 is crucial to regulate the expression of ABA-responsive genes, but how plants precisely regulate the transcriptional activation activity of ABI5 for the downstream genes especially during seed germination remains to be further elucidated. Here, we provide extensive evidence demonstrating that CycC1;1 functions in ABA-inhibited seed germination and cotyledon greening processes by interacting with and inhibiting ABI5. CycC1;1 interacts with ABI5 in a de-phosphorylated form of Ser-42 to occupy the promoters of ABI5-targeted genes, thus interfering ABI5 transcriptional activation effects for the downstream target genes including *ABI5* itself under low ABA conditions; when ABA level is increased in plants, phosphorylation of ABI5 at Ser-42 relieves CycC1;1 repression of ABI5 by dampening their physical interaction, thereby promoting the expression of ABA response and inhibiting seed germination. These results illustrate a CycC1;1-involved regulatory network that precisely regulates plant ABI5 activity and expression at multiple layers, and also provide insights into the understanding of ABA–ABI5 module-mediated regulation of seed germination (Figure 8).

In this study, we identified and employed two previously-reported T-DNA insertion lines, SALK_053291 and SALK_039400 (Zhu et al., 2014; Chen et al., 2019), where the T-DNA is inserted in the second intron of *CycC1;1* and the intergenic region between *CycC1;2* and *CycC1;1*, resulting in reduced expression of *CycC1;1* alone or both genes, respectively (Figure 2, A, B, and F), and our results showed that both the mutants are hypersensitive to ABA in terms of seed germination and cotyledon greening rate. Although we could not obtain the T-DNA insertion mutant that only affects the expression of *CycC1;2*, the ABA insensitive phenotype of the *CycC1;2*-overexpressing lines (Figure 2, G–I) and the inhibitory effects of CycC1;2 on ABI5 transactivation of targeted genes (Figures 3, A and B, 4F, and 6, D and E) also clearly support that *CycC1;2* negatively regulates ABA-inhibited seed germination through interfering ABI5 activity via protein physical interaction in a similar way to *CycC1;1*.

Mediator is a large protein complex comprised with multiple subunits, and CycC1;1/CycC1;2 together with MED12, MED13, and CDK8 belong to the kinase module that can be associated with or separated away from the core part of Mediator. Our study showed that CycC1;1 can physically interact with ABI5 whereas CDK8 has no interaction with ABI5, suggesting that CycC1;1 possibly modulates ABI5 activity for ABA response in a CDK8-independent manner. This hypothesis is at least partially supported by the findings that *cdk8* mutant is insensitive to ABA as reported by a previous study (Zhu et al., 2020) while *cycc1;1* mutant is hypersensitive to ABA as shown in our study (Figure 2). In addition to the regulation of plant ABA response, CycC1;1 and CDK8 also have complicated relationships in different biological processes, including growth, development, and stress responses. For example, it has been reported that the *ckd8* and *cycc1;1/1;2* mutants exhibits similar susceptibility to necrotrophic pathogen *Alternaria brassicicola* but different resistance against another necrotrophic pathogen *Botrytis cinerea* (Zhu et al., 2014), suggesting that CDK8 and CycC have both convergent and divergent functions in plant response to different pathogens infection. Interestingly, CDK8 and CycC also elicit similar roles in the regulation of plant flowering as a recent study reported that both the mutants of *CDK8* and *CycC* showed delayed flowering and prolonged reproductive phase in pea (*Pisum sativum* L.) (Hasan et al., 2020). Based on these reports and our findings, we infer that CycC regulates plant growth, development, and environmental responses in a CDK8-dependent and CDK8-independent manners. Identification of more CDK8- and CycC-implicated biological processes and unraveling the underlying mechanisms will shed more light on how CDK8 and CycC work together or separately in plants.

In addition to the kinase module, the core module of Mediator complex also functions in plant response to ABA. For instance, MED25, a subunit of Mediator tail module, can also interact with ABI5 and negatively regulate the transcriptional activation activity of ABI5 for the downstream target genes and *ABI5* itself (Chen et al., 2012), which is very similar to the role of CycC1;1 in the regulation of ABI5 for plant ABA response. However, we have noticed that MED25 interacts with the C3 domain of ABI5 (Chen et al., 2012) but CycC1;1 interacts with its C1 domain (Figure 7, A–C). Also, phospho-mimic mutation of Ser-42 with C1 domain of ABI5 blocks its interaction with CycC1;1, whereas phospho-mimic mutation of Thr-201 within C3 domain of ABI5 did not alter its interaction with CycC1;1 (Figure 7, D–E), further suggesting distinct mechanisms of CycC1;1 and MED25 in the regulation of ABI5 activity through their interaction during plant response to different doses of ABA. From this point of view, CycC1;1 likely plays its role in the regulation of ABI5 activity for ABA response independently of MED25.

Interestingly, ABI5 can stimulate expression of its own gene through binding to the ABRE sites on its own gene promoter, forming a positive feedback regulatory loop in ABA-ABI5 module-regulated processes (Xu et al., 2014; Bai et al., 2019). It is noteworthy that the expression of *LUC* driven by *ABI5* native promoter in *N. benthamiana* leaves could be suppressed by CycC1;1 (Figure 4, D and E), and the ChIP-qPCR experiments demonstrated that CycC1;1 could mainly associate with *ABI5* promoter regions to restrict recruitment of RNAP II (Figure 4, A–C). Therefore, we infer that CycC1;1 as a subunit of Mediator complex interacts with ABI5 to form a repressor complex to repress *ABI5* expression by associating with ABRE site on *ABI5* promoter. The observation of higher *ABI5* expression in the *cycc1;1* mutant than the WT by the RT-qPCR, GUS staining, and immunoblotting analysis (Figure 5, A–D) can also be ascribed to more RANP II recruitment to *ABI5* promoter caused by the absence of CycC1;1 in

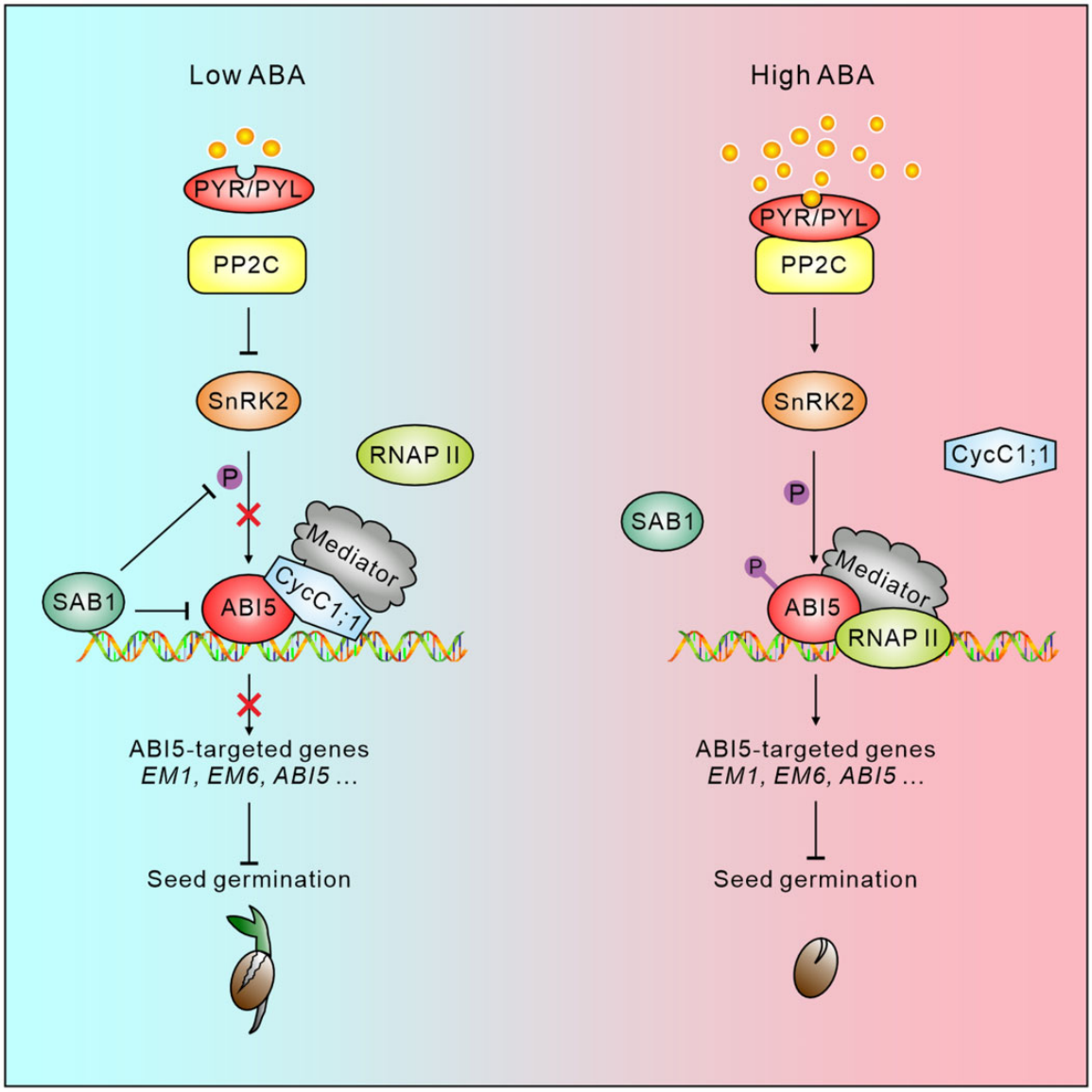

Figure 8 A simplified model showing the role of CycC1;1 in ABA-inhibited seed germination by interacting with and modulating ABI5. Under low ABA conditions, CycC1;1, as a subunit of the Mediator complex interacts with de-phosphorylated ABI5 to form a transcriptional repressor complex and occupy the promoters of ABI5-targeted downstream genes including *ABI5* itself, therefore interfering the RNAP II recruitment and suppressing the transcriptional activation activity of ABI5 for the expression of *ABI5* and ABI5 downstream genes; Under high ABA conditions, ABA-induced phosphorylation of ABI5 at Ser-42 reduces CycC1;1 interaction with ABI5 to relieve its repression of ABI5 transcriptional activation activity, and thus the expression of *ABI5* and ABI5 downstream genes was promoted and seed germination was inhibited.

the *cycc1;1* mutant. Similarly, CycC1;1 can also associate to the ABRE sites of other ABI5-targeted genes promoter including *EM1* and *EM6* through interacting with ABI5 (Figure 6, C–F), which might disrupt the RNAP II recruitment to these genes and thus repressing their transcription. In addition, ABA treatment strongly repressed CycC1;1 interaction with ABI5 and CycC1;1 association with *EM1* and *EM6* promoters at the ABRE sites (Figure 6, C–F). Different from those previously reported transcription factors regulating *ABI5* expression or interacting partners affecting ABI5 activity, our results reveal a previously unidentified mechanism of ABA-mediated ABI5-activated expression of *EM1* and *EM6*, where CycC1;1 interacts with ABI5 to form a transcriptional repressor to modulate ABA signaling during seed germination while the effects of such complex are dynamically modulated by ABA, conferring plants flexible adaptive responses to the internal and external cues. From another point of view, it is worthy of further explorations to study whether and how CycC1;1 modulates plant growth and development because the trade-off between plant growth and stress response is tightly modulated in plants under changing environments (Liu et al., 2022a).

Structural analysis of ABI5 reveals that ABI5 contains four conserved regions and a bZIP domain for DNA binding. We identified that the C1 region of ABI5 provides the interface for CycC1;1 physical interaction (Figure 4, A–C). Further

study found that CycC1;1 could interact with dephosphorylated or phospho-dead ABI5 at Ser-42, but phospho-mimicking Asp-42 destroyed its interaction with CycC1;1 (Figure 4, D and E). Considering that Ser-42 is one of the major phosphorylation sites mediated by SnRK2s and that SnRK2 kinase activity is activated by increased ABA accumulation in plants (Wang et al., 2013; Zhou et al., 2015), we speculated that changes of phosphorylation status of Ser-42 in ABI5 caused by ABA/SnRK2s act as a molecular switch for its interaction with CycC1;1 and subsequent ABI5 activation. In addition to Ser-42, Ser-145, and Thr-201 of ABI5 can also be phosphorylated by SnRK2s (Lopez-Molina et al., 2002; Wang et al., 2013; Zhou et al., 2015), whereas neither phospho-dead nor phospho-mimic mutations of these two amino acids affect ABI5 interaction with CycC1;1 (Figure 4D), which is due to that the C2C3 regions of ABI5 covering Ser-145 and Thr-201 had no interaction with CycC1;1 (Figure 4, A–C). We have noticed that Ser-145 is the major site for its interaction with SAB1, a previously reported negative regulator of ABI5 stability and transcriptional binding activity (Ji et al., 2019). The different interaction sites of ABI5 for CycC1;1 an SAB1 suggest distinct regulatory mechanisms of CycC1;1 and SAB1 on ABI5 activity in ABA-mediated seed germination. Apart from post-translational regulation by CycC1;1 and SAB1 on ABI5 activity, they also negatively regulate *ABI5* expression at the transcription level with contrasting mechanisms: SAB1 is an RCC1 family protein and downregulates *ABI5* expression by increasing the level of histone H3K27me2 in the *ABI5* promoter (Ji et al., 2019), while CycC1;1 as a subunit of the Mediator complex reduces *ABI5* transcription by disrupting RNAP II association with its genomic DNA. These multiple layers of regulation on ABI5 mediated by CycC1;1 as well as other factors should enable plants sophisticated and accurate responses to the external surroundings during seed germination and seedling establishment.

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 *cycc1;1/1;2* (SALK_039400) mutants were obtained from the Arabidopsis Biological Resource Center. The *abi5-7* mutant (Zhao et al., 2020) and *ABI5::GUS* transgenic line were used in our study. The *cycc1;1 abi5-7* double mutant and *ABI5::GUS* line in the *cycc1;1* mutant and *CycC1;1*-overexpressing background plants were obtained by genetic cross. Plants were grown at 22°C under a 16-/8-h light/dark photoperiod at 100 μmol m^{-2} s^{-1} on a half-strength Murashige and Skoog (0.5 MS) medium containing 0.8% (w/v) agar.

To generate transgenic overexpression plants, the full-length CDSs of *CycC1;1* and *CycC1;2* cloned into the pEGAD and pCambia1300 vectors at the *BamH*I and *Sal*I sites, respectively, under the control of the cauliflower mosaic virus *35S* promoter. The constructs were used to transform the WT Arabidopsis plants by *Agrobacterium tumefaciens*-mediated transformation. The expression of *CycC1;1* and *CycC1;2* of the transgenic plants was examined by RT-qPCR. T4 homozygous transgenic lines were used for subsequent analysis. The primers used are listed in Supplemental Table S1.

To generate the complementation (Com) lines of the *cycc1;1* mutant, the genomic sequence of 1,030 bp upstream of *CycC1;1* translation start codon (ATG) and the full length CDS of *CycC1;1* were amplified, and cloned into pCambia1381 vector at the *Pst*I and *Hind*III sites, respectively. The resulting plasmid was introduced into the *cycc1;1* mutant via *A. tumefaciens*-mediated floral transformation. Com T4 transgenic plants were used for phenotypic analysis.

Seed germination assays

Mature Arabidopsis seeds were harvested and dried at room temperature for 3 weeks, and then used for the germination assays. Sterilized seeds were plated on 0.5 MS medium without or with the indicated concentration of ABA (Sigma, St. Louis, Missouri, USA). Following stratification at 4°C for 3 days in the dark, the plates were transferred to a growth chamber with a 16-h light/8-h dark cycle at 23°C. Seeds were identified as germinated when a radicle had emerged from the seed coat (Nie et al., 2022).

RT-qPCR analysis

Treated or untreated germinating seeds or seedlings were collected for total RNA isolation, first-strand cDNA synthesis, and RT-qPCR as we described previously (Wang et al., 2021). The constitutively expressed *ACTIN2* gene was used as an internal control. The primers used for RT-qPCR are listed in Supplemental Table S1.

Y2H assay

The full-length CDS of *CycC1;1* or *CycC1;2* was cloned into pGBKT7 vector. The full-length and truncated forms of *ABI5* were cloned into pGBKT7 vector. The yeast transformation and growth were carried out with the Matchmaker system (Clontech, Mountain View, California, USA). Yeast cells were selected on double dropout medium lacking Leu and Trp (-LW) to select transformants, triple dropout medium lacking Leu, Trp, and His (-LWH) or quadruple dropout medium lacking Leu, Trp, His, and Ade (-LWHA) to analyze protein interactions. Primer sequences are listed in Supplemental Table S1.

LCI assay

The CDS of *ABI5* and *CycC1;1* or *CycC1;2* were cloned into the JW772 or JW771 vector containing the N-terminal of LUC (nLUC) and or the C-terminal of LUC (cLUC) at the *BamH*I and *Sal*I sites. The resultant plasmids were transformed into *A. tumefaciens* and used to infiltrate *N. benthamiana* leaves. Three days after infiltration, the leaves were incubated in PBS solution containing 150 μg/mL D-Luciferin potassium salt in the dark for 10 min before luminescence assay. The LUC image was captured by NightSHADE LB 985 according to the manufactory's instructions. Primer sequences are listed in Supplemental Table S1.

BiFC assays

The CDS of *ABI5* and *CycC1;1* or *CycC1;2* were cloned into the pSPYNE or pSPYCE vector containing the N-terminal of YFP (nYFP) and or the C-terminal of YFP (cYFP), respectively. nYFP-ABI5 and cYFP-CycC1;1 or cYFP-CycC1;2 were co-expressed in *N. benthamiana* leaves. YFP fluorescence (excitation 488 nm, emission 543 nm) was detected under a Zeiss LSM980 laser scanning confocal microscope. H2B-mCherry (Rosa et al., 2014) was used as a nuclear marker, and the red fluorescence (excitation 561 nm, emission 600–630 nm) was also detected by Zeiss LSM980 laser scanning confocal microscope. Primer sequences are listed in Supplemental Table S1.

Dual-LUC reporter gene assay

The dual-LUC reporter gene assay was performed according to the previous reports (Liu et al., 2022b). The plasmid containing the *EM1* promoter upstream of the *LUC* gene was used as the reporter, and the REN under the control of *35S* promoter was used as the internal control. The reporter and internal control were transiently co-expressed with pCambia1307-ABI5, pEGAD-CycC1;1, or pCambia1300-CycC1;2 in *N. benthamiana* leaves, and then the activities of LUC and REN were detected.

To assay the role of CycC1;1 or CycC1;2 in the regulation of *ABI5* expression, the genomic DNA sequence of 2,024 bp upstream of *ABI5* translation start codon (ATG) was cloned into pGreenII0800-LUC vector at the *BamH*I site, resulting in the reporter ABI5::LUC. The reporter and internal control were transiently co-expressed with pEGAD-*CycC1;1* or pCambia1300-*CycC1;2* in *N. benthamiana* leaves, and the activities of LUC and REN were then detected. Primer sequences are listed in Supplemental Table S1.

To test the effects of CycC1;1 and CycC1;2 on ABI5-activated *EM1* expression using *ABI5* genomic DNA sequence, the genomic sequence of 2,024 bp upstream of *ABI5* translation start codon (ATG) was cloned into pCambia1381 vector at the *EcoR*I site, and then the DNA sequence from the translation start codon (ATG) to the end of terminator of *ABI5* was cloned into the above plasmid at the *Hind*III site, resulting in ABI5pro::ABI5g-Ter. The reporter and internal control were co-expressed with ABI5pro::ABI5g-Ter, pEGAD-*CycC1;1* or pCambia1300-*CycC1;2* in *N. benthamiana* leaves, and then the activities of LUC and REN were detected. The primers used for these plasmids construction are listed in Supplemental Table S1.

Co-IP assays

The Co-IP experiment was performed according to our previously reported methods with some modifications (Liu et al., 2022a; Zhang et al., 2022a). Total proteins were isolated from 7-day-old *35S::ABI5-Myc* transgenic seedlings (Zhang et al., 2022b) treated with or without 10 μM ABA for 12 h, and then proteins were immunoprecipitated by the anti-CycC1;1 antibody. After immunoprecipitation, the precipitated proteins were separated on SDS-PAGE gel, and ABI5-Myc protein was immunoblotted by anti-Myc antibody (#AE060, ABclonal, China). The anti-CycC1;1 polyclonal antibody was generated in GenScript (Nanjing, China) using the peptide (VDVVHDLDKERGISC) specific for CycC1;1 protein, and verified by immunoblot analysis using the proteins isolated from the WT and *cycc1;1* mutant seedlings.

ChIP-qPCR analysis

To assay whether CycC1;1 was associated with *ABI5* genomic DNA, *35S::GFP-CycC1;1* transgenic lines was were used for ChIP analysis according to previously reported method (Zhu et al., 2014). In brief, chromatin was isolated from 7-day-old *35S::GFP-CycC1;1* transgenic plant seedlings. Monoclonal anti-GFP antibody (#AE064; ABclonal, Wuhan, China) was used for protein immunoprecipitation. DNA fragments in both input and immunoprecipitated samples were quantified by qPCR.

To examine the effect of CycC1;1 on RNAP II recruitment to the *ABI5* promoter, Chromatin was extracted from 7-day-old WT and *cycc1;1* mutant seedlings and precipitated with anti-RPB2 antibody (ABclonal) raised against the C-terminal domain of RNAP II. DNA fragments in both input and immunoprecipitated samples were quantified by qPCR. The *ACTIN7* was used as a reference genes. At least three independent experiments were performed. The primers used for ChIP-qPCR are listed in Supplemental Table S1.

GST pull-down assay

The CDS of full-length or truncated *ABI5* and *CycC1;1* or *CycC1;2* was in cloned into the pGEX4t-1 and pET28a vectors at the *Sma*I and *BamH*I sites, respectively, and the GST-tagged ABI5 or truncated ABI5 and 6 × His-CycC1;1 were purified from *E. coli*. 6 × His-CycC1;1 was mixed with GST alone, GST-tagged ABI5, or truncated ABI5 on ice for 1 h. The protein mixture was then incubated with glutathione sepharose beads at 4°C for 3 h. After washing, the eluted proteins with Elution Buffer (10 mM GSH in 50 mM Tris–HCl, pH 8.0) were detected with anti-His (#E12-004-3; EnoGene) and anti-GST (#E12-007; EnoGene) antibodies, respectively. Primer sequences are listed in Supplemental Table S1.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers_CycC1;1 (AT5G48640), CycC1;2 (AT5G48630), ABI5 (AT2G36270), EM1 (AT3G51810), EM6 (AT2G40170), RD29A (AT5G52310), ABF3 (AT4G34000), and CDK8 (AT5G63610).

Supplemental data

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

Supplemental Figure S1. The interaction of CycC1;2 with ABI5 and CDK8 with ABI5 in yeast cells.

Supplemental Figure S2. CycC1;1 suppresses ABA-inhibited seed germination through ABI5.

Supplemental Figure S3. Analysis of the ABA sensitivity of the *cycc1;1/1;2* mutant and *CycC1;2*-overexpression lines.

Supplemental Figure S4. ABA represses the expression of CycC1;1 and CycC1;2.

Supplemental Table S1. List of the primers used in this study.

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