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The DELLA-ABI4-HY5 module integrates light and gibberellin signals to regulate hypocotyl elongation

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ABSTRACT

Plant growth is coordinately controlled by various environmental and hormonal signals, of which light and gibberellin (GA) signals are two critical factors with opposite effects on hypocotyl elongation. Although interactions between the light and GA signaling pathways have been studied extensively, the detailed regulatory mechanism of their direct crosstalk in hypocotyl elongation remains to be fully clarified. Previously, we reported that ABA INSENSITIVE 4 (ABI4) controls hypocotyl elongation through its regulation of cell-elongation-related genes, but whether it is also involved in GA signaling to promote hypocotyl elongation is unknown. In this study, we show that promotion of hypocotyl elongation by GA is dependent on ABI4 activation. DELLAs interact directly with ABI4 and inhibit its DNA-binding activity. In turn, ABI4 combined with ELONGATED HYPOCOTYL 5 (HY5), a key positive factor in light signaling, feedback regulates the expression of the *GA2ox* GA catabolism genes and thus modulates GA levels. Taken together, our results suggest that the DELLA-ABI4-HY5 module may serve as a molecular link that integrates GA and light signals to control hypocotyl elongation.

Key words: light signal, GA signal, hypocotyl elongation, DELLA, ABI4, HY5

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INTRODUCTION

Plant growth is tightly regulated by multiple environmental and endogenous signals such as light, temperature, and phytohormones. How the input of these signals within a response network affects a common biological output remains an important question in plant biology (Halliday and Fankhauser, 2003; Franklin, 2009; Lau and Deng, 2010; de Lucas and Prat, 2014; de Wit et al., 2016; Ku et al., 2018). In particular, a hormone signaling cascade typically involves feedback regulation through adjustment of its own biosynthesis. However, how this mechanism affects the complex interactions between multiple signaling pathways requires further investigation (Silverstone et al., 1998; Sun and Gubler, 2004; Nonogaki et al., 2014; Zong et al., 2016; Takato et al., 2017).

Among the environmental signals, light is the most influential environmental factor for plant growth and development. Plants are sessile organisms, and their development undergoes critical changes during the switch from germination in subterranean darkness to emergence through the soil surface into the light (Bae and Choi, 2008; Kami et al., 2010; Yang and Liu, 2020). In the dark, germinated seedlings develop skotomorphogenically and are characterized by long hypocotyls and closed cotyledons that contain undifferentiated chloroplasts. When seedlings are exposed to light as they emerge from the soil, they undergo a series of photomorphogenic responses,

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including inhibition of hypocotyl elongation, expansion of cotyledons, and functional chloroplast development (Kaiser et al., 2019; Armarego-Marriott et al., 2020; Cackett et al., 2022). The crucial regulation by light is mediated by a number of photoreceptors and downstream signaling components (Chen et al., 2004; Wu, 2014; Cheng et al., 2021). Among them, CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) functions as a RING finger E3 ubiquitin ligase and facilitates the degradation of a number of positive signaling factors through the ubiquitin-26S proteasome pathway, thus acting as a central negative regulator of photomorphogenesis (Lau and Deng, 2012; Han et al., 2020; Ponnu and Hoecker, 2021). A group of basic helix-loop-helix (b-HLH) transcription factors called PHYTOCHROME-INTERACTING FACTORS (PIFs) also repress photomorphogenesis. PIFs are active in the dark to promote skotomorphogenesis but are degraded in the light through the activation of phytochromes (Lee and Choi, 2017; Paik et al., 2017; Pham et al., 2018). In contrast to PIFs, the bZIP transcription factor ELONGATED HYPOCOTYL5 (HY5) plays key roles in promoting photomorphogenesis by directly regulating the expression of a wide range of genes under various light regimes (Gangappa and Botto, 2016; Paik et al., 2017; Xiao et al., 2021). HY5 is degraded by COP1-mediated ubiquitination in the dark, whereas it accumulates in the light to promote photomorphogenesis (Holm et al., 2002; Marzi et al., 2020).

It is well established that gibberellin (GA) signals and light signals have opposite effects on seedling photomorphogenesis (Xu et al., 2014). Mutants deficient in GA display de-etiolation in the dark, suggesting that GA inhibits photomorphogenesis (Alabadi et al., 2004). As negative master regulators of GA signaling, DELLAs appear to be pivotal factors in the integration of light and GA signaling pathways for seedling photomorphogenesis. In *Arabidopsis*, there are five members of the DELLA protein family, GA INSENSITIVE (GAI), REPRESSOR OF *ga1-3* (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3, that have overlapping or distinct biological functions in plant development (Gao et al., 2008; Ito et al., 2018; Wang et al., 2020). GA promotes plant growth through the degradation of DELLA proteins (Hirano et al., 2008). In the absence of GA, DELLAs accumulate and inhibit the transcriptional activity of PIF3/4 by sequestering their DNA-recognition domains, thereby abrogating PIF-mediated hypocotyl elongation (de Lucas et al., 2008; Feng et al., 2008). A recent study has also revealed that DELLAs promote PIF1/3/4/5 degradation via the ubiquitin-proteasome system (Li et al., 2016). In addition to the DELLA-PIFs module, light signals may directly or indirectly modulate genes involved in GA biosynthesis and metabolism, including *GA20ox* and *GA3ox* genes, which convert GA12 into active GA1 and GA4, and *GA2ox* genes, which convert active GA1 and GA_4 into inactive GA_8 and GA_{34} (Garcia-Martinez and Gil, 2001; Yamaguchi 2008; Weller et al., 2009; Rizza et al., 2017; Stawska and Oracz, 2019). Nevertheless, how the endogenous GA level is finely modulated to coordinate distinct cellular responses remains largely unknown.

The AP2/ERF transcription factor ABI4 was found to participate in the regulation of endogenous GA content. ABI4 mediates the balance of ABA and GA biogenesis by regulating the transcription of ABA and GA metabolic genes in opposite ways during primary seed dormancy and post-germination stages (Shu et al., 2013, 2016, 2018). Previously, we reported that ABI4 directly regulates a set of genes involved in hypocotyl elongation, such as *EXP2* (*EXPANSIN2*), *XTH5* (*XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 5*), and *XTH21*, which promote cell wall loosening and cell elongation (Cho and Kende, 1997; Rose et al., 2002; Sánchez et al., 2004; Shin et al., 2005; Miedes et al., 2013; Xu et al., 2016; Wu et al., 2022). However, it remains unclear whether ABI4 is involved in GA signaling for the promotion of hypocotyl elongation. In this study, we found that ABI4 is required for GA promotion of hypocotyl elongation. Further analysis suggested that DELLAs inhibit the DNA-binding activity of ABI4 through direct physical interaction. In addition, DELLAs, ABI4, and HY5 can form coherent feedback loops to regulate the *GA2ox* GA catabolism genes and thus finely modulate GA levels to coordinate these cellular responses. These results revealed a direct interaction between GA and light signals mediated by a DELLA-ABI4-HY5 module. Therefore, this study not only identifies a molecular module by which plants optimize their growth in response to light and GA signals but also provides insights into the mechanisms by which the biosynthesis of GA is modulated by a complex signaling network in plants.

RESULTS

ABI4 is required for GA promotion of hypocotyl elongation

Our previous study reported that the *abi4* mutant has a short hypocotyl in low white light, whereas seedlings that overexpress *ABI4* (*ABI4OE*) have long hypocotyls (Xu et al., 2016). Further biochemical and genetic studies demonstrated that ABI4 promotes hypocotyl elongation by enhancing the expression of cell-elongation-related genes (Xu et al., 2016). The fact that GA signals also exert a similar effect on plant growth led us to ask whether GA treatment could rescue the hypocotyl defect of the *abi4* mutant. Our results showed that GA effectively induced hypocotyl elongation in the wild type (WT), whereas the *abi4* mutant was partially resistant to GA (Figure 1A–1C). We also found that the response to increasing GA concentration was reduced in the *abi4* mutant, whereas *ABI4OE* seedlings were hypersensitive to GA (Figure 1B and 1C). However, when seedlings were treated with the GA biosynthesis inhibitor paclobutrazol (PAC), the *abi4* mutant seedlings showed a hypersensitive response, whereas the *ABI4OE* seedlings were partially resistant to this inhibitor (Figure 1A, 1D, and 1E). These results suggest an important role for ABI4 in the promotion of hypocotyl elongation by GA.

RGA physically interacts with ABI4

To further explore the molecular mechanism by which GA promotion of hypocotyl elongation depends on ABI4, we measured ABI4 protein levels after GA treatment and found no obvious change (Supplemental Figure 1A). Then we performed chromatin immunoprecipitation (ChIP) assays using *35S:ABI4-GFP* transgenic seedlings to determine whether GA treatment altered ABI4-DNA binding activity. The results showed that the interaction of ABI4 with its targets *EXP2*, *XTH5*, and *XTH21* was significantly enhanced in seedlings treated with GA in which DELLA proteins were destabilized (Supplemental Figure 1B).

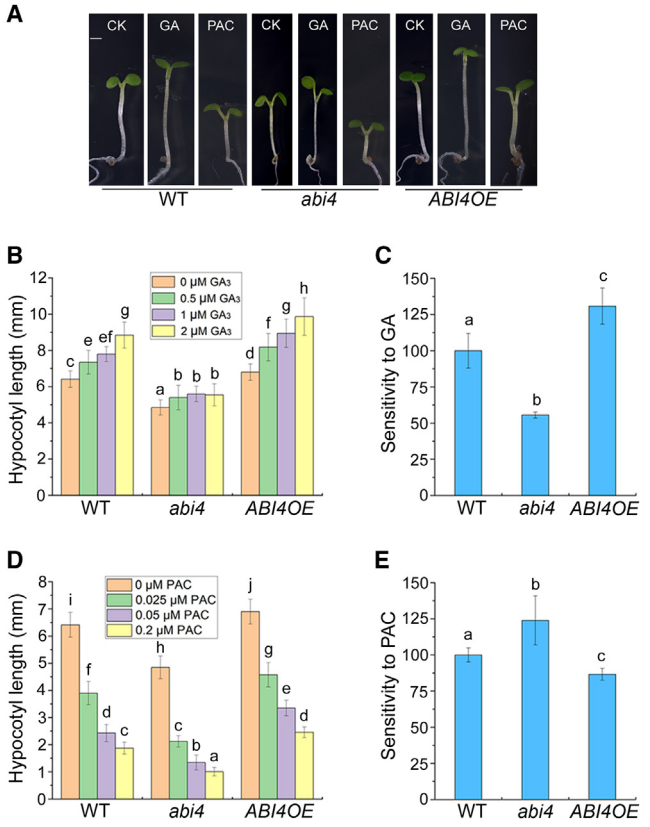

Figure 1. ABI4 is required for GA promotion of hypocotyl elongation.

(A) Phenotypes of WT, *abi4*, and *ABI4OE* seedlings in response to GA and PAC treatments. Seedlings were grown on medium without (CK) or with 0.5 μM GA_3 or with 0.025 μM PAC (an inhibitor of GA biosynthesis) in continuous low white light (10 μmol m^{-2} s^{-1}) for 5 days. Scale bar, 1 mm.

(B) Hypocotyl lengths of WT, *abi4*, and *ABI4OE* seedlings grown with increasing concentrations of GA_3 (0, 0.5, 1, and 2 μM). Different lowercase letters indicate significant differences (one-way ANOVA, $P < 0.05$) among different groups, and the same convention is used below.

(C) Relative response to GA treatment of WT, *abi4*, and *ABI4OE* seedlings shown in **(B)**.

(D) Hypocotyl lengths of WT, *abi4*, and *ABI4OE* seedlings grown with increasing concentrations of PAC (0, 0.025, 0.05, and 0.2 μM).

(E) Relative response to PAC treatment of WT, *abi4*, and *ABI4OE* seedlings shown in **(D)**.

DELLA proteins have been shown to interact with several transcription factors such as PIF3/4, BZR1, and HAT1 and block their transcriptional activity (de Lucas et al., 2008; Feng et al., 2008; Bai et al., 2012; Tan et al., 2021). Given our results that the DNA-binding activity, but not the protein level, of ABI4 increased after GA treatment, we speculated that DELLAs may inhibit ABI4 transcriptional activity through direct interaction. To test this possibility, we first performed yeast two-hybrid (Y2H) analysis. Because full-length *DELLA* genes show strong autoactivation when fused with the binding domain of GAL4 (Zhang et al., 2018), we used N-terminally truncated RGA, GAI, RGL1, RGL2, and RGL3 that lacked the DELLA domain as baits for the Y2H assay. Direct interactions between ABI4 and all five members of the DELLA family were observed (Figure 2A). Similar results were obtained in bimolecular fluorescence complementation (BiFC) assays using *Nicotiana benthamiana* leaves (Supplemental Figure 2).

RGA has been shown to act as a master repressor of hypocotyl elongation; we therefore examined the direct interaction between RGA and ABI4 *in vitro*. Consistent with the Y2H assays, pull-down assays showed that ABI4-maltose-binding protein (MBP) was able to pull down RGA-glutathione S-transferase (GST) but not GST alone, and vice versa, indicating an *in vitro* interaction between RGA and ABI4 (Figure 2B). To confirm the direct interaction between ABI4 and RGA *in vivo*, we carried out co-immunoprecipitation (coIP) assays using protein extracts from *N. benthamiana* leaves agroinfiltrated with ABI4-GFP and RGA-FLAG constructs. The results indicated that RGA was able to precipitate ABI4, and vice versa (Figure 2C). Additional evidence for the RGA-ABI4 interaction was obtained by BiFC assays in *Arabidopsis* protoplasts. As shown in Figure 2D, a very strong yellow florescent protein (YFP) signal was observed in the nucleus when ABI4-nYFP and RGA-cYFP were co-transformed into *Arabidopsis* protoplasts. These data suggest that RGA physically interacts with ABI4 both *in vitro* and *in vivo*.

RGA inhibits the DNA-binding activity of ABI4

To investigate whether the interaction between RGA and ABI4 blocks the DNA-binding activity of ABI4, we performed electrophoretic mobility shift assays (EMSAs). As shown in Figure 3A, ABI4-MBP, but not MBP alone or RGA-MBP, bound the *EXP2* promoter. However, incubation of ABI4-MBP with RGA-MBP markedly diminished this binding activity.

To determine whether RGA inhibits ABI4 transcriptional activity, we performed transient expression assays using a *LUC* (luciferase) fusion to the *EXP2* promoter as a reporter for ABI4 transcriptional activity. As shown in Figure 3B, the expression level of luciferase driven by the *EXP2* promoter was increased by ABI4, but this increase was clearly abolished by co-expression of RGA and ABI4, indicating that RGA blocks ABI4 transcriptional activity. Furthermore, GA treatment suppressed the inhibitory effect of RGA, probably because GA induced RGA degradation. Consistent with these results, Δ17-RGA, a stable version of the RGA protein that lacks 17 amino acids and is resistant to GA destabilization, inhibited ABI4 transcriptional activity more extensively, and this inhibition could not be reversed by GA treatment (Figure 3B). These results suggest that DELLA proteins interact with ABI4 and repress its transcriptional activity.

To further investigate the genetic relationship between RGA and ABI4, we generated lines expressing *35S: Δ17-RGA-FLAG* in the WT and *ABI4OE* backgrounds. As expected, *35S: Δ17-RGA-FLAG* seedlings showed short hypocotyls, and this growth limitation was partially rescued by *ABI4* overexpression. In addition, GA treatment did not promote hypocotyl growth of either *35S: Δ17-RGA-FLAG* or *35S: Δ17-RGA-FLAG/ABI4OE* seedlings, which simulated a stable form of RGA (Figure 3C). These results support a positive function of ABI4 in hypocotyl growth and the attenuation of ABI4 activity by DELLA proteins.

Consistent with this mode of action, ChIP analysis showed that, during seedling de-etiolation, the binding of ABI4 to the *EXP2*, *XTH5*, and *XTH21* promoters was strikingly suppressed,

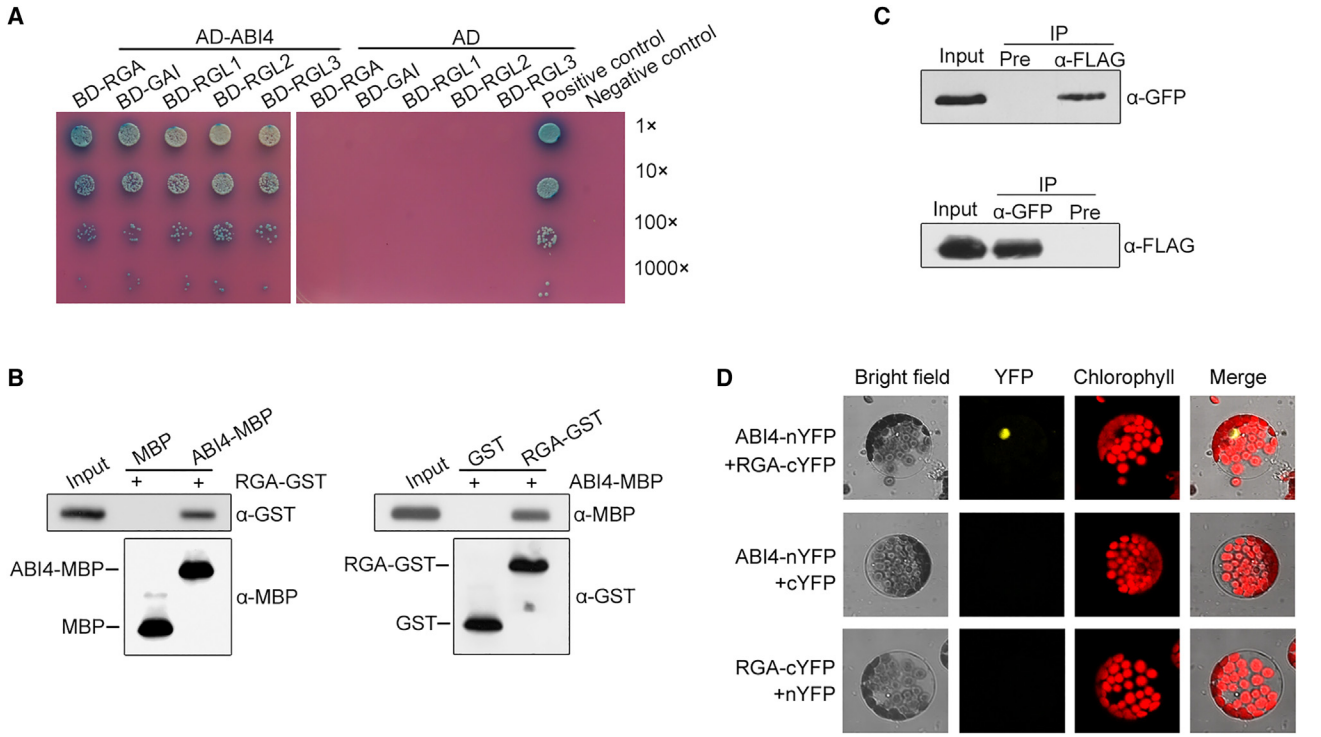

Figure 2. DELLA interacts with ABI4 *in vitro* and *in vivo*.

(A) Y2H assays for the interaction between N-terminally truncated DELLA fused with the GAL4 DNA binding domain (BD) and ABI4 fused with the GAL4 activation domain (AD). Yeast co-transformed with pGBKT7-53 and PGBKT7-Lam was used as a negative control, and yeast co-transformed with pGBKT7-53 and pGADT7-T was used as a positive control.

(B) Pull-down assays showing interaction of MBP-tagged ABI4 and GST-tagged RGA. In the left panel, full-length ABI4-MBP bound to amylose resin was used as a bait, MBP was used as a control, and full-length RGA-GST was used as a prey. Input, 4% of the prey protein RGA-GST. In the right panel, full-length RGA-GST bound to GST resin was used as a bait, GST was used as a control, and full-length ABI4-MBP was used as a prey. Input, 4% of the prey protein ABI4-MBP.

(C) CoIP analysis of the interaction between ABI4 and RGA. ABI4-GFP and RGA-FLAG constructs were co-transformed into *N. benthamiana* leaves, and proteins were extracted and immunoprecipitated with anti-FLAG antibody (the upper panel) or anti-GFP antibody (the lower panel), respectively, and analyzed by immunoblotting with anti-GFP (the upper panel) or anti-FLAG antibody (the lower panel). Pre-immune antiserum (Pre) was used as a negative control. Input indicates that 10 μg of proteins were loaded on the gel; input in the upper panel indicates ABI4-GFP, and input in the lower panel indicates RGA-FLAG.

(D) BiFC analysis for the interaction between ABI4 and RGA in *Arabidopsis* protoplasts.

probably because of the decreased active GA levels. When seedlings were exposed to light for 4 h, the DNA-binding activity of ABI4 was reduced to only 10%–30% of that in the dark (Figure 3D). Our previous study showed that COP1 targets ABI4 for ubiquitin-proteasome degradation during seedling de-etiolation (Xu et al., 2016). However, after a 4-h illumination, the remaining ABI4 protein level was still 60% of that in the dark, suggesting a much faster rate of de-activation than degradation of ABI4 protein.

ABI4 feedback modulates GA metabolism

Endogenous GA levels must be tightly regulated during plant growth (Yamaguchi and Kamiya, 2000; Garcia-Martinez and Gil, 2001; Yamaguchi, 2008). We therefore measured active GA levels in WT and *abi4* mutant seedlings. Interestingly, we found that the active GA content of the *abi4* mutant was decreased to only about 40%–60% that of the WT, suggesting a feedback regulation of ABI4 on GA accumulation (Figure 4A). To study how ABI4 influences GA content, we tested the expression of GA biosynthesis and catabolic genes. *GA2ox1*, *4*, and *7* transcript levels were more than 1.5-fold higher in the *abi4* mutant than in the WT but were clearly lower in the *ABI4OE* seedlings (Figure 4B and Supplemental Figure 3). These results indicated that ABI4 inhibits the expression of the *GA2ox* GA catabolism genes and thus increases endogenous GA levels.

To test whether ABI4 directly binds to the promoter regions of the *GA2ox* genes, we first performed yeast one-hybrid (Y1H) assays. GAD-ABI4 (fused with the GAL activation domain) activated the *LacZ* reporter gene driven by the promoters of *GA2ox1*, *4*, and *7* but not by the promoters of other *GA2ox* genes (Figure 5A). Next, a 50-bp double-stranded fragment that included the putative ABI4-binding site CCAC motifs (−1145 to −1096 bp) in the *GA2ox7* promoter was selected as a probe for EMSA assays (Chandrasekaran et al., 2020). As shown in Figure 5B, recombinant MBP-tagged ABI4 bound to the 50-bp *GA2ox7* promoter fragment. This protein–DNA interaction was fully dependent on the presence of ABI4, as demonstrated by use of MBP

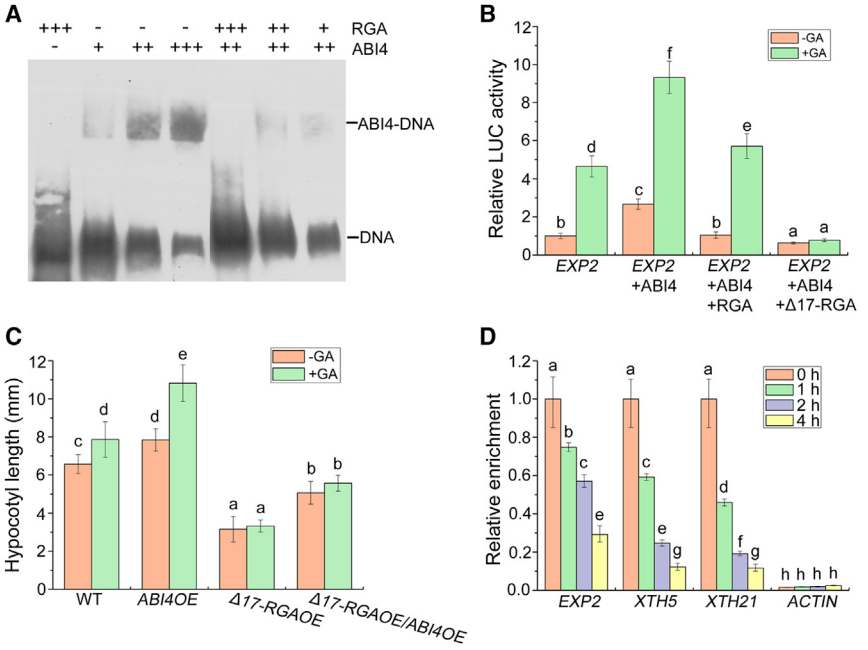

Figure 3. RGA inhibits the DNA-binding activity of ABI4.

(A) EMSA assays examining the effect of RGA on ABI4 binding to the *EXP2* promoter using MBP-tagged ABI4, GST-tagged RGA, and the *EXP2* promoter fragment with CCAC motifs. "+", "++", and "+++" indicate 1-fold, 2-fold, and 4-fold quantities of ABI4-MBP or RGA-GST protein.

(B) Transient assays for ABI4 transcriptional activity in *Arabidopsis* protoplasts. The luciferase reporter gene was driven by the *EXP2* promoter. Protoplasts were incubated with or without 50 μM GA_3. *35S:GUS* was used as the internal control for transformation, and relative activities were quantified as the ratio of LUC to GUS. Different lowercase letters indicate significant differences (one-way ANOVA, $P < 0.01$) among different groups, and the same convention is used below.

(C) Hypocotyl lengths of WT, *ABI4OE*, *Δ17-RGAOE*, and *Δ17-RGAOE/ABI4OE* seedlings grown on medium with or without GA for 5 days.

(D) ChIP-qPCR assays for the binding activity of ABI4 on *EXP2*, *XTH5*, and *XTH21* promoters during seedling de-etiolation. The 5-day-old *35S:ABI4-GFP* etiolated plants were transferred into light for 0, 1, 2, and 4 h, DNA was isolated, and GFP antibodies were used for precipitation. The relative DNA enrichment was determined by qPCR using specific primers. Quantification of *ACTIN* was used as a negative control.

alone. The specificity of the binding was corroborated by using excess unlabeled probe as a specific competitor, but no competition was observed when the CCAC motif was mutated, supporting the necessary role of the CCAC motif in the interaction between ABI4 and the *GA2ox7* promoter *in vitro* (Figure 5B).

Next, we performed ChIP assays using *35S:ABI4-GFP* transgenic seedlings to examine whether ABI4 binds to the promoters of *GA2ox* genes *in vivo*. Quantitative PCR analysis revealed that the P1 region of the *GA2ox1* promoter, the P5 region of the *GA2ox4* promoter, and the P6 region of the *GA2ox7* promoter, which contained CCAC motifs, were enriched in the anti-GFP samples but not in the pre-immune serum samples (Figure 5D and 5E). These results suggested a direct interaction between ABI4 and the promoters of the *GA2oxs*.

ABI4 and HY5 antagonistically regulate *GA2ox* expression

Previous studies have documented that light signals also play important roles in regulating GA contents (Weller et al., 2009; Rizza et al., 2017; Stawska and Oracz, 2019). The promoters of *GA2ox* genes contain several ACE-box (ACGT) or G-box (CACGTG) motifs, which are characteristic binding sites for HY5, a central transcription factor in the light signaling pathway (Gangappa and Botto, 2016). We therefore speculated that HY5 is also involved in the direct regulation of *GA2ox* expression. Y1H, EMSA, and ChIP assays indicated that HY5 directly binds to the promoters of these *GA2ox* genes both *in vitro* and *in vivo* (Figure 5A, 5C, 5D, 5F). Moreover, in contrast with lower active GA content in the *abi4* mutant, *hy5* had higher GA levels than the WT, and the higher level of GA in the *hy5* mutant was largely suppressed by the *abi4* mutation in the *abi4hy5* double mutant (Figure 6A). Consistent with this result, qRT-PCR analysis showed lower *GA2ox* expression in the *hy5* mutant, and the lower transcript levels of *GA2oxs* in the *hy5* mutant were partly rescued by the *abi4* mutation in the *abi4hy5* double mutant (Figure 6B).

We next performed transient expression assays with a *LUC* reporter gene driven by the *GA2ox7* promoter. Effector constructs for the ABI4 and HY5 proteins were expressed under the control of the 35S promoter and co-transformed together with the reporter construct and the *35S:GUS* internal control into *Arabidopsis* protoplasts. As shown in Figure 6C, *GA2ox7* expression was inhibited by ABI4 but stimulated by HY5. Co-expression of ABI4 and HY5 greatly suppressed the activation effect of HY5 on the *GA2ox7-LUC* reporter, providing evidence that ABI4 and HY5 act antagonistically to regulate *GA2ox* gene expression.

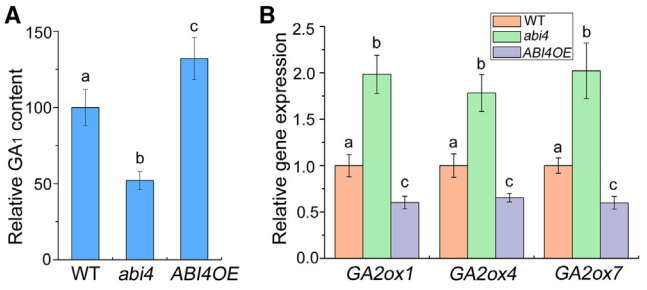

Figure 4. ABI4 feedback modulates expression of GA metabolism genes and GA content.

(A) Relative endogenous GA_1 levels in WT, *abi4*, and *ABI4OE* seedlings grown in continuous low white light (10 μmol m^{-2} s^{-1}) for 5 days. Different lowercase letters indicate significant differences (one-way ANOVA, $P < 0.01$) among different groups, and the same convention is used below.

(B) qRT-PCR analysis for transcript levels of *GA2ox1*, *GA2ox4*, and *GA2ox7* genes in WT, *abi4*, and *ABI4OE* seedlings grown in continuous low white light (10 μmol m^{-2} s^{-1}) for 5 days.

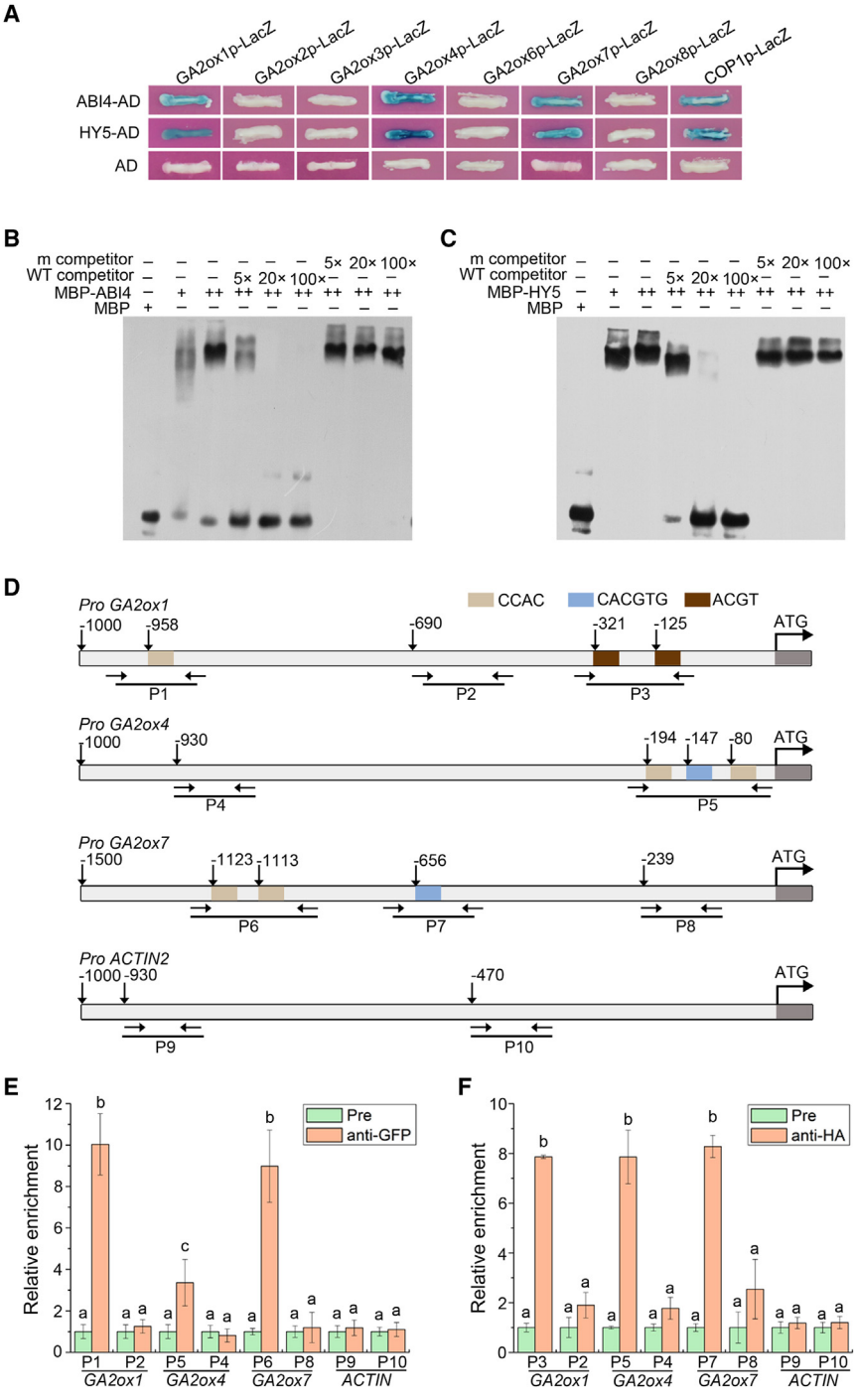

Figure 5. ABI4 and HY5 bind directly to the promoters of *GA2ox* genes.

(A) Y1H assays of ABI4 and HY5 binding to the promoters of *GA2ox1*, *GA2ox4*, and *GA2ox7* genes.

(B) EMSA analysis for the interaction between the MBP-tagged ABI4 and a *GA2ox7* promoter fragment probe containing CCAC motifs. "m" indicates that the CCAC motifs in the probe are mutated to TATA. "+" and "++" indicate 1-fold and 2-fold quantities of MBP-ABI4 protein.

(C) EMSA analysis for the interaction between MBP-tagged HY5 and a *GA2ox7* promoter fragment probe containing the G-box (CACGTG). "m" indicates that the G-box in the probe is mutated to TATATA. "+" and "++" indicate 1-fold and 2-fold quantities of MBP-HY5 protein.

(D) Schematic representation of the *GA2ox1*, *GA2ox4*, *GA2ox7*, and *ACTIN2* promoters.

(E) ChIP-qPCR assays of ABI4 binding to the promoters of *GA2ox1*, *GA2ox4*, and *GA2ox7* genes using *35S:ABI4-GFP* seedlings grown in continuous low white light for 5 days. Pre-immune antiserum (Pre) and *ACTIN* were used as negative controls. Different lowercase letters indicate significant differences (one-way ANOVA, $P < 0.01$) among different groups, and the same convention is used below.

(F) ChIP-qPCR assays of HY5 binding to the promoters of *GA2ox1*, *GA2ox4*, and *GA2ox7* genes using *35S:HY5-HA* seedlings grown in continuous low white light for 5 days. Pre-immune antiserum (Pre) and *ACTIN* were used as negative controls.

In order to demonstrate the relationship between ABI4/HY5 and the *GA2ox7* gene genetically, we introduced *35S:GA2ox7* into WT, *ABI4OE*, and *hy5* seedlings. Strikingly, we observed that *GA2ox7OE* seedlings contained less GA and exhibited a more pronounced GA-deficient phenotype, including dwarfism and late flowering, and this defective phenotype was effectively rescued by exogenous GA treatment (Supplemental Figure 5A–5C). Moreover, hypocotyl length was markedly shorter in *GA2ox7OE* than in the WT (Supplemental Figure 5D). It is noteworthy that overexpression of *GA2ox7* reversed the longer hypocotyl of *ABI4OE* and *hy5* seedlings, and GA treatment could also rescue this short-hypocotyl phenotype (Supplemental Figure 5D). These genetic results support the notion that *GA2ox7* acts downstream of ABI4 and HY5 to regulate GA content and hypocotyl elongation, suggesting that ABI4 and HY5 antagonistically regulate *GA2ox7* expression and thus GA content.

Consistent with these findings, transient expression analysis in tobacco leaves showed that HY5 could activate the *LUC* gene driven by the *GA2ox1*, *4*, or *7* promoter under low light conditions, whereas ABI4 negatively regulated expression of these genes (Supplemental Figure 4A). However, when tobacco plants were grown under normal light conditions, ABI4 could slightly promote *GA2ox7* expression (Supplemental Figure 4B), consistent with a previous study (Shu et al., 2016). Therefore, it is likely that the effect of ABI4 on *GA2ox7* may be dependent on light conditions and biological processes.

DISCUSSION

Light and GA antagonistically regulate hypocotyl elongation of plant seedlings. In this crosstalk, the interaction between

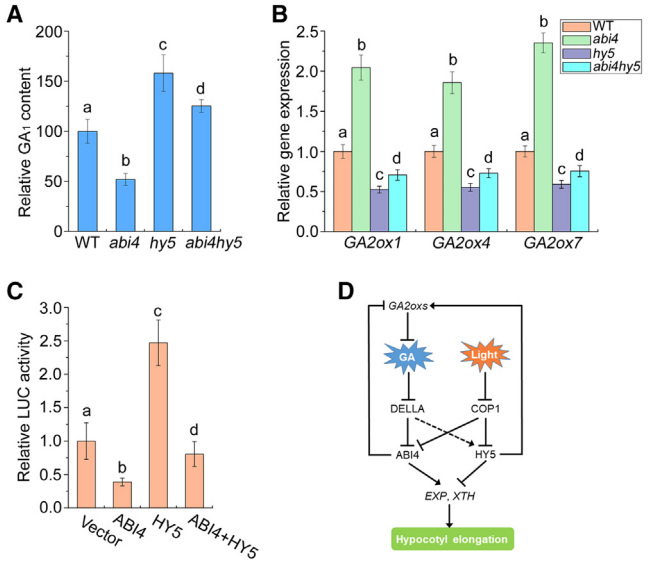

Figure 6. ABI4 and HY5 antagonistically regulate expression of *GA2ox* genes.

(A) Relative endogenous GA_1 levels in WT, *abi4*, *hy5*, and *abi4hy5* seedlings grown in continuous low white light (10 μmol m^{-2} s^{-1}) for 5 days. Different lowercase letters indicate significant differences (one-way ANOVA, $P < 0.01$) among different groups, and the same convention is used below.

(B) qRT-PCR analysis for expression of *GA2ox1*, *GA2ox4*, and *GA2ox7* genes in WT, *abi4*, *hy5*, and *abi4hy5* seedlings grown in continuous low white light (10 μmol m^{-2} s^{-1}) for 5 days.

(C) Transient assays for the capacity of ABI4 and HY5 to regulate expression of the *LUC* reporter gene driven by the *GA2ox7* promoter in *Arabidopsis* protoplasts.

(D) Regulatory model for the DELLA-ABI4-HY5 module that integrates GA and light signals to modulate hypocotyl elongation.

DELLA and PIF3/4 not only inhibits the transcriptional activity of PIF3/4 but also promotes their degradation via the ubiquitin-proteasome system (de Lucas et al., 2008; Feng et al., 2008; Li et al., 2016). In this study, we propose a novel DELLA-ABI4-HY5 module that integrates GA and light signals to regulate hypocotyl elongation (Figure 6D). We show that promotion of hypocotyl elongation by GA is dependent on ABI4 activity (Figure 1). Biochemical, molecular, and genetic analyses demonstrate that association of RGA with ABI4 decreases its DNA-binding activity (Figures 2 and 3). Moreover, DELLA and HY5 have no regulatory effect on ABI4 at the protein level (Supplemental Figure 6), and HY5 does not directly interact with either RGA or ABI4 (Supplemental Figure 7), suggesting that the DELLA effect on ABI4 does not occur through sequestration or competitive binding by HY5. Therefore, ABI4 de-activation by DELLA provides a new regulatory level of ABI4 activity in addition to our previously reported regulation by COP1-mediated ubiquitin-proteasome degradation (Xu et al., 2016). Our data suggest that the de-activation of ABI4 is much faster than its degradation, probably because interaction of DELLAs with ABI4 can immediately sequester its DNA-recognition domain, whereas the degradation of ABI4 depends on the slower-acting ubiquitin-proteasome system (Figure 3D). It appears that ABI4 de-activation by DELLA cannot promote ABI4 degradation, because ABI4 abundance is almost the same after GA treatment, which decreases DELLA levels, suggesting that ABI4 de-activation and degradation are two separate regulatory mechanisms that both contribute to the inhibition of ABI4 activity (Supplemental Figure 1A).

It was reported that the shorter hypocotyl of the *abi4* mutant was observed only under low light conditions but not at higher light intensities (Xu et al., 2016). Similar results were also observed in other studies (Lin et al., 1998; Saijo et al., 2003; Kneissl et al., 2009), probably because increased photoreceptor activity at higher light intensities makes the hypocotyl too short to be distinguishable between WT and mutant seedlings. Moreover, we found that ABI4 promotes GA biosynthesis only under low light conditions (Supplemental Figure 4), which may explain the short hypocotyl of the *abi4* mutant at low light intensities.

Both GA biosynthesis and degradation must be tightly regulated by developmental and environmental signals to maintain a proper GA level and subsequent plant development (Yamaguchi and Kamiya, 2000; Garcia-Martinez and Gil, 2001; Yamaguchi, 2008). In this study, we found that ABI4 feedback increased GA contents by directly regulating expression of *GA2ox1*, *4*, and *7* (Figures 4 and 5), consistent with previous studies that reported a feedback mechanism closely linked to the activity of the GA signaling pathway (Hedden and Kamiya, 1997; Cowling et al., 1998; Silverstone et al., 1998; Sun and Gubler, 2004). It should be noted that ABI4 was also reported to attenuate GA biosynthesis and promote ABA biosynthesis during primary seed dormancy and post-germination stages; however, our results show that ABI4 promotes GA biosynthesis and hypocotyl elongation under low light conditions (Figures 1, 4, 6, and Supplemental Figure 4). These studies therefore suggest diverse and complicated functions of ABI4 in different plant developmental and physiological processes (Cantoro et al., 2013; Shu et al., 2013, 2016). Furthermore, our data show that HY5, the key component of the light signaling pathway, antagonizes ABI4 to control *GA2ox* expression and subsequent GA content and hypocotyl elongation (Figures 5, 6, and Supplemental Figure 5). Our previous study revealed that the ABI4-HY5 transcriptional module antagonistically controls hypocotyl elongation, chlorophyll biosynthesis, ROS homeostasis, and chloroplast development (Xu et al., 2016). This study provides new evidence for the notion that the ABI4-HY5 module functions as an integrator of diverse signals to precisely regulate multiple aspects of developmental responses.

Interestingly, DELLAs promote HY5 protein stability (Alabadi et al., 2008). Our previous study reported that COP1 plays an important role in ensuring the proper interplay of ABI4-HY5 actions (Xu et al., 2016). Thus the present study suggests that in addition to COP1, DELLAs also function to finely control the activity of the ABI4-HY5 transcriptional module and that ABI4-HY5 module feedback regulates GA metabolism to ensure proper GA content. Together with previous results, this study supports a comprehensive mechanistic model in which DELLA-ABI4-HY5 integrate GA and light signals to coordinately regulate environmental and endogenous responses in *Arabidopsis*. As shown in Figure 6D, light-grown seedlings contain low GA levels, resulting in the release of DELLA proteins. The inhibition of ABI4 activity by DELLA proteins, combined with the COP1-mediated degradation of ABI4, blocks the activation of hypocotyl elongation-related genes by ABI4. On the other hand, HY5 activity increases greatly and inhibits cell-elongation-related genes. In turn,

the activated HY5 and deactivated ABI4 induce expression of *GA2ox* genes, resulting in low GA levels, low ABI4 activity, and high HY5 activity and further inhibiting hypocotyl elongation. This model establishes a positive feedback loop between light and the GA cascade signaling pathway that ensures proper plant growth.

METHODS

Plant materials and growth conditions

All mutants and transgenic plants used in this study have been described previously (Xu et al., 2016). Seedlings were grown at 22°C on Murashige and Skoog (MS) medium (M519, Phytotech) containing 2% sucrose (V900116, Sigma-Aldrich) and 0.8% agar (A7002, Sigma-Aldrich) with or without GA_3 (G7645, Sigma-Aldrich) under specific light conditions as described in the text. Seedlings were photographed and hypocotyl lengths were measured using ImageJ software.

Generation of transgenic plants and double mutants

To generate *GA2ox7OE* transgenic plants, the coding region of *GA2ox7* was amplified from WT cDNA using the primers listed in Supplemental Table 1, digested with BamHI and SacI, and ligated into the pSN1301 binary vector under the control of a CaMV35S promoter. The *pSN1301-GA2ox7* construct was electroporated into *Agrobacterium* strain C58C1 and transformed into the WT by floral infiltration. The transformants were identified by screening for hygromycin resistance, and the expression levels of the transgene were determined by qRT-PCR analysis.

The *ABI4OE* transgenic plants were generated as previously described (Xu et al., 2016). To generate *GA2ox7OE/ABI4OE* transgenic plants, *GA2ox7OE* was crossed with *ABI4OE* transgenic plants. *GA2ox7OE* and *ABI4OE* were identified by PCR and transgene expression. To generate *GA2ox7OE/hy5* transgenic plants, *GA2ox7OE* was crossed with *hy5* plants. *GA2ox7OE* was identified by screening for hygromycin resistance and transgene expression, and the *hy5* mutation was identified by PCR of genomic DNA.

Y2H assays

Y2H assays were performed using the Matchmaker Gold Yeast Two-Hybrid System (Clontech) according to the manufacturer's instructions. In brief, truncated RGA (RGA-ΔN) was cloned into the pGBK vector, and ABI4 and PIF3 were cloned into the pGAD vector. The fusion constructs were co-transformed into Y2H Gold yeast cells and then plated on SD/-Leu-Trp-His (630318, Clontech) dropout plates containing 5-bromo-4-chloro-3-indolyl-a-D-galactopyranoside (X-a-Gal) (630463, Clontech) for color development.

Pull-down assays

RGA-GST and ABI4-MBP fusion proteins were induced by 0.4 mM isopropyl β-D-1-thiogalactopyranoside (9030, Takara) and expressed in *E. coli* cells. The recombinant proteins were purified using GST-bind resin (A10018S, Abmart) and amylose resin (E8021S, NEB), respectively. The purified recombinant bait, prey proteins, and GST-bind or amylose resin were incubated in binding buffer containing 10 mM Tris-HCl (pH 7.5) (0497, Amresco), 1 mM EDTA (0322-1KG, Amresco), 1 mM EGTA (324 626, Sigma-Aldrich), 0.5% Nonidet P-40, 1% Triton X-100 (A600198, BBi), 150 mM NaCl (V900058, Sigma-Aldrich), and 1 mM DTT (20290, Pierce) at 4°C for 4 h. Then the bound proteins were eluted and separated by SDS-PAGE and evaluated by immunoblotting.

CoIP assays

Agrobacteria suspensions containing ABI4-GFP and RGA-FLAG constructs were injected into *N. benthamiana* leaves. The plants were kept in the greenhouse for 2 days, and total protein was extracted from the transfected leaves with buffer containing 50 mM HEPES–KOH (pH 7.5) (0511, Amresco), 100 mM NaCl (V900058, Sigma-Aldrich), 0.1% Triton X-100 (A600198, BBi), 5% glycerol (G8190, Solarbio), and protease inhibitor (4693132001, Roche). The protein extract was incubated overnight with FLAG (M185-3L, MBL) or GFP (D153-3, MBL) antibodies immobilized on protein A/G agarose beads (A10001M, Abmart). After incubation, the beads were washed four times to remove the non-specifically bound proteins, and the eluted samples were subjected to immunoblot analysis using GFP and FLAG antibodies, respectively.

BiFC assay

For BiFC assays using *Arabidopsis* protoplasts, the ABI4-nYFP and RGA-cYFP constructs were co-transformed into protoplasts prepared from *Arabidopsis* leaves, and YFP signals were observed with a confocal laser scanning microscope.

For BiFC assays using tobacco cells, agrobacteria suspensions containing ABI4-nYFP and GAI-cYFP, ABI4-nYFP and RGA-cYFP, ABI4-nYFP and RGL1-cYFP, ABI4-nYFP and RGL2-cYFP, or ABI4-nYFP and RGL3-cYFP constructs were injected into *N. benthamiana* leaves, and YFP signals were observed with a confocal laser scanning microscope.

EMSA

Recombinant ABI4-MBP, HY5-MBP, and RGA-GST proteins purified from *E. coli* were used for EMSA assays. The synthetic *EXP2* or *GA2ox7* promoter probes were labeled with biotin and incubated with the recombinant proteins. Then the protein–DNA signals were detected using the Light Shift Chemiluminescent EMSA Kit (20148, Thermo Fisher). Primers for the plasmids and DNA probes used in EMSA are provided in Supplemental Table 1.

Transient luciferase expression assay

The *LUC* reporter construct, the effector plasmid, and the *35S:GUS* internal control plasmid were co-transformed into *Arabidopsis* protoplasts. After incubation overnight, the protoplasts were pelleted and proteins were isolated by resuspending the cells in lysis reagent (N1610, Promega). Activities of LUC luminescence and GUS fluorescence were measured using a Modulus luminometer/fluorometer (Promega). The relative activity was quantified as the ratio of LUC/GUS.

ChIP assay

The *35S:ABI4-GFP* and *35S:HY5-HA* transgenic seedlings were grown in corresponding conditions as described in the text and were treated with 1% formaldehyde in buffer containing 10 mM sodium phosphate (pH 7), 50 mM NaCl, and 0.1 M sucrose for 0.5 h under vacuum to cross-link the protein–DNA complexes.

Then chromatin extracts were sonicated using a Branson sonicator 450 (VWR) to achieve an average fragment size of 250 bp. The chromatin samples were divided into two tubes: one tube was incubated with anti-GFP (D153-3, MBL) or anti-HA antibody (SAB2702249, Sigma-Aldrich) for 4 h, and the other tube was incubated with a serum control for 4 h and then incubated with protein A agarose beads (A10001M, Abmart) to precipitate the chromatin complexes. The immunoprecipitated DNA was analyzed by qPCR using the specific primers listed in Supplemental Table 1. Quantification of *ACTIN* served as a control.

Y1H assay

The ABI4 or HY5-GAD fusion effectors and the GA2ox-LacZ reporter plasmids were co-transformed into yeast strain EGY48. Transformants were first selected and grown on SD/-Trp-Ura (630427, Takara) dropout medium and then grown on SD/-Trp-Ura dropout medium supplied with 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (9031, Takara) for color development.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

AUTHOR CONTRIBUTIONS

X.X. conceived the project. X.X., H.X., D.L, Z.L., J.W., X.N., W.L., and Z.B. performed the experiments. X.X., C.Z., Y.S., D.L., W.C., and L.Z. analyzed the data. X.X. and L.Z. wrote the manuscript.

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