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ABI4 mediates antagonistic effects of abscisic acid and gibberellins at transcript and protein levels

Kai Shu[1,2,†], Qian Chen[2,3,†], Yaorong Wu[2], Ruijun Liu[2,3], Huawei Zhang[2], Pengfei Wang[2], Yanli Li[2], Shengfu Wang[2], Sanyuan Tang[2], Chunyan Liu[2], Wenyu Yang[1], Xiaofeng Cao[2], Giovanna Serino[4,*] and Qi Xie[2,*]

[1]*Key Laboratory of Crop Ecophysiology and Farming System in Southwest China, Institute of Ecological Agriculture, Sichuan Agricultural University, Chengdu 611130, China,*

[2]*State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China,*

[3]*University of Chinese Academy of Sciences, Beijing 100049, China, and*

[4]*Department of Biology and Biotechnology 'Charles Darwin', Sapienza University, Rome 00185, Italy*

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*For correspondence (e-mail qxie@genetics.ac.cn; giovanna.serino@uniroma1.it).
†These authors contributed equally to this work.

SUMMARY

Abscisic acid (ABA) and gibberellins (GAs) are plant hormones which antagonistically mediate numerous physiological processes, and their optimal balance is essential for normal plant development. However, the molecular mechanism underlying ABA and GA antagonism still needs to be determined. Here, we report that ABA-INSENSITIVE 4 (ABI4) is a central factor in GA/ABA homeostasis and antagonism in post-germination stages. *ABI4* overexpression in Arabidopsis (*OE-ABI4*) leads to developmental defects including a decrease in plant height and poor seed production. The transcription of a key ABA biosynthetic gene, *NCED6*, and of a key GA catabolic gene, *GA2ox7*, is significantly enhanced by *ABI4* overexpression. ABI4 activates *NCED6* and *GA2ox7* transcription by directly binding to the promoters, and genetic analysis revealed that mutation in these two genes partially rescues the dwarf phenotype of *ABI4* overexpressing plants. Consistently, *ABI4* overexpressing seedlings have a lower GA/ABA ratio than the wild type. We further show that ABA induces *GA2ox7* transcription while GA represses *NCED6* expression in an ABI4-dependent manner; and that ABA stabilizes the ABI4 protein whereas GA promotes its degradation. Taken together, these results suggest that ABA and GA antagonize each other by oppositely acting on *ABI4* transcript and protein levels.

Keywords: ABA, GA, ABI4, antagonism, transcription factor.

INTRODUCTION

Plant growth and development is the end result of the interaction of diverse endogenous signals with environmental cues (Rymen and Sugimoto, 2012). Each phytohormone acts at low concentrations to regulate numerous aspects of plant development, with distinct or synergistic functions (Gray, 2004; Vanstraelen and Benkova, 2012). Many elegant studies have demonstrated that optimal hormone levels are essential for the achievement of plant normal growth and development (Lee *et al.*, 2002, 2012; Porri *et al.*, 2012).

Gibberellins (GAs) are a large group of tetracyclic diterpenoid plant hormones which regulate diverse developmental processes throughout the plant life cycle, including seed germination, stem elongation, leaf expansion, trichome and root development, and the transition from vegetative growth to reproductive growth (Yamaguchi, 2008; Nelissen *et al.*, 2012; Porri *et al.*, 2012). A number of genes have been implicated in the GA metabolism pathway: bioactive GAs are synthesized by GA3 and GA20 oxidases (GA3ox and GA20ox) and catabolized by a group of catabolic GA2 oxidases (GA2ox) (Rieu *et al.*, 2008b; Porri *et al.*, 2012). In line with these findings, plants overexpressing *GA2ox* genes have a reduced content of bioactive GAs and show GA-deficient phenotypes similar to those displayed by mutants deficient in GA biosynthesis (Lee *et al.*, 2002; Schomburg *et al.*, 2003; Magome *et al.*, 2008; Rieu *et al.*, 2008a; Porri *et al.*, 2012). These phenotypes include dwarf or semi-dwarf stature (Magome *et al.*, 2008; Porri *et al.*,

2012). On the contrary, plants with an elevated GA content have, among other defects, a taller stature (Busov *et al.*, 2008). Therefore, the endogenous GA level must be precisely regulated in order to achieve proper plant development.

Gibberellins positively regulate plant growth by promoting the degradation of a group of DELLA proteins, which inhibit plant growth and act as negative regulators in the GA signaling pathway (Peng *et al.*, 1997; Lee *et al.*, 2002, 2012; Feng *et al.*, 2008). The Arabidopsis genome encodes five DELLA proteins: GA INSENSITIVE (GAI), REPRESSOR OF GA1-3 (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3, which possess distinct or synergistic biological functions during different stages of plant development (Lee *et al.*, 2002; Cheng *et al.*, 2004; Piskurewicz and Lopez-Molina, 2009). The Arabidopsis ubiquitin ligase complexes SCF^{SLY1} and SCF^{SNE1} target the DELLA protein(s) for 26S proteasome-mediated degradation, and the interaction between GA and its receptor GID1 promotes degradation of DELLAs (Ueguchi-Tanaka *et al.*, 2005; Willige *et al.*, 2007; Murase *et al.*, 2008).

Abscisic acid (ABA) regulates a variety of developmental processes including seed dormancy and germination, root development, stomatal movement and adaptive stresses responses (Nambara and Marion-Poll, 2005; Cutler *et al.*, 2010). Extensive studies have demonstrated that ABA generally regulates development by retarding plant growth (Barrero *et al.*, 2005; Nambara and Marion-Poll, 2005; Fujii and Zhu, 2009; Cutler *et al.*, 2010). Consistently, a constitutively elevated ABA level in plant causes severe growth defects (Fan *et al.*, 2009). Therefore it is not surprising that, similar to GAs, the level of endogenous active ABA must be strictly determined by tight control of its rate of biosynthesis and catabolism (Nambara and Marion-Poll, 2005).

Abscisic acid and GA antagonistically regulate many physiological processes including seed germination and plant growth, as well as their own metabolic processes. A low ABA level promotes GA biosynthesis (Seo *et al.*, 2006) and vice versa (Oh *et al.*, 2007). However, the precise molecular mechanism by which ABA and GA antagonize each other has long eluded researchers. Nevertheless, several factors have been isolated which might mediate – at least partially – the antagonistic effects of these two hormones. For example, GA has been shown to inhibit ABA biogenesis by repressing the expression of *XERICO*, which encodes an E3 ubiquitin ligase that enhances ABA biosynthesis by promoting the expression of the ABA biosynthesis gene *NCED3* (Ko *et al.*, 2006; Zentella *et al.*, 2007). In addition, the transcription factor FUS3 has been shown to inhibit GA biosynthesis by directly binding to the *GA3ox2* promoter, while promoting the accumulation of ABA with an as yet uncharacterized mechanism (Curaba *et al.*, 2004; Gazzarrini *et al.*, 2004). Recently, the transcription factor OsAP2-39 was demonstrated to play a key role in mediating the GA/ABA balance in rice (Yaish *et al.*, 2010). Several studies also point to a key role of an APETALA2 (AP2) family member, ABA-INSENSITIVE 4 (ABI4), in the control of GA/ABA homeostasis: we have recently shown that ABI4 controls primary seed dormancy by regulating the balance between ABA and GA metabolism (Shu *et al.*, 2013). In addition, we and others have shown that ABI4, by positively regulating ABA signaling, is also involved in the control of other aspects of plant development in addition to seed dormancy and germination (Finkelstein, 1994; Finkelstein *et al.*, 1998; Soderman *et al.*, 2000; Shu *et al.*, 2013). These novel aspects include lipid mobilization from the embryo (Penfield *et al.*, 2006), glucose signaling (Arenas-Huertero *et al.*, 2000; Laby *et al.*, 2000), the salt stress response (Quesada *et al.*, 2000), regulation of plant male sterility (Shu *et al.*, 2014) and the mitochondrial and chloroplast–nucleus retrograde signaling pathways (Koussevitzky *et al.*, 2007; Giraud *et al.*, 2009; Sun *et al.*, 2011). Recently, ABI4 was also shown to regulate ABA and cytokinin-mediated inhibition of lateral roots by impairing polar auxin transport (Shkolnik-Inbar and Bar-Zvi, 2010). Furthermore, ABI4 is downstream of both ABA- and jasmonic acid (JA)-dependent signaling pathways (Kerchev *et al.*, 2011), and mediates plant responses to both sugar and ABA signaling (Li *et al.*, 2014). Therefore ABI4 seems to be a highly versatile factor which may function in diverse signaling pathways.

Here, we report that ABI4 is a key factor in the modulation of GA/ABA homeostasis and antagonism. We show that ectopic expression of *ABI4* (*OE-ABI4*) leads to pleiotropic phenotypic defects including dwarf stature and poor seed production. *OE-ABI4* lines have a lower GA/ABA ratio than the wild type, and ABI4 directly promotes the expression of the ABA biosynthetic gene *NCED6* and the GA catabolic gene *GA2ox7*. In line with these results, mutations in *nced6* and *ga2ox7* can partially rescue the dwarf phenotype of *OE-ABI4*. Furthermore, ABA-mediated induction of *GA2ox7* and GA-mediated inhibition of *NCED6* both depend on ABI4. At the protein level, ABA stabilizes ABI4 whereas GA promotes its degradation. Taken together, our results suggest that ABI4 is not only a key regulator of GA/ABA homeostasis but also a key target of GA/ABA antagonism.

RESULTS

Overexpression of *ABI4* causes pleiotropic phenotypes

A previous study demonstrated that *ABI4* is expressed at higher levels in maturing and germinating seeds and at lower levels in almost all tissues during vegetative growth (Soderman *et al.*, 2000) and in previous work we have shown that ABI4 regulates seed dormancy (Shu *et al.*, 2013). In this work we further dissect the role of ABI4 in post-germination stages. We took advantage of a gain-of-function approach and analyzed the phenotypes due to

ABI4 overexpression. We used the same two independent *OE-ABI4* transgenic lines (named OE1 and OE2) that we employed in our previous work (Shu *et al.*, 2013) and compared their phenotypes with those of the *abi4* mutant (Finkelstein, 1994; Shu *et al.*, 2013).

At an early growth stage (6-day-old seedlings), both *abi4* and *OE-ABI4* plants showed abnormal leaf size: the true leaves were slightly larger in *abi4* seedlings and significantly smaller in *OE-ABI4* seedlings when compared with the wild-type (WT) control (Figure 1a and Figure S1 in the Supporting Information). *abi4* mutant plants had no detectable defects at later growth stages, as also indicated by previous reports (Soderman *et al.*, 2000). On the contrary, adult *OE-ABI4* plants showed pleiotropic defects, including shorter petioles, smaller rosettes and fewer and shorter siliques, and consequently reduced seed production (Figure 1). *OE-ABI4* plants were also dwarf and much shorter than the WT control. In detail, in *OE-ABI4* plants, height was reduced by 43–67%, rosette size by 58–68% and the silique number per plant by 15–30% when compared with the WT (Figure S1). The seed yield of the transgenic plants was about 20–26% of the WT yield (Figure S1), and since the 1000-grain weight was not significantly affected by *ABI4* overexpression, the difference in seed yield was most likely due to a reduction in seed number rather than seed size (Figures S1 and S2). These defects were not detected in other *OE-ABI4* transgenic lines with lower *ABI4* expression levels, suggesting that they are due to *ABI4* overexpression, and that strict regulation of *ABI4* transcription is essential for normal plant development.

ABI4 affects the transcription of several ABA and GA metabolism genes

The dwarf phenotype of *OE-ABI4* lines is reminiscent of the phenotypes of plants with defects in the GA or ABA pathways, suggesting that GA and ABA metabolism and/or signaling might be altered in these lines (Fan *et al.*, 2009; Porri *et al.*, 2012).

To explore this possibility, we first examined the expression of selected GA catabolic genes (*GA20ox2* and *GA2ox7*) in the two *OE-ABI4* lines, the *abi4* mutant and their WT control by quantitative reverse transcription PCR (qRT-PCR) using two reference genes (*18S* and *ACTIN*). Only genes showing a significant result with both normalizers and, in the case of *OE-ABI4*, in both overexpressing lines, were considered to be differentially expressed. While *GA20ox2* expression was not changed in the overexpressing lines according to our criterion (Figures 2a and S4a), *GA2ox7* was significantly upregulated in both *OE-ABI4* lines (Figures 2b and S4b). In particular the *GA2ox7* transcript level was 5-fold and 26-fold higher than the WT in OE-1 and OE-2, respectively. On the contrary, these genes were not

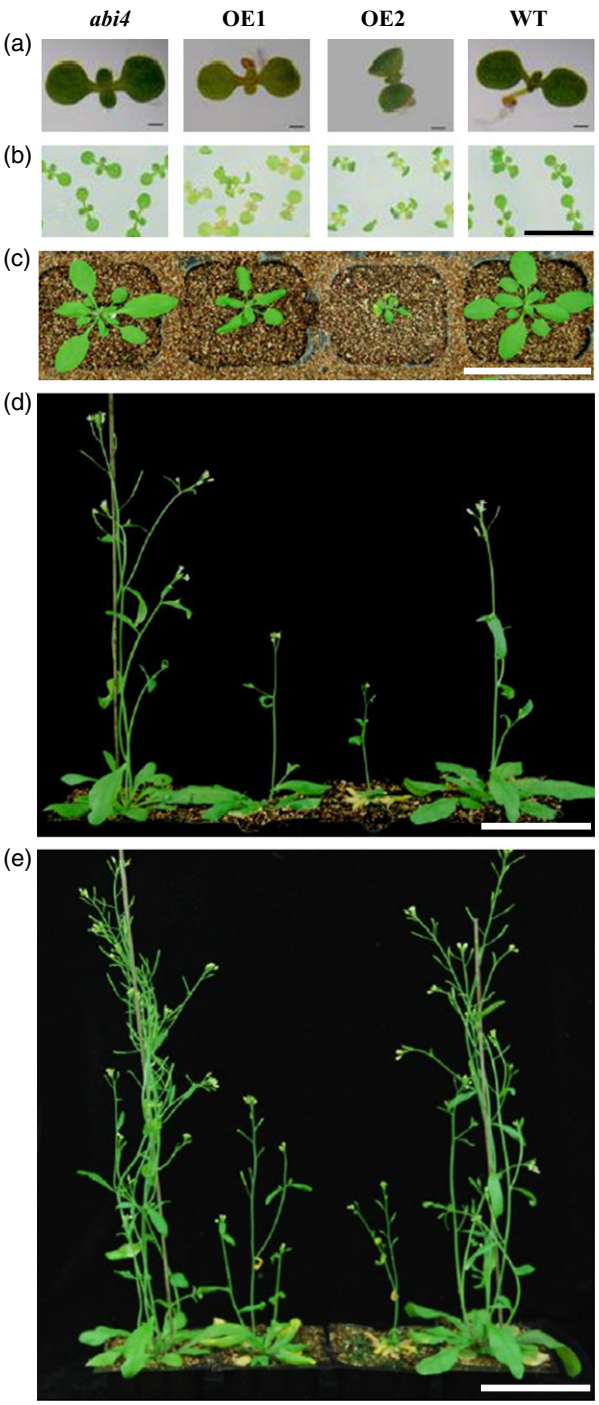

Figure 1. Post-germination phenotypes of *abi4*, wild-type (WT) and OE-*ABI4* plants at different developmental stages.
OE1 and OE2 represent two independent *OE-ABI4* transgenic lines.
(a) Six-day-old seedlings, bar = 0.5 mm.
(b) Ten-day-old seedlings, bar = 5 mm.
(c) Four-week-old plants, bar = 20 mm.
(d) Five-week-old plants, bar = 20 mm.
(e) Seven-week-old plants, bar = 20 mm.

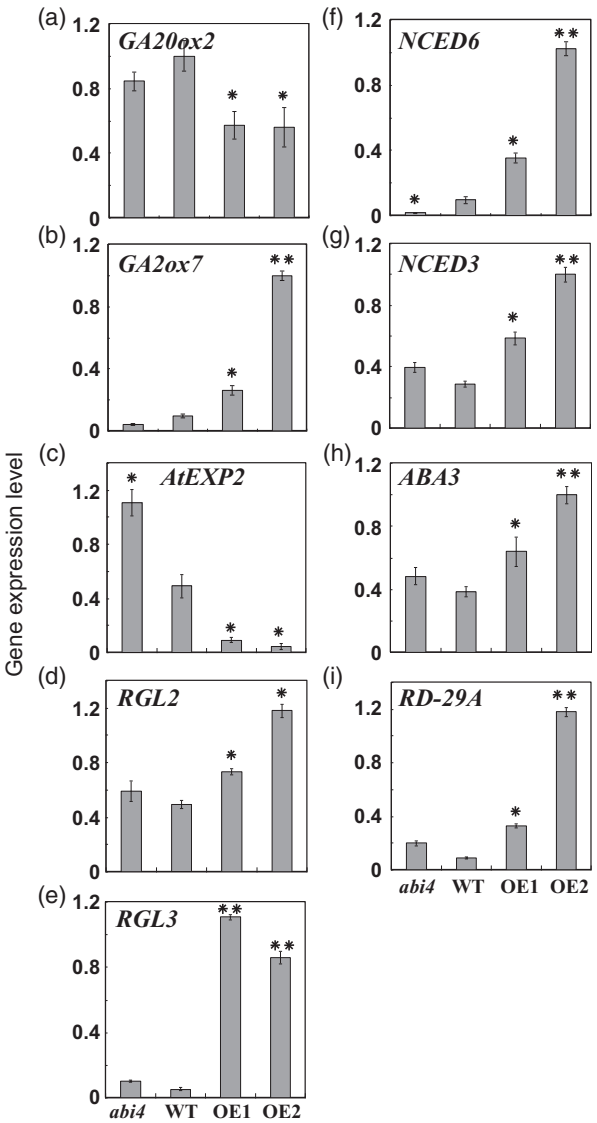

Figure 2. ABI4 regulates the transcription profiles of specific gibberellin (GA) and ABA metabolism genes.

Quantitative RT-PCR analysis of *GA20ox2* (a), *GA2Ox7* (b), *AtEXP2* (c), *RGL2* (d), *RGL3* (e), *NCED6* (f), *NCED3* (g), *ABA3* (h) and *RD-29A* (i), in 2-week-old *abi4, OE-ABI4* and wild-type (WT) seedlings.

OE1 and OE2 represent two independent *OE-ABI4* transgenic lines. The *18S* rRNA was used as the reference gene. The experiments were performed in three replicates and one typical experiment is shown. Asterisks indicate statistically significant differences from WT (*$P < 0.05$; **$P < 0.01$).

significantly affected in the *abi4* mutant (Figures 2a,b and S4a,b), consistent with the weak phenotype of these mutant seedlings. However, these results, together with our finding that *abi4* seedlings showed instead a significant increase in the expression of *GA20ox1* with both normalizers (Figures S3a and S4i), suggest that the endogenous GA level might be altered in *OE-ABI4* transgenic plants.

To test this hypothesis, we analyzed the expression level of genes known to be regulated by GA, such as *AtEXP2*, *RGL2* and *RGL3* (Lee *et al.*, 2010; Yamauchi *et al.*, 2004). Indeed, while the *AtEXP2* transcript level was decreased in *OE-ABI4* plants when compared with the WT (Figure 2c), the level of expression of *RGL2* and *RGL3* was significantly higher in *OE-ABI4* plants than in the WT. Expression of these genes, however, was not significantly altered in the *abi4* mutant (Figures 2c–e and S4c,d), again consistent with the weak phenotype of these mutants. Taken together, these results further strengthened our hypothesis that *OE-ABI4* plants might contain a reduced amount of active GA, and that this might be responsible for the differential expression of GA-regulated genes.

Given the antagonistic effect of ABA and GA in the control of plant growth and development (Vanstraelen and Benkova, 2012), we also investigated whether *ABI4* overexpression affected the expression of selected genes involved in ABA anabolism, catabolism or response pathways. As reported in Figures 2(f–h) and S4(e–g), the transcript levels of the ABA biosynthesis genes *NCED6*, *NCED3* and *ABA3* were significantly increased in *OE-ABI4* plants compared with the WT (Figures 2f–h and S4e–g). The level of transcription of *NCED6* in particular was not only remarkably enhanced in the *OE-ABI4* lines but also decreased in the *abi4* mutant (Figures 2f and S4e). Furthermore, to our surprise, the expression of the ABA catabolic genes *CYP707A2* and *CYP707A3* was also significantly upregulated in *OE-ABI4* seedlings (Figure S3b,c), and, in the case of *CYP707A2*, downregulated in the *abi4* mutant, further indicating that ABA metabolism might be altered by non-physiological levels of *ABI4*.

To address whether the altered expression of ABA metabolism genes in *OE-ABI4* plants also affected the ABA response, we monitored the expression of *RD29A,* an ABA-inducible gene that contains ABA-responsive elements in its promoter (Shinozaki and Yamaguchi Shinozaki, 1997; Xiong *et al.*, 2001), in *OE-ABI4* transgenic lines. While no significant difference in *RD29A* expression was observed between the *abi4* mutant and the WT, the *RD29A* transcript level was significantly increased in *OE-ABI4* (Figures 2i and S4h) when compared with the WT, suggesting that the complex expression profile of genes involved in ABA catabolism and biosynthesis might result in a higher ABA content in *OE-ABI4* plants, which in turn could lead to enhanced expression of *RD29A*.

ABI4 enhances ABA biosynthesis and GA catabolism in seedlings

To confirm the hypothesis that *OE-ABI4* might contain abnormal levels of GA and ABA, we next measured the endogenous GA and ABA content using our previously reported assay (Chen *et al.*, 2011; Shu *et al.*, 2013). Because of the phenotypic similarity between the two *OE-ABI4* lines (Figures 1, 2 and S1), only one line (OE1) was chosen for these experiments. Consistent with our

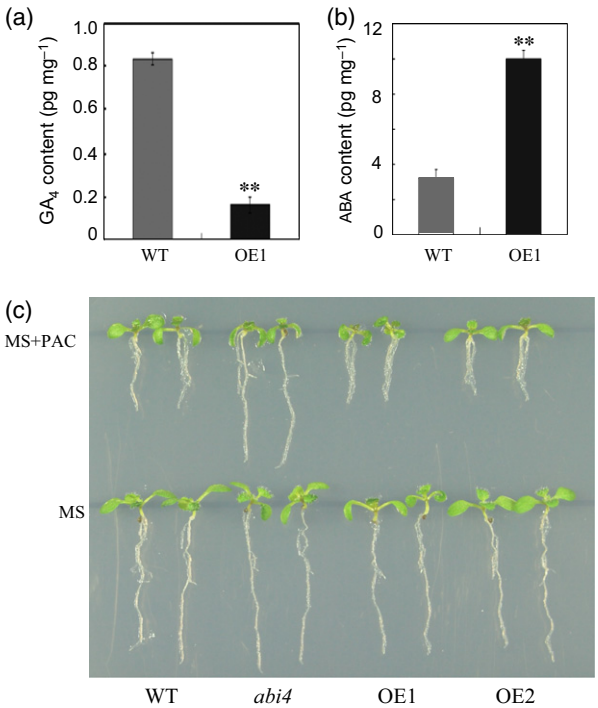

Figure 3. *ABI4* overexpressing lines have a lower gibberellin (GA) and higher ABA content.
(a) The GA content of 2-week-old wild-type (WT) and *OE-ABI4* line 1 (OE1) seedlings.
(b) The ABA content of 2-week-old WT and *OE-ABI4* line 1 (OE1) seedlings.
(c) Phenotypic comparison of 2-week-old *abi4*, WT and *OE-ABI4* seedlings grown in the presence (top) or absence (bottom) of paclobutrazol (PAC).
OE1 and OE2 represent two independent *OE-ABI4* transgenic lines. Asterisks indicate statistically significant differences between *OE-ABI4* and WT (**$P < 0.01$).

hypotheses, the GA content was lower and the ABA content higher in the *OE-ABI4* plants than in the WT (Figure 3a,b). To further confirm the GA measurement results, we analyzed the root growth pattern of *abi4* and *OE-ABI4* seedlings in response to paclobutrazol (PAC), an inhibitor of GA biosynthesis. Our results showed that while root growth of *OE-ABI4* seedlings was slightly sensitive to PAC treatment, *abi4* seedlings were resistant to PAC (Figure 3c). Because it has been shown that seedlings with a higher GA content are more resistant to PAC and seedlings with lower GA content are more sensitive to it (Zhang *et al.*, 2011b), our results are consistent with a lower GA content in *OE-ABI4* lines (Figure 3a).

We also hypothesized that the higher ABA level in *OE-ABI4* plants might be the cause of the upregulation of the catabolic genes *CYP707A3* and *CYP707A2* (*18S* for Figure S3b,c; *ACTIN* for Figure S4j,k). This upregulation could be due to feedback regulation by the higher ABA content in these plants. Indeed, information retrieved from the public Arabidopsis microarray database (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi) revealed that exogenous ABA treatment strongly induces transcription of *CYP707A2* (Figure S5a) but has no obvious effect on *CYP707A3* expression (Figure S5b), and our qRT-PCR analysis (Figure S5c,d) showed that *CYP707A2* at least is progressively upregulated by an increasing concentration of exogenous ABA. Similar qRT-PCR results were also obtained when the *ACTIN* reference gene was employed (Figure S5e,f).

ABI4 directly binds to the *GA2ox7* and *NCED6* promoters *in vivo*

ABI4 is an AP2-domain-containing transcription factor, and previous studies have demonstrated that ABI4 regulates gene expression by binding to a CCAC motif within the promoters of target genes (Acevedo-Hernandez *et al.*, 2005; Koussevitzky *et al.*, 2007). Because *ABI4* overexpression leads to misexpression of a series of ABA and GA metabolism genes, we investigated whether ABI4 directly regulates the transcription of these genes. First, we analyzed the promoter sequences of the genes whose transcription is under ABI4 control (Figures 2 and S3). We found that the promoters of both *GA2ox7* and *NCED6* contain putative ABI4-binding motifs: the *GA2ox7* promoter contains 5 CCAC motifs while the *NCED6* promoter contains 10 motifs (Figure 4a,b), indicating that ABI4 might directly bind to these promoters. However, another transcription factor, DDF1, is known to directly bind to the *GA2ox7* promoter and enhance its expression (Magome *et al.*, 2008). Thus, to exclude the possibility that ABI4 regulates *GA2ox7* transcription indirectly by affecting *DDF1* expression, we analyzed the *DDF1* transcript level in the *abi4* and *OE-ABI4* lines and did not observe any noticeable change (Figure S6).

We next tested if ABI4 binds directly to the *GA2ox7* and *NCED6* promoters by performing chromatin immunoprecipitation (ChIP)-qPCR assays on a *35S-ABI4-GFP* transgenic line (Shu *et al.*, 2013). The *ABI5* promoter was used as a positive control since it was already known to be directly bound by ABI4 (Bossi *et al.*, 2009). Protein and DNA complexes were immunoprecipitated using an antibody against the GFP, and enriched DNA was amplified by qPCR using specific primers sets that anneal to the CCAC motifs (P1–3 on *GA2ox7*; P5–8 on *NCED6*) or to CCAC-poor regions (P4 on *GA2ox7*) present in the *GA2ox7* and *NCED6* promoters (Figure 4a,b). As shown in Figure 4(c), we found enrichment of the P1 and P3 regions of the *GA2ox7* promoter and of the P5 and P7 regions of the *NCED6* promoter, suggesting that *GA2ox7* and *NCED6* are direct targets of ABI4. Similar results were obtained using two independent *35S-ABI4-GFP* transgenic lines, which indicated that ABI4 promotes *GA2ox7* and *NCED6* transcription by directly binding to their promoters. However, we did not detect an enrichment of ABI4 on *ABA3* and *NCED3* promoters, although they also contained a series of CCAC

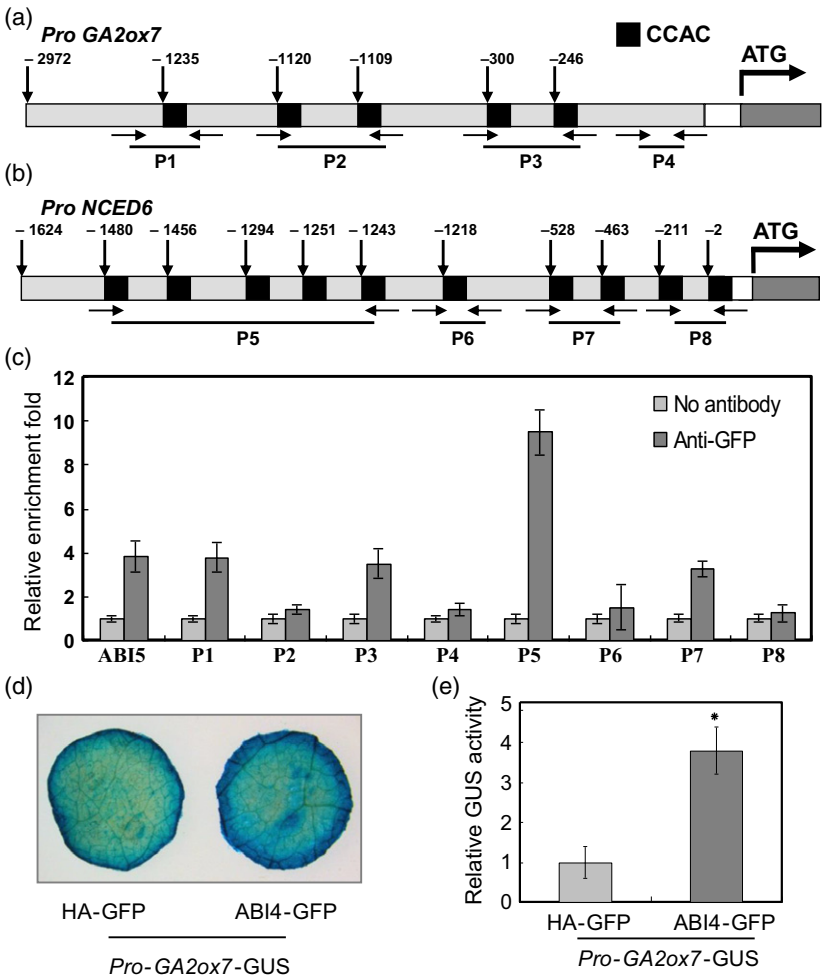

Figure 4. ABI4 binds directly to the *GA2ox7* and *NCED6* promoters *in vivo*.
(a), (b) Schematic representation of the *GA2ox7* (a) and the *NCED6* (b) promoters. Black boxes indicate the position of the CCAC motif. The arrows indicate the positions of the primers used in part (c).
(c) Chromatin immunoprecipitation–quantitative PCR analysis conducted using the specific primers pairs indicated in (a). *TUB4* was used as an internal control and a specific region of the *ABI5* promoter as a positive control. The experiments were conducted in three replicates on two independent *35S-ABI4-GFP* lines (OE1 and OE2), and one typical experiment result is shown.
(d), (e) Histochemical assay (d) and quantitative analysis (e) of *N. benthamiana* leaves transformed with the constructs indicated in the figure. (d) Representative GUS-staining images of samples taken from *N. benthamiana* leaves at 3 days after infiltration. (e) Quantitative analysis of relative GUS activity from samples taken from the same leaves shown in (d). Activity units are given in nmol methyl-umbelliferone (mg protein)$^{-1}$ min^{-1}. The experiments were performed in three biological replicates and one typical experiment is shown. Asterisks indicate statistically significant differences between HA-GFP- and ABI4-GFP-transformed leaves (*$P < 0.05$).

motifs (11 for *NCED3* promoter and 8 for *ABA3* promoter, respectively).

Taken together, the results presented in Figures 2(b,f) and 4(c) suggest that ABI4 might promote *GA2ox7* and *NCED6* expression by directly binding to their promoters. To assess this hypothesis, we employed a transient expression system in tobacco (Liu *et al.*, 2010). Two reporter plasmids (*Pro-GA2ox7-GUS*, *Pro-NCED6-GUS*) were separately transformed in *Nicotiana benthamiana* leaves with or without the *pCanG-ABI4-GFP* or *pCanG-HA-GFP* (negative control) effector plasmids, and GUS levels were detected by a qualitative (Figure 4d) and a quantitative (Figure 4e) assay. Leaves co-transformed with the *pCanG-ABI4-GFP* and *Pro-GA2ox7-GUS* constructs showed a significant increase in GUS levels in both assays when compared with leaves co-transformed with the *pCanG-HA-GFP* and *Pro-GA2ox7-GUS* constructs. Similar effects of ABI4 on the expression of *NCED6* were also detected using the same system (Figure S7a,b). These results confirm that ABI4 has a direct effect on *GA2ox7* and *NCED6* transcription *in vivo*.

GA2ox7 and *NCED6* mutations partially rescue the dwarf phenotype of *OE-ABI4*

Our finding that *OE-ABI4* transgenic plants have a decreased GA/ABA ratio suggests that their phenotypic defects might be related to this hormonal imbalance. To confirm this hypothesis, we genetically dissected the relationship between *GA2ox7*, *NCED6* and *ABI4*.

To this end, we introduced the *35S-ABI4-GFP* construct into the knock-out mutants *ga2ox7* (SALK_055721C) and *nced6* (CS852600) (Magome *et al.*, 2008; Toh *et al.*, 2008) and subjected them to phenotypic analysis. As shown in Figure 5(a,b), neither *ga2ox7* nor *nced6* mutant seedlings have a noticeable phenotype when compared with the WT. However, the *ga2ox7* mutation partially rescued the dwarf phenotype of adult *OE-ABI4* plants (Figure 5a, top panel). Similarly, the *nced6* mutation partially restored the WT phenotype of *OE-ABI4* transgenic plants (Figure 5b, top panel). This partial phenotypic rescue does not seem to be due to altered expression of the transgene, since the fusion protein is expressed at comparable levels in all lines exam-

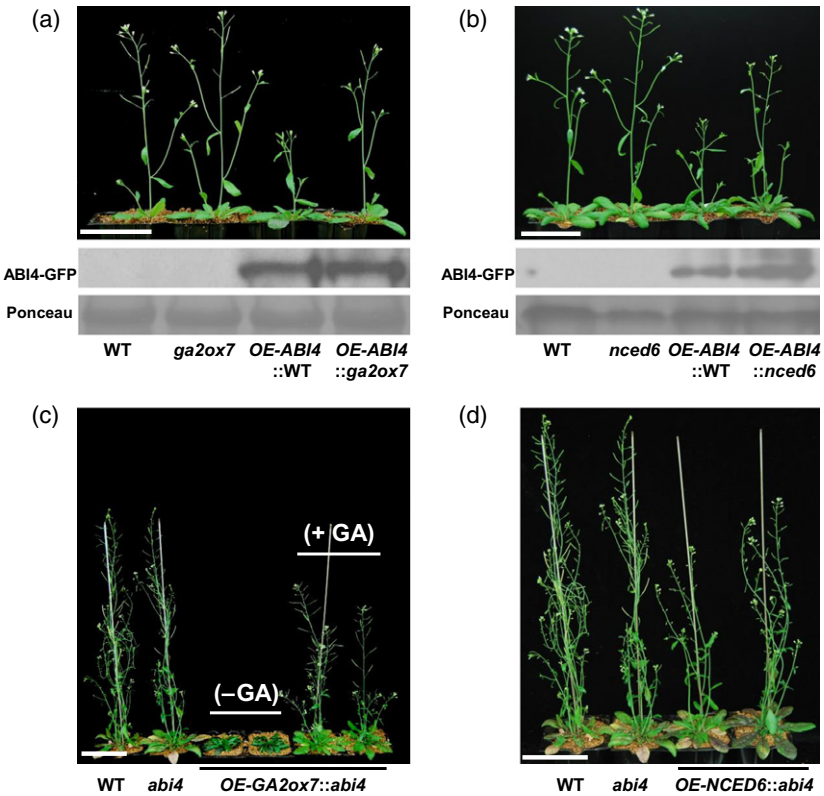

Figure 5. *ABI4* acts genetically upstream of *GA2ox7* and *NCED6*.
(a) Phenotypic comparison (top panel) and relative immunoblot analysis (bottom panel) of 35-day-old plants from WT (wild type), *ga2ox7, OE-ABI4* and *OE-ABI4* in a *ga2ox7* background (*OE-ABI4*::*ga2ox7*).
(b) Phenotypic comparison (top panel) and relative immunoblot analysis (bottom panel) of 35-day-old plants from WT, *nced6*, *OE-ABI4*,and *OE-ABI4* in a *nced6* background (*OE-ABI4*::*nced6*).
(c) Phenotypic comparison of WT, *abi4* and OE-*GA2ox7*::*abi4* plants (7 weeks old), grown with or without 100 μM GA.
(d) Phenotypic comparison of WT, *abi4* and OE-*NCED6*::*abi4* plants (7 weeks old).
Bar = 20 mm.

ined (Figure 5a,b, lower panels). Taken together, these genetic analyses indicate that the dwarf stature of *OE-ABI4* plants is partially dependent on the ABI4-induced expression of *GA2ox7* and *NCED6* and suggest that *GA2ox7* and *NCED6* may act genetically downstream of *ABI4*.

To further evaluate this hypothesis, we analyzed the phenotype of *abi4* mutant plants overexpressing *GA2ox7*. As shown in Figure 5(c), ectopic *GA2ox7* expression in the *abi4* background greatly reduced plant height (Figure 5c). This phenotype mimics the phenotypes of *GA2ox7* overexpressing plants or of the *ga1-3* mutant (Lee *et al.*, 2002) (Porri *et al.*, 2012). Indeed, similar to *ga1-3* and to WT plants overexpressing *GA2ox7* (*OE-GA2ox7*::WT), the *OE-GA2ox7*::*abi4* transgenic plants could not bolt unless treated with exogenous GA (Figure 5c). Similar phenotypes were also detected in *abi4* plants overexpressing *NCED6* (Figure 5d). These experiments support the hypothesis that *GA2ox7* and *NCED6* act genetically downstream of *ABI4*.

The dwarf phenotype of *OE-ABI4* is caused by *ABI4* rather than by *ABI5*

It has been shown that ABI4 directly promotes the transcription of *ABI5* (*ABA- INSENSITIVE 5*), another central factor in the ABA signaling pathway (Bossi *et al.*, 2009). Indeed, we have already shown that the *ABI5* expression level increased significantly in *OE-ABI4* transgenic plants (Shu *et al.*, 2013). To exclude the possibility that the observed dwarf phenotype of *OE-ABI4* is a result of ABI4-induced ectopic expression of *ABI5*, we overexpressed *ABI4* in the *abi5-7* mutant background, which has no obvious phenotype under normal growth conditions, compared with its relative WT (Col-0; Figure S8a, and Chen *et al.*, 2012). Among the resulting *OE-ABI4::abi5-7* transgenic plants, two independent lines were confirmed to express high levels of *ABI4* by qRT-PCR analysis and were thus selected for phenotypic analysis (Figure S8b). As shown in Figure 6(a–c), overexpression of *ABI4* in the *abi5-7* mutant background also resulted in a dwarf stature in both the seedling (Figure 6a,b) and the adult stage (Figure 6c; compare this with Figure 1d). This evidence demonstrates that the dwarf phenotype of *OE-ABI4* transgenic plants is directly caused by *ABI4*, rather than by *ABI4 ABI5*.

ABA and GA have opposite effects on *ABI4* expression and protein stability

Our data demonstrate that ABI4 enhances ABA biogenesis while suppressing GA biogenesis (Figures 2 and 3), and suggest that ABI4 might regulate GA/ABA homeostasis. We thus speculated that GA and ABA might in turn control ABI4 itself. To assess this hypothesis, we asked whether GA and ABA affect *ABI4* transcription and protein stability.

To this end, we first monitored the effects of ABA and GA on *ABI4*, *GA2ox7* and *NCED6* transcription over time. As shown in Figure 7, we found that *ABI4* transcription is

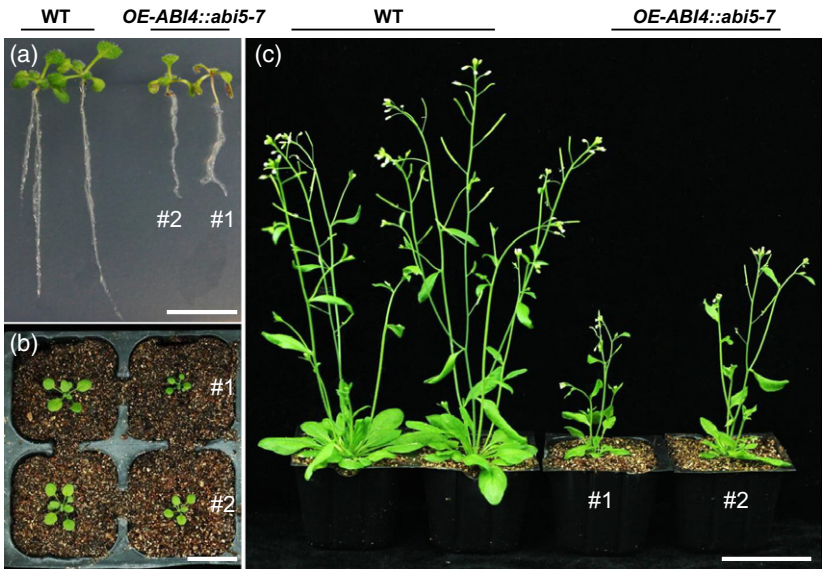

Figure 6. The dwarf phenotype of *OE-ABI4* is caused by *ABI4* rather than by *ABI5*.
Post-germination phenotypes of wild-type (WT) and *OE-ABI4::abi5-7* plants at different developmental stages.
(a) Sixteen-day-old seedlings, bar = 10 mm.
(b) Eighteen-day-old seedlings, bar = 20 mm.
(c) Forty-day-old plants, bar = 50 mm.
#1 and #2 represent two independent *OE-ABI4::abi5-7* transgenic lines.

rapidly induced by ABA and suppressed by GA (Figure 7a, b). Abscisic acid also significantly induces *GA2ox7* expression (Figure 7c) while GA represses *NCED6* transcription (Figure 7d). In addition, both ABA-mediated induction of *GA2ox7* and GA-mediated inhibition of *NCED6* require ABI4. In detail, both transcriptional induction and inhibition were significantly decreased in the *abi4* mutant compared with the WT (Figure 7c–f). Similar results were also obtained when we employed a second reference gene in the qRT-PCR analysis (*ACTIN*; Figure S9). Because ABI4 directly activates the transcription of *GA2ox7* and *NCED6* (Figures 2 and 4), it is possible that ABI4 might partially mediate a cascade amplification effect of ABA and GA through the transcription of these two genes.

As a previous study has demonstrated that the level of ABI4 protein is regulated – at least partially – by the ubiquitin 26S proteasome system (UPS) (Finkelstein *et al.*, 2011), we next monitored the effects of ABA and GA on the stability of ABI4 protein over time by incubating seedlings in a medium containing GA or ABA and cycloheximide (CHX; an inhibitor of protein synthesis). As shown in Figure 7(g, h), ABI4 protein was already degraded after incubation for 30 min in the presence of GA. On the contrary, ABA was able to induce stabilization of ABI4 over an incubation time of 45 minutes, while the level of a control protein (Myc-GFP) remained stable over the same time frame (Figure S10). These results conclusively indicate that ABA stabilizes ABI4 while GA enhances its degradation.

DISCUSSION

Abscisic acid and GA are well known to antagonistically regulate diverse plant growth and development processes (Gale and Marshall, 1973; Ho *et al.*, 1981; Schomburg *et al.*, 2003; Porri *et al.*, 2012). The negative effect of ABA and the positive effect of GA on plant growth and development are well described in the literature (Nambara and Marion-Poll, 2005; Yamaguchi, 2008; Cutler *et al.*, 2010; Porri *et al.*, 2012). However, the factors regulating the balance between ABA and GA, as well as the molecular mechanism of their antagonism, are still not completely clear. Our present study reveals that ABI4 could be part of this mechanism, since it promotes ABA biosynthesis while inhibiting GA biosynthesis by directly regulating the transcription of specific hormone metabolism genes. Further, our study shows that ABI4 also is a key factor that mediates GA/ABA antagonism, since ABA and GA have opposite effects on *ABI4* transcription and protein stability.

ABI4 promotes ABA biosynthesis and represses GA biosynthesis

ABI4 has mainly been described as a positive regulator of the ABA signaling pathway (Finkelstein, 1994; Finkelstein *et al.*, 1998; Soderman *et al.*, 2000). *ABI4* is highly expressed in maturing and germinating seeds as well as in early seedlings, while it is expressed at relatively low levels during vegetative growth (Soderman *et al.*, 2000). Consistent with this expression pattern, *abi4* mutant seedlings develop normally (Soderman *et al.*, 2000), thus preventing a more detailed characterization of the function of ABI4.

To circumvent this problem, we have employed a gain-of-function approach to dissect the role of ABI4 in plant growth and development. Similarly to the *ABI4* overexpressing plants described by Soderman *et al.* (2000), our *ABI4* overexpressing plants have decresed height, lower seed production and other developmental defects (Figures 1 and S1). *OE-ABI4* seedlings also have a decreased GA/ABA ratio, which suggests that this imbalance might

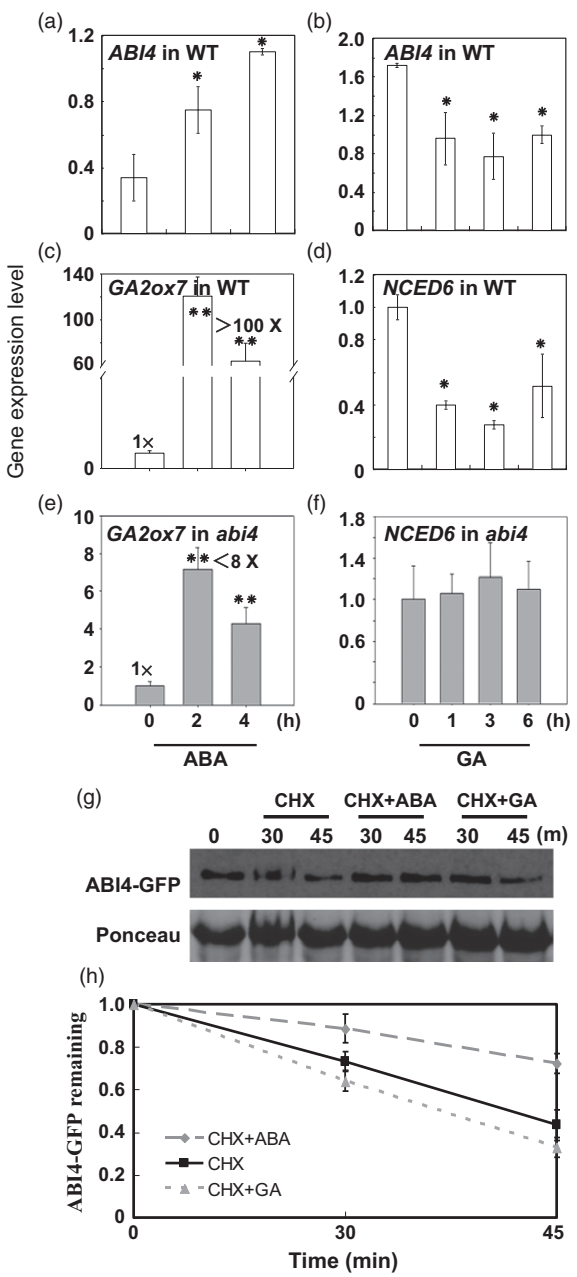

Figure 7. Opposite effect of ABA and gibberellin (GA) on *ABI4* transcription and protein stability.
(a)–(f) Effect of ABA and GA on *ABI4* transcription. Quantitative RT-PCR analysis of *ABI4* (a, b), *GA2ox7* (c, e), and *NCED6* (d, f) transcript levels in 2-week-old wild type (WT) or *abi4* seedlings. Seedlings were treated with ABA (a, c, e) or GA (b, d, f), at the concentrations indicated. Asterisks indicate statistically significant differences from the beginning of the experiment (0) (*$P < 0.05$; **$P < 0.01$). (g) Effect of ABA and GA on ABI4 protein levels. Immunoblot analysis (top panel) of 2-week-old homozygous *35S-ABI4-GFP* seedlings treated with 50 μM ABA or 50 μM GA plus or minus 80 μM CHX. Antibodies to GFP were used to detect the ABI4-GFP fusion protein. The Ponceau staining (bottom panel) indicates the loading control. The experiment was run in three biological replicates and a typical result is shown. (h) Densitometric analysis of ABI4-GFP degradation shown in (g). The detailed protocol for quantification and normalization is described in the Experimental Procedures. Data are the mean from three biological replicates. Error bars represent SD.

be responsible for the phenotypic defects observed in *OE-ABI4* transgenic plants.

Previous studies have demonstrated that ABI4, in addition to ABA signaling, is also involved in other pathways, including glucose, cytokinin and JA signaling (Finkelstein, 1994; Finkelstein *et al.*, 1998; Arenas-Huertero *et al.*, 2000; Laby *et al.*, 2000; Soderman *et al.*, 2000; Shkolnik-Inbar and Bar-Zvi, 2010; Kerchev *et al.*, 2011). Here, we further show that ABI4 regulates ABA and GA biogenesis in seedlings (Figure 3). Other AP2 family members seem to share similar functions; Yaish and colleagues have recently demonstrated that – similar to ABI4 – another AP2-like transcription factor, OsAP2-39 from rice (*Oryza sativa* L.), also enhances ABA biosynthesis and suppresses GA biosynthesis, leading to a decrease in biomass and seed yield (Yaish *et al.*, 2010). A second AP2 family member, DDF1, is a positive regulator of *GA2ox7* expression, and, when overexpressed, leads to lower GA levels and dwarfism (Magome *et al.*, 2008). Finally, CHOTTO1, a putative double AP2 repeat transcription factor, represses GA biosynthesis during seed germination (Yano *et al.*, 2009).

Combined with these studies, our results suggest that at least some AP2 family members possess specific yet little characterized roles in ABA and GA biogenesis. Recently, by analyzing the phylogenetic history of ABI4 homologs from published proteomes and genomes (a total of 33 species from Phytozome or Plaza, including *Arabidopsis thaliana*, *Thellungiella halophila*, *Zea mays* and *Glycine max*), a new 'ABI4 motif' (LRPLLPRP) was found, which is conserved across angiosperms (Wind *et al.*, 2013). This remarkable conservation might reflect a role for this domain in mediating the biological functions of ABI4. Further experiments will be required to assess this hypothesis.

ABI4 oppositely regulates the transcription of ABA and GA metabolism genes

Our data indicate that ABI4 regulates the levels of ABA and GA by binding to the promoters of the *NCED6* and *GA2ox7* genes and promoting their transcription (Figures 4 and S7). This conclusion is further supported by the partial rescue of the *OE-ABI4* dwarf phenotype by the *ga2ox7* and *nced6* mutations (Figure 5).

GA2ox7 overexpression has been shown to significantly reduce the levels of bioactive GA, resulting in a dwarf phenotype in Arabidopsis and tobacco (*N. tabacum*) (Schomburg *et al.*, 2003; Magome *et al.*, 2008; Tong *et al.*, 2009; Porri *et al.*, 2012). Furthermore, constitutive overexpression of *NCED1* in tomato (*Solanum lycopersicum*) and of *NCED2*, *NCED3* and *NCED5* in Arabidopsis increased the ABA content in transgenic plants and caused similar phenotypes (Thompson *et al.*, 2000; Fan *et al.*, 2009). *NCED6* overexpressing transgenic plants also showed a reduced rosette size due to an increase in the level of ABA (Lefebvre

et al., 2006). In agreement with these studies, we show that *GA2ox7* and *NCED6* are upregulated in *OE-ABI4* plants, and consequently that the GA and ABA contents are altered in the opposite manner (Figures 2 and 3). We also provide molecular and genetic evidence that *GA2ox7* and *NCED6* are direct targets of ABI4 (Figures 4, 5 and S7). Taken together, our results demonstrate that ABI4 mediates GA/ABA homeostasis by directly regulating the transcription of specific genes involved in the GA and ABA metabolism pathways.

ABA and GA antagonize each other by oppositely affecting *ABI4* transcription and protein stability

The antagonistic relationship between GA and ABA has been extensively studied (Rymen and Sugimoto, 2012; Vanstraelen and Benkova, 2012). However, the detailed molecular mechanisms underlying the antagonistic effect between these two hormones are still largely unknown.

A possible target of ABA and GA is *miR159*, which regulates the expression of the *MYB33* gene (Achard *et al.*, 2004; Reyes and Chua, 2007). However, both ABA and GA induce accumulation of *miR159* – and consequently downregulation of *MYB33* – thus promoting ABA responses in seeds and GA responses in flowers (Gocal *et al.*, 2001; Reyes and Chua, 2007). This implies that MYB33 is not a likely mediator of ABA/GA antagonism, but rather a common effector of the two hormones in two different developmental processes. On the contrary, in our present study we demonstrate that ABA and GA exert opposite effects on *ABI4* transcript and protein levels; ABA induces *ABI4* transcription while GA inhibits it (Figure 7a,b): ABI4 is stabilized by ABA, while GA promotes its degradation (Figure 7g,h).

We also show that ABA induces transcription of *GA2ox7* while GA inhibits expression of *NCED6* in an ABI4-dependent manner (Figure 7c–f). Based on these results, we propose the working model shown in Figure 8. Briefly, ABA induces transcription of *ABI4* and protein stabilization; the resulting higher level of ABI4 promotes expression of *GA2ox7*, thus downregulating GA biogenesis. In contrast, GA inhibits the expression of *ABI4* and enhances degradation of its protein, thus inhibiting transcription of *NCED6* and impairing ABA biosynthesis. Therefore, ABA and GA antagonize each other by altering both transcription of *ABI4* and its protein levels, and, consequently, transcription of *GA2ox7* and *NCED6*. ABI4 could possibly represent a key target of GA/ABA antagonism, and provide one of the missing links between ABA production and GA inactivation.

ABI4 transcript and protein levels are tightly regulated

Our working model shown in Figure 8 depicts positive feedback between ABA biogenesis, *ABI4* transcription and the level of ABI4 protein. On the other hand, the model predicts that higher ABA levels further inhibit GA biosynthesis through activation of *GA2ox7* expression, thus explaining the dwarf stature of *ABI4* overexpression lines (Figures 1 and 8). Because optimal hormone levels are essential for normal plant development, the ABA- and GA-mediated regulation of ABI4 might represent a key mechanism for finely regulating the plant life cycle. However, the molecular mechanism through which ABA and GA regulate levels of ABI4 transcript and protein still requires further characterization. Recently, members of the PTM (PHD-type transcription factor with transmembrane domains) transcription factor family were identified as activators of *ABI4* expression (Sun *et al.*, 2011). Furthermore, it has been shown that ABI4 also promotes its own transcription by binding directly to its promoter (Bossi *et al.*, 2009). On the other hand, repressors of *ABI4* expression have been recently found: the transcription factors WRKY40, WRKY18 and WRKY60 bind to the *ABI4* promoter and suppress its expression (Shang *et al.*, 2010; Liu *et al.*, 2012). However, although these WRKY transcription factors directly inhibit expression of ABI4, introduction of the *abi4* mutation into the *wrky18* and *wrky60* mutants led to double mutants with an *abi4* phenotype on ABA-containing medium (Liu *et al.*, 2012). These results demonstrate that *ABI4* is a target of these two WRKY transcription factors, but do not give full insight into the molecular mode of action of ABI4.

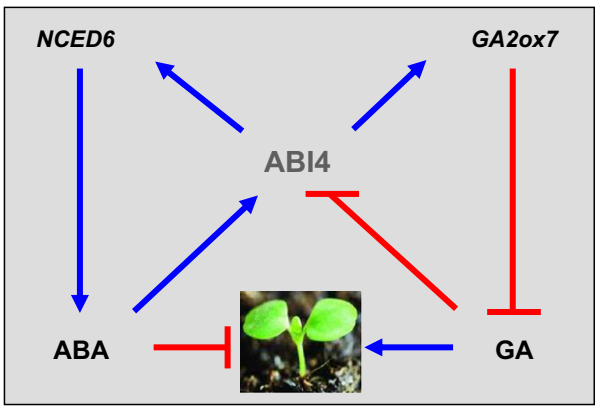

Figure 8. Proposed working model of the antagonistic effects of gibberellin (GA)/ABA on ABI4.
ABI4 promotes ABA biosynthesis and represses GA biogenesis by the direct activation of *NCED6* and *GA2ox7*, and therefore negatively regulates plant growth and development. Conversely, ABA induces *GA2ox7* expression and GA inhibits *NCED6* transcription in an ABI4-dependent manner. In addition, ABA maintains a proper level of ABI4 protein, while GA promotes degradation of ABI4. This model proposes that ABA antagonizes GA by promoting *ABI4* transcription and protein stabilization, thus enhancing transcription of *GA2ox7* and eventually decreasing GA biogenesis. Vice versa, GA antagonizes ABA by inhibiting *ABI4* expression and enhancing degradation of its protein, thus, attenuating expression of *NCED6* and impairing biogenesis of ABA. Blue lines ending with arrows denote positive regulation, while red lines ending with bars denote negative regulation.

As for the ABI4 protein level, a previous study has already shown that ABI4 degradation is at least partially mediated by the UPS (Finkelstein *et al.*, 2011). In line with this result, our present study shows that ABI4 is degraded via the UPS: however, the specific E3 ubiquitin ligase(s) responsible for ABI4 ubiquitination have not yet been found. Therefore, the regulatory mechanism through which GA and ABA precisely regulate the optimal levels of *ABI4* mRNA and protein still awaits further investigation. Clearly, a genetic screen for suppressors of the *abi4* mutant phenotype could represent a worthwhile future study.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The Arabidopsis ecotype Col-0 was used as the WT in this study. The point mutant *abi4-1* (CS8104) and the *ga2ox7* (SALK_055721C) and *nced6* (CS852600) mutants were ordered from the Arabidopsis Biological Resource Center (ABRC; https://abrc.osu.edu/). The *abi5-7* mutant is of the Col-0 ecotype and was kindly provided by Dr Chuanyou Li, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Arabidopsis seeds were surface-sterilized with 10% bleach and washed at least four times with sterile water. Seeds were suspended in 0.2% agarose and sowed on 1/2 MS solid medium plus 1% sucrose. Plates were stratified under darkness for 3 days in a 4°C cold room and then transferred to the tissue culture room at 22°C under a 16-h light/8-h dark photoperiod. After about 2 weeks, seedlings on 1/2 MS were potted in soil and placed in a growth chamber (16-h light/8-h dark photoperiod) at 22°C and 70% relative humidity condition. The 1/2 MS medium was supplemented with PAC (product number 46046, Sigma-Aldrich, http://www.sigmaaldrich.com/) as indicated in the text. For spraying the plants, 100 μM GA was used.

Constructs and transgenic plants

Transgenic plants constitutively expressing *ABI4* in the WT background were described in Shu *et al.* (2013). Transgenic plants overexpressing *ABI4-GFP* (*35S-ABI4-GFP*) (Shu *et al.*, 2013), *GA2ox7-GFP* (*OE-GA2ox7-GFP*) or *NCED6-GFP* (*OE-NCED6-GFP*) in the WT or in the *abi5-7* background were generated by PCR amplification of the coding sequences of *ABI4*, *GA2ox7* and *NCED6,* followed by cloning into the binary vector pCanG-HA-GFP under the control of the CaMV 35S promoter. Transformation of Arabidopsis was conducted by the vacuum infiltration method using *Agrobacterium tumefaciens* strain *EHA105* (Bechtold and Pelletier, 1998). T_2 seeds were germinated on MS plates containing 50 mg ml^{-1} kanamycin, and the resistant seedlings were transferred to soil to obtain homozygous T_3 seeds. The expression levels of the transgenes were determined by qRT-PCR analyses. Independent T_3 homozygous lines containing a single insertion were employed in the subsequent phenotypic and physiological analyses.

To generate *ga2ox7* and *nced6* plants overexpressing *ABI4-GFP,* the *35S-ABI4-GFP* construct was crossed into the *ga2ox7* and the *nced6* background, respectively. The F_2 progenies were tested by PCR using the specified primers for *ga2ox7* and *nced6* genotyping. The ABI4-GFP protein level was monitored through immunoblot analysis for all lines, and the lines which possessed a comparable ABI4-GFP protein level were selected and used for further genetic analysis.

Gene expression analyses

Total RNA preparation from about 2-week-old seedlings, first-strand cDNA synthesis and qRT-PCR were performed following a previous protocol (Cui *et al.*, 2012). DNaseI-treated total RNA (2 μg) was denatured and subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase (200 units per reaction; Promega, http://www.promega.com/). Quantitative RT-PCR was performed using the SsoFast™ EvaGreen Supermix (Bio-Rad, http://www.bio-rad.com/) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Gene expression was quantified at the logarithmic phase using expression of the housekeeping *18S* RNA as an internal control.

Genetic and immunoblot analyses

For total protein extracts, 2-week-old seedlings were ground in liquid nitrogen and extracted with 4 M urea buffer. Crude extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were stained with 0.2% Ponceau S, with Rubisco functioning as an internal control. The antibody to GFP was purchased from Santa Cruz Biotechnology, Inc. (http://www.scbt.com/). For the experiment on the effect of ABA and GA on ABI4 protein stability, 2-week-old homozygous *35S-ABI4-GFP* seedlings were treated with 50 μM ABA plus 80 μM CHX, or 50 μM GA plus 80 μM CHX, for the time indicated in the text.

Protein quantification and normalization

To quantify and normalize the amount of protein in the protein degradation assay, we employed the standard software ImageJ (National Institutes of Health, http://imagej.nih.gov/ij/) according to a previous protocol (Zhang *et al.*, 2011a). The percentage of ABI4-GFP remaining in the degradation assay was normalized by the formula $P_t = (C_t/L_t) \times 100\%$, where P_t is the percentage of the test protein remaining in each sampling time point, C_t is the quantified content of the test protein in each sampling time point and L_t is the loading content in each sampling time point. In particular, $P_0 = (C_0/L_0) \times 100\%$, where P_0 is the percentage of the test protein remaining at 0 h, C_0 is the quantified content of the test protein at 0 h and L_0 is the loading content at 0 h. P_0 was normalized as 1.00, and the values of different P_ts were calculated.

Quantification of ABA

Two-week-old seedlings were ground in liquid nitrogen, and 250 mg frozen powder was homogenized and extracted for 24 h in methanol containing D6-ABA (purchased from OIChemIm Ltd, http://www.olchemim.cz/) as an internal standard. Purification was performed with an Oasis Max solid phase extract cartridge (150 mg/6 cm^3; Waters, http://www.waters.com/) and eluted with 5% formic acid in methanol. The elution was dried, reconstituted and finally injected into a liquid chromatography–tandem mass spectrometry system consisting of an Acquity ultra performance liquid chromatograph (Acquity UPLC; Waters) and a triple quadrupole tandem mass spectrometer (Quattro Premier XE; Waters). Three biological replicates were performed.

Quantification of GA

The endogenous GA content was determined in the Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), Wuhan University, China, by a previous method (Chen

et al., 2011). Briefly, Arabidopsis seedlings (100 mg) were frozen in liquid nitrogen, ground to a fine powder and extracted with 80% (v/v) methanol. Before grinding GA isotope standards were added to the plant samples. The crude extracts were purified by reverse-phase solid-phase extraction, ethyl ether extraction and derivatization. The resulting mixture was injected into a capillary electrophoresis-mass spectrometer (CE-MS) for quantitative analysis.

Chromatin immunoprecipitation-qPCR assay

Chromatin Immunoprecipitation was performed as previously described with minor modifications (Lu *et al.*, 2011). *35S-ABI4-GFP* transgenic seedlings grown on 1/2 MS plates for about 2 weeks were sampled (1.5 g) and cross-linked by 1% formaldehyde for 30 min in a vacuum, and stopped by 0.125 M glycine. Seedlings were ground in liquid nitrogen and nuclei were isolated. Immunoprecipitations were performed with anti-GFP antibody and protein G beads. DNA was precipitated by isopropanol, washed by 70% ethanol and then dissolved in 10 μl water within 20 μg ml^{-1} RNase. Absence of anti-GFP functions as a control. Quantitative PCR analysis was performed using specific primers corresponding to different promoter regions of *GA2ox7* and *NCED6*. *TUB4* was used as an internal control. Because it is known that ABI4 binds directly to the promoter of *ABI5* (Bossi *et al.*, 2009), we employed the *ABI5* promoter as a positive control.

In vivo transient analysis of *GA2ox7* and *NCED6* promoter activity by ABI4

Native *GA2ox7* and *NCED6* promoters (Pro- *GA2ox7* and Pro-*NCED6*) were amplified by PCR from genomic DNA. Primer sequences are listed in Table S1. Fragments of both promoters were cloned in the *pCambia1300-221* vector by replacing the original CaMV 35S promoter, thus generating the *Pro-GA2ox7-GUS* and *Pro-NCED6-GUS* constructs. The effector construct *pCanG-ABI4-GFP* was generated in our previous study (Shu *et al.*, 2013). *Agrobacterium tumefaciens*-mediated tobacco transient transformation was performed according to our previous protocol (Liu *et al.*, 2010). Briefly, *Agrobacterium* cells containing the appropriate construct combinations were cultured at 28°C overnight, collected, re-suspended with infiltration buffer and infiltrated into healthy tobacco (*N. benthamiana*) leaves. Total proteins were extracted from the infiltrated leaves and GUS activity was determined using the protocol described previously using 4-methylumbelliferyl-β-D-glucuronide (Sigma-Aldrich) as a substrate (Jefferson *et al.*, 1987). The total protein was quantified using the Bradford protein assay kit method (Bio-Rad). At the same time, histochemical staining for GUS was performed on leaves at 3 days after infiltration using a hole punch according to a protocol published elsewhere (Stalberg *et al.*, 1993). Photographs were taken using a Leica MZ16 FA stereomicroscope (Leica Company, http://www.leica.com/).

ACCESSION NUMBERS

Arabidopsis Genome Initiative locus identifiers for the major genes mentioned in this article are as follows: *ABI4* (AT2G40220), *ABI5* (AT2G36270), *GA2ox7* (AT1G50960), *NCED6* (AT3G24220), *GA20ox1* (AT4G25420), *GA20ox2* (AT5G51810), *AtEXP2* (AT5G05290), *NCED3* (AT3G14440), *ABA3* (AT1G16540), *RGL2* (AT3G03450), *RGL3* (AT5G17490), *DDF1* (AT1G12610), *CYP707A2* (AT2G29090) and *CYP707A3* (AT5G45340). The stock numbers of the mutants used in this study are as follows: *abi4* (CS8104), *ga2ox7* (SALK_055721C) and *nced6* (WiscDsLox356H02, also named CS852600).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Quantification of selected phenotypes of wild-type, *abi4* and *OE-ABI4* plants at different developmental stages.

Figure S2. Size analysis of wild-type, *abi4* and *OE-ABI4* seeds.

Figure S3. Effects of ABI4 loss or overproduction on selected gibberellin and ABA biogenesis genes.

Figure S4. ABI4 regulates the transcription profiles of specific gibberellin and ABA biogenesis genes.

Figure S5. Effect of exogenous ABA on *CYP707A2* and *CYP707A3* transcription.

Figure S6. ABI4 does not affect *DDF1* transcription.

Figure S7. ABI4 activates *NCED6* transcription *in vivo*.

Figure S8. Confirmation of *OE-ABI4::abi5-7* transgenic lines by quantitative PCR.

Figure S9. Opposite effect of ABA and gibberellin on *ABI4* transcription.

Figure S10. Exogenous ABA and gibberellin have no effect on the stability of Myc-GFP protein.

Table S1. Primers used in this study.

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