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Abscisic Acid Antagonizes Ethylene Production through the ABI4-Mediated Transcriptional Repression of *ACS4* and *ACS8* in *Arabidopsis*

Zhijun Dong[1,2,3,4], Yanwen Yu[2,4], Shenghui Li[2,4], Juan Wang[2,3], Saijun Tang[1,*] and Rongfeng Huang[2,3,*]

[1]College of Biological Sciences, China Agricultural University, Beijing 100193, China

[2]Plant Molecular Biology Department, Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China

[3]National Key Facility of Crop Gene Resources and Genetic Improvement, Beijing 100081, China

[4]These authors contributed equally to this article.

***Correspondence: Rongfeng Huang (rfhuang@caas.cn), Saijun Tang (saijuntang@gmail.com)**

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ABSTRACT

Increasing evidence has revealed that abscisic acid (ABA) negatively modulates ethylene biosynthesis, although the underlying mechanism remains unclear. To identify the factors involved, we conducted a screen for ABA-insensitive mutants with altered ethylene production in *Arabidopsis*. A dominant allele of *ABI4*, *abi4-152*, which produces a putative protein with a 16-amino-acid truncation at the C-terminus of ABI4, reduces ethylene production. By contrast, two recessive knockout alleles of *ABI4*, *abi4-102* and *abi4-103*, result in increased ethylene evolution, indicating that ABI4 negatively regulates ethylene production. Further analyses showed that expression of the ethylene biosynthesis genes *ACS4*, *ACS8*, and *ACO2* was significantly decreased in *abi4-152* but increased in the knockout mutants, with partial dependence on ABA. Chromatin immunoprecipitation–quantitative PCR assays showed that ABI4 directly binds the promoters of these ethylene biosynthesis genes and that ABA enhances this interaction. A fusion protein containing the truncated ABI4-152 peptide accumulated to higher levels than its full-length counterpart in transgenic plants, suggesting that ABI4 is destabilized by its C terminus. Therefore, our results demonstrate that ABA negatively regulates ethylene production through ABI4-mediated transcriptional repression of the ethylene biosynthesis genes *ACS4* and *ACS8* in *Arabidopsis*.

Keywords: ABA, ABI4, ethylene biosynthesis, stress response, transcriptional regulation

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INTRODUCTION

Gaseous ethylene is an important phytohormone in the regulation of plant development (e.g., floral organs, fruit ripening, and senescence) and stress response (Guo and Ecker, 2004; Ji and Guo, 2013). Ethylene biosynthesis consists of three major steps. First, the ethylene precursor methionine is converted to *S*-adenosylmethionine (S-AdoMet). Then, 1-aminocyclopropane-1-carboxylic acid (ACC) is synthesized by ACC synthase (ACS) using S-AdoMet as a substrate. Finally, ethylene is released from ACC by ACC oxidase (ACO) (Yang and Hoffman, 1984). Ethylene production can be induced by floral organ development, ripening, senescence, and stress (Yamagami et al., 2003; Tsuchisaka and Theologis, 2004; Lin et al., 2009). Moreover, a variety of transcription factors regulate the transcriptional levels of the *ACS* and *ACO* genes. For example, the transcription factor hypocotyl elongation 5 (HY5), an integrator of light- and abscisic acid (ABA)-induced responses (Chen et al., 2008), participates in the regulation of ethylene biosynthesis through transcriptional modulation of the ethylene response factor (ERF) repressor AtERF11, which further suppresses the expression of *ACS2/5* and ethylene production (Li et al., 2011).

Increasing evidence has shown that the stability of ACS proteins is regulated by their C-terminal regions. MPK6-mediated phosphorylation of the C-terminal amino acid residues in ACS2/ACS6 significantly increases the levels of these proteins and ethylene production (Hernandez Sebastia et al., 2004; Liu and

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A Segregation of seed germination in response to ABA in F_2 population from the cross between *abi152* and col-0

Combination	Total number of seeds	Insensitive	Sensitive	Expected rate	χ^2
abi152/Col-0	326	251	72	3:1	0.7000

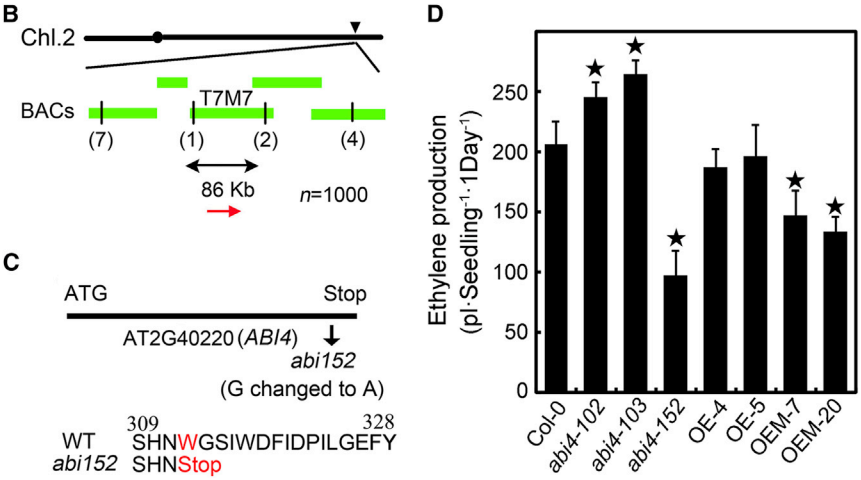

Figure 1. *abi4-152* Is a Dominant *ABI4* Mutant that Shows Decreased Ethylene Emission.

(A) Genetic segregation assay in response to ABA. Insensitive and sensitive indicate that the seeds were germinated or not germinated in 0.5 μM ABA containing MS medium, respectively.

(B) Physical map of the *abi152* locus. The numbers of recombinants are shown in parentheses.

(C) Mutation site of *ABI4* in *abi152*.

(D) Ethylene emission in 4-day-old seedlings. The values are the means ± SE (n = 3). P values (★: mutant versus Col-0) were determined with a two-tailed Student's t-test at $P < 0.05$.

Zhang, 2004). Similarly, *Arabidopsis* ethylene overproduction in *eto2* and *eto3* mutants was caused by disrupted C-terminal extensions in ACS5 and ACS9, respectively. These mutant proteins are more stable because they are more resistant to degradation by the 26S proteasome (Vogel et al., 1998a; Chae et al., 2003; Joo et al., 2008). ETO1, a component of the E3-ligase complex, directly interacts with ACS5, and mutated forms of ETO1 lead to increased stability of the ACS5 protein and the overproduction of ethylene (Guzman and Ecker, 1990; Wang et al., 2004). ABA treatment prevents the induction of ethylene biosynthesis (Wright, 1980; Li et al., 2011), whereas ethylene production is increased in the ABA-deficient mutant *aba2-1* compared to the wild type (LeNoble et al., 2004). Moreover, the ABA-activated CDPK protein kinases CPK4 and CPK11 can stabilize ACS6 by phosphorylating its C-terminus, promoting ethylene biosynthesis (Luo et al., 2014), and ABI1 regulates ozone-induced ethylene biosynthesis by affecting the ACS6 phosphorylation level, which is controlled by MPK6 (Ludwikow et al., 2014). These findings reveal an antagonistic interaction between ABA and ethylene biosynthesis that is regulated by protein phosphorylation. However, the mechanism of how ABA represses ethylene biosynthesis at the transcriptional level remains to be investigated.

ABI4 is a transcription factor involved in many aspects of plant development and stress responses (Finkelstein et al., 1998; Söderman et al., 2000; Penfield et al., 2006; Kerchev et al., 2011; Lee et al., 2015). This protein functions as both a repressor and an activator (Wind et al., 2013), and is partially regulated by the 26S proteasomal pathway (Finkelstein et al., 2011). In the present study, we identified *abi4-152*, a dominant mutation that converts Trp 313 to a premature termination codon in ABI4, resulting in a 16-amino-acid truncation of the C terminus. The fusion of ABI4-152 peptide (a truncated version of ABI4 in *abi4-152*) accumulated to higher levels than full-length ABI4 in plant cells, indicating that these 16 amino acids affect the protein's stability. The *abi4-152* mutant was insensitive to low concentrations of ABA and produced less ethylene, revealing both the repressor and activator biochemical function of ABI4. Thus, our results demonstrate that ABA negatively regulates ethylene production in *Arabidopsis* by repressing the expression of the ethylene biosynthesis genes *ACS4* and *ACS8*.

RESULTS

ABI4 Is a Negative Regulator in Ethylene Production

To identify factors in ABA-regulated ethylene biosynthesis, we screened ethyl methanesulfonate (EMS)-mutagenized seeds for mutants that could germinate on 0.5 μM ABA, which is inhibitory to the wild-type seeds (Supplemental Figure 1A). Four independent ABA-insensitive mutants were selected for further investigation. Relative to the wild type, the *abi227* and *abi304* mutants produced significantly more ethylene, whereas *abi81* and *abi152* produced only half that of wild type (Supplemental Figure 1B).

Although the *Arabidopsis* mutants *eto1*, *eto2*, *eto3*, *hy5*, and *xbat32* produce elevated levels of ethylene (Guzman and Ecker, 1990; Woeste et al., 1999; Chae et al., 2003; Li et al., 2011; Lyzenga et al., 2012), no single mutant with reduced ethylene production has been previously reported. Therefore, we focused our experiments on the mutant *abi152*, which showed reduced ethylene production. Phenotypic segregation experiments indicated that *abi152* is a dominant allele (Figure 1A), and genetic mapping revealed that the mutation is between the single nucleotide polymorphism markers S76 and S84 on the long arm of chromosome 2 (Figure 1B). DNA sequencing analysis of the genomic DNA in this interval revealed a single nucleotide change of G to A in the *ABI4* gene in *abi152*, which was identical to that of *abi81*, as noted above. This mutation converts Trp 313 to a premature termination codon, resulting in a 16-amino-acid truncation of the ABI4 C terminus (Figure 1C). ABI4 is a member of the ERF family and is an important element in the ABA signaling pathway (Finkelstein et al., 1998), and several recessive loss-of-function

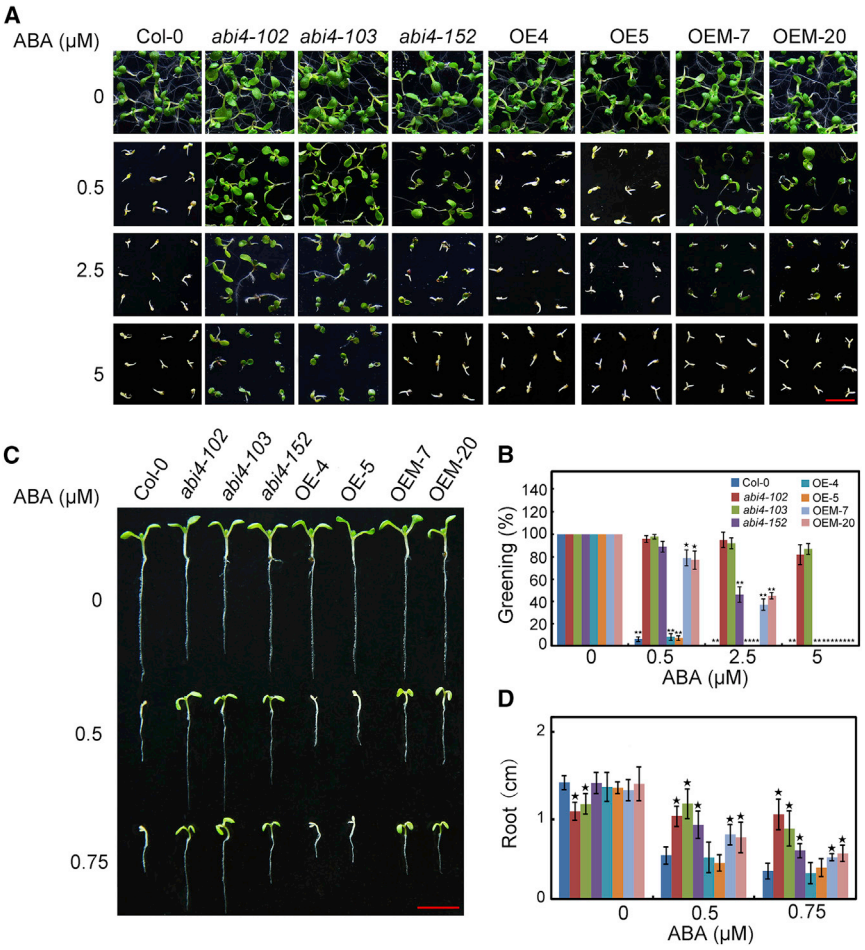

Figure 2. *abi4-152* Is Not Resilient to High Concentrations of ABA.

Phenotypic images **(A)** and statistical data **(B)** of seedling greening in response to ABA treatment for 7 days. Seedling growth **(C)** and statistical data **(D)** of root length in response to ABA treatment for 4 days. Greening and root lengths were determined with an average of >50 seedlings from three independent experiments. The values are expressed as the mean ± SE (n = 3). P values in **(B)** (★, treatment versus the corresponding control) and **(D)** (★, mutant versus Col-0 with the corresponding treatment) were determined with a two-tailed Student's t-test at $P < 0.05$ (★) or $P < 0.01$ (★★). Scale bar in **(A)** and **(C)** represents 0.5 cm.

abi4 alleles have been isolated (Finkelstein et al., 1998; Arenas-Huertero et al., 2000). Thus, the *abi152* mutant was renamed *abi4-152*.

To determine whether the C-terminal truncation of ABI4 is responsible for reduced ethylene production and the ABA-resistant phenotype in *abi4-152* mutants, constructs containing the *ABI4* full-length cDNA, or ABI4-152 peptide cDNA under the control of a 2× cauliflower mosaic virus 35S promoter, were generated. The resulting transgenic plants overexpressing the full-length *ABI4* were designated OE, and the lines overexpressing the ABI4-152 peptide were designated OEM. An analysis of the transgenic plants showed that, compared with that of Col-0, ethylene emission was significantly reduced in OEM transgenic lines but was not obviously reduced in OE lines (Figure 1D). We next investigated whether ethylene production was altered in the recessive *abi4* knockouts *abi4-102* and *abi4-103*, which bear nonsense mutations (Supplemental Figure 2) (Söderman et al., 2000). Ethylene evolution was increased in the two knockouts compared with Col-0 (Figure 1D), demonstrating that ABI4 is a negative regulator of ethylene production.

It has previously been reported that the C-terminal half of the ABI4 protein has several features, such as an acidic domain and a proline-rich domain, suggesting that ABI4 might be a transcriptional activator Söderman et al., (2000). However, increasing evidence has revealed that ABI4 functions as both a repressor and an activator, depending on the target genes (Wind et al., 2013). Considering that the ABI4-152 peptide is shortened by only 16 amino acids at its C terminus, we investigated whether the ABI4-152 peptide in *abi4-152* retains the function of its full-length counterpart. To test this hypothesis, various ABI4 fragments were fused to the GAL4 DNA-binding domain (GAL4-BD) to detect their ability to activate β-galactosidase in yeast. Consistent with a previous report that ABI4 has transcriptional activation functions at its C terminus (Söderman et al., 2000), we found that a fusion protein containing the full-length ABI4 activated β-galactosidase in yeast. Removal of the acidic N-terminal domain (amino acids 1–187) had no influence on its transcriptional activation. However, deletion of the amino acids between 188 and 328 or between 188 and 307 in ABI4 resulted in almost complete loss of its transcriptional activity, whereas deletion of the C-terminal 16 amino acids (313–328) had no effect on the protein's activation function (Supplemental Figure 3). These findings indicated that the *abi4-152* might not affect the transcriptional activity of full-length ABI4.

abi4 Alleles Display Altered ABA and Ethylene Responses

Compared with Col-0, *abi4-152* and OEM were less sensitive to low levels of ABA (less than 2.5 μM). In contrast, mutants with loss-of-function *abi4* alleles were insensitive to high concentrations of ABA (more than 2.5 μM) during seed germination (Supplemental Figure 4A) and seedling growth (Figure 2A and 2B). A similar trend was observed for root elongation in Murashige and Skoog (MS) medium containing less than 0.75 μM ABA (Figure 2C and 2D) and high salinity (100 mM NaCl) (Supplemental Figure 4B and 4C). These observations indicated that *abi4-152* is more sensitive than *abi4-102* and *abi4-103* to ABA. We noted that these physiological responses were unaltered in the OEM lines compared with *abi4-152*, suggesting that the endogenous level of the ABI4-152 peptide

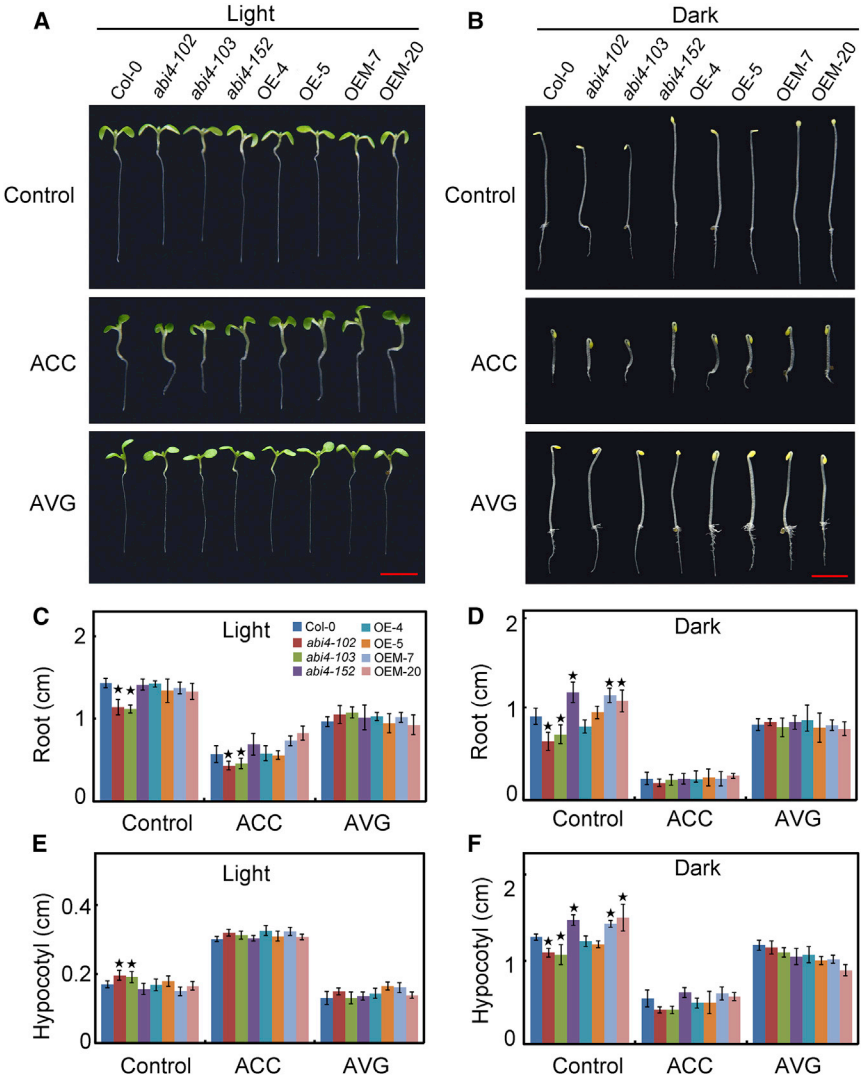

Figure 3. The Loss of *ABI4* Function Results in an Ethylene Response.

Representative phenotypic images of **(A)** light-grown and **(B)** dark-grown seedlings, and the statistical data of root **(C and D)** and hypocotyl **(E and F)** lengths after 4 days of growth on MS plates with or without 5 μM ACC or 0.2 μM AVG. Hypocotyl and root lengths were determined with an average of >50 seedlings from three independent experiments. The values are the means ±SE (n = 3). P values (★, mutant versus Col-0 with the corresponding treatment) were determined with a two-tailed Student's t-test at $P < 0.05$. Scale bar in **(A)** and **(B)** represents 0.5 cm.

is sufficient to saturate and block the ABI4-mediated ABA response. The regulation of the ABI4-152 peptide in reducing ABA sensitivity, resembling the recessive loss-of-function *abi4* alleles, reveals that the *abi4-152* may function as a dominant-negative allele in ABA response.

We next observed the ethylene response in different genotypes (Zhong et al., 2012; Yu et al., 2013). Compared with Col-0 under normal growth conditions, the root length was shorter in the light-grown and dark-grown *abi4-102* and *abi4-103* seedlings but longer in the *abi4-152* and transgenic OEM lines in dark-grown seedlings, although the root growth did not change in the light-grown *abi4-152* OEM and OE seedlings or in the dark-grown OE seedlings (Figure 3A–3D). Similarly, the hypocotyl length of the light-grown seedlings was longer in *abi4-102* and *abi4-103* but did not change in *abi4-152* and transgenic OEM or OE lines relative to Col-0 (Figure 3A and 3E). In contrast, the hypocotyl length of dark-grown seedlings was shorter in the knockouts, longer in *abi4-152* and the transgenic OEM lines, and unchanged in the OE lines relative to Col-0 (Figure 3B and 3F). These data suggest that ABI4 affects the ethylene response.

When the ethylene precursor ACC or an inhibitor of ethylene production, aminoethoxyvinylglycine (AVG), was added to the medium, all of the genotypes displayed root and hypocotyl lengths similar to those of Col-0, but the *ABI4* knockouts displayed much shorter root lengths after the addition of ACC in the light-grown seedlings (Figure 3). This result indicated that ABI4 affects ethylene biosynthesis, and specifically affects the growth of the hypocotyl and root. The results in Figure 3A and 3C also suggest that root elongation in the light-grown *abi4-152*, OE and OEM seedlings was slightly more resistant to inhibition by ACC and that the loss-of-function allelic mutants showed the opposite response. This complementarity provides solid evidence for the role of ABI4 in root elongation, which is consistent with the observation that the inhibitory effect of ABA on root growth is mediated by the regulation of ethylene biosynthesis (Luo et al., 2014).

We then tested whether the ethylene-responsive genes *ERF1* and *ERF5* (Solano et al., 1998; Moffat et al., 2012) were transcriptionally altered in the different genotypes. In the absence of ABA, the transcript levels of *ERF1*, but not *ERF5*, were higher in the two knockouts, lower in *abi4-152* and OEM, and unchanged in OE. Although ABA treatment significantly decreased the expression of *ERF1* and *ERF5* in Col-0 and OE, the transcripts remained much higher in the knockouts and were lower in *abi4-152* and OEM (Supplemental Figure 5), suggesting that the regulation of these two ethylene-responsive genes by ABI4 is ABA dependent.

ABI4 Represses the Expression of *ACS4/8* and *ACO2*

We then asked whether the regulation of ABI4 in ethylene production is dependent on ABA. In the absence of ABA, ethylene production was significantly increased in the loss-of-function mutants, decreased in *abi4-152* and OEM, and unchanged in the OE lines. Although ABA treatment led to a general decrease in ethylene production, the loss-of-function mutants were the

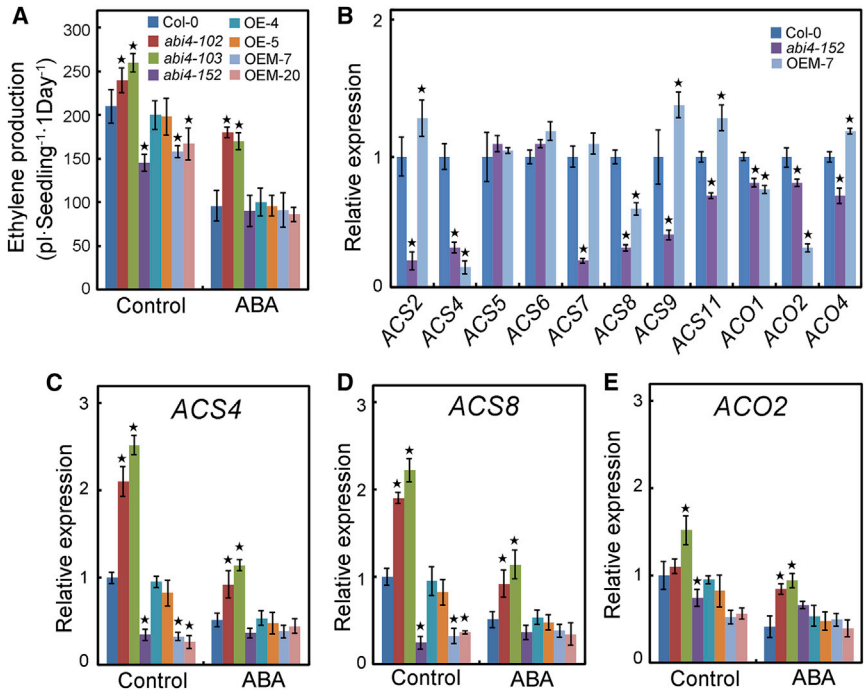

Figure 4. ABA-Mediated ABI4 Reduces Ethylene Production by Repressing the Expression of *ACS4*, *ACS8*, and *ACO2*.

(A) The suppressive effects of ABA on ethylene emission.

(B) The screen to identify ethylene biosynthesis genes repressed by ABI4.

(C–E) Repressive effects of ABA on the expression of ethylene biosynthesis genes in different phenotypes. Seedlings were grown for 4 days and were then treated with or without 5 μM ABA for 4 h. The transcripts were quantified by qPCR and normalized to *TUB4*. The values represent the means ± SE (n = 3). P values (★, mutant versus Col-0 with the corresponding treatment) were determined with a two-tailed Student's t-test at $P < 0.05$.

most resilient to this ABA effect (Figure 4A), demonstrating that ABI4 is required for the ABA-mediated repression of ethylene production.

We next speculated that ABI4 represses the expression of ethylene biosynthesis genes. To address this query, we first quantified the transcripts of all of the ethylene biosynthesis genes and found that the expression of *ACS4*, *ACS8*, and *ACO2* was significantly reduced in *abi4-152* and OEM7 (Figure 4B). This repression by ABI4 was further confirmed in different genotypes; specifically, the expression of *ACS4* and *ACS8* was significantly enhanced in the knockouts, greatly reduced in *abi4-152* and OEM, and unchanged in the OE lines (Figure 4C and 4D). ABA treatment significantly decreased the expression of *ACS4* and *ACS8* in Col-0 to nearly that of *abi4-152*, OEM, and OE, but the transcription of these two genes in the knockouts was not significantly decreased (Figure 4C and 4D). There was no significant difference in *ACO2* expression in the different genotypes except in the OEM lines in the absence of ABA. However, after ABA addition, the transcripts of *ACO2* were higher in the two knockouts but were not further reduced in *abi4-152*, OEM, and OE (Figure 4E). These results indicated that the *ACS4*, *ACS8*, and *ACO2* transcripts in *abi4-152* and OEM were reduced and that the loss-of-function mutants showed the opposite response, demonstrating that the ABA-mediated transcriptional repression of ethylene biosynthesis genes, such as *ACS4*, *ACS8*, and *ACO2*, takes place through the function of ABI4. In addition, the mutation of *abi4-152* allele behaves genetically as dominant-positive regulator in suppressing ethylene biosynthesis.

ABI4 is a versatile transcription factor that can promote or inhibit the expression of multiple genes by directly binding to the CACCG motif or the CCAC element (Koussevitzky et al., 2007; Bossi et al., 2009). We investigated whether ABI4 can directly bind to the upstream sequences of the *ACS* and *ACO* genes. Sequence analysis indicated that there are five, six, and four CCAC motifs upstream of the *ACS4*, *ACS8*, and *ACO2* coding regions, respectively (Figure 5A). We conducted chromatin immunoprecipitation (ChIP)–quantitative PCR (qPCR) with the transgenic lines, using the *ABI4* and *ABI5* promoters as positive controls because ABI4 binds directly to these sequences (Bossi et al., 2009). Whereas ChIP–qPCR analysis with chromatin precipitated from OE transgenic lines did not yield any signals, pretreatment of the OE and OEM seedlings with MG132 yielded binding of ABI4 or the ABI4-152 peptide to the upstream sequences of *ACS4*, *ACS8*, *ACO2*, *ABI4*, and *ABI5* in all transgenic lines. When the plant materials were treated with 5 μM ABA, the binding activity was increased relative to that in the control conditions (Figure 5B and 5C). In the absence of a functional *ABI4*, we did not detect the same sequences in the immunoprecipitates (Figure 5B and 5C). Because the upstream regions of *ACS* and *ACO* genes in *Arabidopsis* that have demonstrated promoter activities (Tsuchisaka and Theologis, 2004) overlap with those bound by ABI4, our results suggested that ABI4 directly interacts with the promoter elements to repress the expression of *ACS4*, *ACS8*, and *ACO2*.

The C-Terminal Region Contributes to ABI4 Stability

Extensive studies have shown that ABI4 is regulated post-transcriptionally. In one study, the transcript was expressed constitutively at high levels, but no protein was detected in the ABI4 transgenic plants (Finkelstein et al., 2011). Thus, we tested whether the ABI4-152 peptide was more stable than its full-length counterpart. To address this question, we first confirmed the transcription of ABI4 in different transgenic plants using RT–PCR (Supplemental Figure 6A) and qPCR (Supplemental Figure 6B). The *ABI4* transcripts were undetectable in Col-0 but were greatly increased in all of the transgenic plants (Supplemental Figure 6A and 6B). Next, the transgenic plants expressing both full-length ABI4 and the ABI4-152 peptide fused to GFP were used to compare the fluorescence strength. In our assays, the general translational inhibitor cycloheximide (CHX) was used to pretreat the seedlings to exclude *de novo* fusion protein synthesis. Then the GFP fluorescence in the

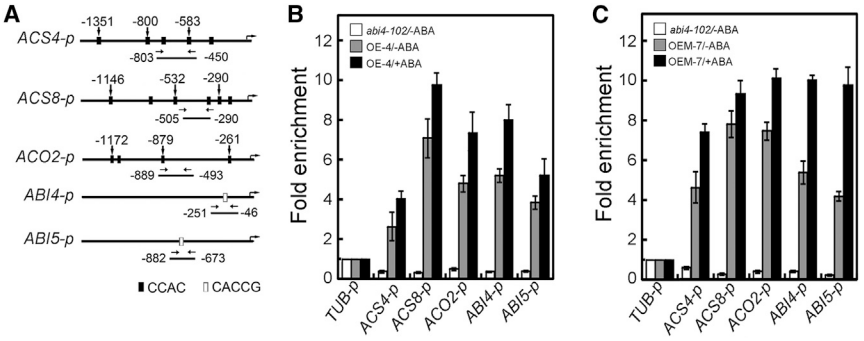

Figure 5. ABI4 Directly Binds to the Promoter Sequences of *ACS4*, *ACS8*, and *ACO2*.

(A) Upstream sequence analysis of *ACS4*, *ACS8*, and *ACO2*. Fragments from −1 to −1500 bp upstream of ATG were chosen as the promoter regions.

(B and C) Representative results of ChIP–qPCR with chromatin precipitated from transgenic plants pretreated for 6 h with 50 μM MG132 with 5 μM ABA or 50 μM MG132 alone, respectively. *TUB4-P* was employed as an internal control. The promoters of *ABI4* and *ABI5* were used as positive controls. The experiments were performed with three biological replicates, which showed similar results.

roots was imaged using a confocal laser-scanning microscope. We observed that the fluorescence of the ABI4 fusion protein 35S:ABI4-GFP was weak after 6 h, whereas the fluorescence of the ABI4-152 peptide (ABI4M) fusion 35S:ABI4M-GFP persisted 12 h after the addition of the protein synthesis inhibitor (Figure 6A). These results indicated that the ABI4-152 fusion peptide is more stable in plants than its full-length counterpart. This fluorescence-based interpretation was further supported by immunoblotting (Figure 6B). These results suggest that the 16 amino acids at the C-terminal region of ABI4 are critical for the stability of the ABI4 protein.

DISCUSSION

Ethylene biosynthesis is highly regulated by multiple exogenous and endogenous factors (Yamagami et al., 2003; Tsuchisaka and Theologis, 2004; Peng et al., 2005), and the crosstalk of ABA and ethylene is important for multiple physiological processes (Beaudoin et al., 2000; Ghassemian et al., 2000; Luo et al., 2014). Although ABA treatment reduces ethylene production (Wright, 1980; Li et al., 2011), until now the underlying mechanism has remained uninvestigated. In this paper, we report that *abi4-152* is the first identified single allele that disrupts ethylene production by reinforcing the function of the wild-type protein. Also, our experimental data demonstrated the transcriptional connection between ABA and ethylene biosynthesis through ABI4. Moreover, the novel *abi4-152* allele revealed biochemical functions of the ABI4 protein as both a repressor and an activator, supporting the dominant-positive versus dominant-negative interpretations of the mutation depending on whether the protein is associated with ABA sensitivity or ethylene production. We also showed that the ABI4 C-terminal segment controls the stability of the ABI4 protein, which is in contrast to previous claims (Finkelstein et al., 2011; Gregorio et al., 2014).

The *eto1*, *eto2*, and *eto3* mutants are disrupted in their mechanism that inhibits ethylene production, leading to ethylene overproduction (Guzman and Ecker, 1990; Vogel et al., 1998a; Chae et al., 2003; Wang et al., 2004; Joo et al., 2008). Except for the mutation of multiple *ACS* genes in one plant (Tsuchisaka et al., 2009), single mutations causing reduced ethylene production have not been previously reported. In the pentuple mutant *acs2 acs4 acs5 acs6 acs9*, ethylene production is inhibited by approximately 40% in 5-day-old, light-grown seedlings and by up to almost 80% in 1-month-old plants. Moreover, low ethylene production in the pentuple mutant is accompanied by earlier flowering and greater height (Tsuchisaka et al., 2009). In our report, we found that the single mutant *abi4-152* displayed reduced ethylene production in 4-day-old, light-grown seedlings. In addition, the hypocotyl length in light-grown and etiolated seedlings was greatly affected by the altered ethylene emission in different alleles of *ABI4*. Although ABI4 was initially found to play a role in ABA signaling, substantial experimental evidence has demonstrated its function in other biological events (Finkelstein et al., 1998; Acevedo-Hernandez et al., 2005; Lee et al., 2015). For example, ABI4 is essential for the regulation of ascorbate-dependent growth (Kerchev et al., 2011) and salt tolerance (Shkolnik-Inbar et al., 2012). ABI4 also functions in retrograde signaling to the nucleus from both chloroplasts and mitochondria (Koussevitzky et al., 2007; Giraud et al., 2009; Zhang et al., 2013). In this research we further found that the *ABI4* knockouts showed increased, while dominant allele *abi4-152* decreased, ethylene emission and ethylene response, demonstrating the essential regulatory role of ABI4 in ethylene biosynthesis. Moreover, ABI4-152 peptide suppresses ethylene evolution more strongly than the wild-type counterpart because the peptide retains the DNA-binding activity and is stable. Moreover, the expression of ethylene biosynthesis genes, including *ACS4*, *ACS8*, and *ACO2*, was significantly reduced in *abi4-152* and OEM but was greatly increased in ABI4 knockouts. It seems that the ABI4-152 peptide is a "stronger" suppressor relative to its wild-type counterpart because of the increased stability of the truncated peptide. The "gain" in stability of the ABI4-152 peptide is the characteristic that gives this mutation its genetic dominance, revealing that *abi4-152* behaves as a dominant-positive mutation in suppressing ethylene production.

The transcriptional activation assay in yeast showed that the ABI4-152 peptide and its full-length counterpart both function as transcriptional activators. Moreover, other studies have demonstrated that ABI4 decreases the expression of the sodium transporter HKT1 in salt tolerance (Shkolnik-Inbar et al., 2012). The data presented here suggest that ABI4 represses the expression of the *ACS4* and *ACS8* genes by directly binding to their promoters, highlighting the transcriptional connection between ABA and ethylene biosynthesis through transcription factor ABI4, with ABA as the trigger. This novel discovery is also in line with a recent report that the phosphorylation of ACS6 by ABA-activated CDPK protein kinases affects the

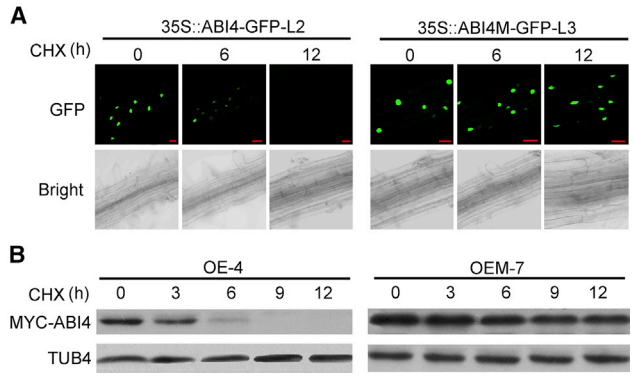

Figure 6. The C Terminus of ABI4 Confers Stability to the ABI4 Protein.

(A) Confocal microscopic images of GFP fluorescence in the roots. Scale bars represent 5 μm.

(B) Immunoblotting of the fusion protein in 4-day-old seedlings pretreated with 100 μM CHX. Ten micrograms of protein extracts was used for immunoblotting with anti-Myc and anti-tubulin antibodies (as a loading control). These images were selected as representatives of at least three biological replicates with similar results.

stability of the ACS6 protein and, subsequently, the promotion of ethylene biosynthesis (Luo et al., 2014). The evidence in this report, which pertains to phosphorylation-based regulatory mechanisms, provides a solid link between ABA and ethylene biosynthesis via the transcriptional repression of ABI4.

Accumulating evidence has supported the importance of ABI3, ABI4, and ABI5 as transcription factors in ABA signaling (Giraudat et al., 1992; Finkelstein et al., 1998; Miura et al., 2009; Delmas et al., 2013). Both ABI3 and ABI5 proteins are unstable *in vivo*, and the E3 ligases AIP2 and KEG, respectively, target these proteins for post-translational destruction (Zhang et al., 2005; Liu and Stone, 2010). Although the accumulation of ABI4 is tightly regulated post-transcriptionally (Finkelstein et al., 2011), little is known about how ABI4 is regulated at the protein level, owing to the difficulty of detecting the ABI4 protein *in planta*. Previous studies have demonstrated that PEST sequences at the N-terminal region are involved in mediating the instability of the ABI4 protein (Finkelstein et al., 2011; Gregorio et al., 2014). In addition, degradation by the proteasome is independent of the C-terminal half of ABI4 (Finkelstein et al., 2011). In the present report, biochemical and fluorescence assays showed that the C-terminal region contributes to the instability of the ABI4 protein. Coincidentally, the instability of the ABI4 protein has no physiological effect in OE lines, whereas the stable ABI4-152 peptide enhances the phenotypes in *abi4-152* and OEM in comparison with the original Col-0. All of these results converge on the fact that ABI4 is not strongly controlled transcriptionally in those ABA sensitivity tests and ethylene production described in this work, but these responses are regulated largely through its protein stability. Thus, the allele *abi4-152*, in contrast to the other identified *abi4* alleles, provides an excellent opportunity for elucidating how the C-terminal region regulates protein stability.

The ABI4-152 peptide mutants described in this report (*abi4-152* and OEM), which have increased ABI4 stability and retain ABI4 transactivation activity, have reduced ABA sensitivity, as do the *abi4-102* and *abi4-103* knockout mutants. In this case, *abi4-152* and OEM may act in a dominant-negative fashion. The dominant-negative effect also predicts that wild-type ABI4 will be found in a complex involved in the positive regulation of ABA responses. In particular, the ABI4-152 peptide is more stable but functionally compromised compared with its full-length counterpart, implying that the ABI4-152 peptide can form a stable but "poison/abortive" DNA-binding complex that impairs the execution of the downstream ABA response pathway(s). Thus, the opposite genetic behavior of the *abi4-152* allele in ABA sensitivity and ethylene production can be explained by the hypothesis that the C-terminal peptide of ABI4 plays an important role by decreasing the half-life of ABI4 by targeting the protein to the proteasome for degradation. Moreover, the presence of the C terminus was required to trigger the downstream ABA responses, supporting the bifunctionality of ABI4 as both an activator and suppressor of gene expression, and linking ABA sensitivity to ethylene evolution. The ABI4-152 peptide probably binds some unknown partners and interferes with the downstream portion of the pathway. Dissecting the unknown factors will be a key issue in revealing the underlying mechanistic function of ABI4 in ethylene and ABA responses.

METHODS

Mutants, Transgenic Lines, and Plant Growth Conditions

Mutants displaying different levels of ethylene production were generated by EMS mutagenesis in the Columbia ecotype (Col-0) background. The mutants *abi4-102* (CS3837) and *abi4-103* (CS3838) were obtained from the *Arabidopsis* Biological Resource Center. The sequences for full-length *ABI4* (At2g40220) and different fragments of *ABI4* were amplified by PCR using specific primers (Supplemental Table 1) and were subcloned into pCAMBIA1307, a derivative of pCAMBIA1300 carrying the 2× cauliflower mosaic virus 35S promoter, the octopine synthase terminator, and either a 6× Myc epitope tag or a fused GFP cassette. Transgenic plants were obtained by *Agrobacterium tumefaciens*-mediated transformation. The seeds were germinated on MS medium supplemented with 2% sucrose and selected with hygromycin. One-week-old, hygromycin-resistant plants were transferred to soil and grown in a greenhouse. Transgenic plants were verified by qPCR amplification, and approximately 8–15 lines were obtained for each transformation. T_2- or T_3-generation transgenic seeds were used for our assays. The transgenic plants overexpressing ABI4-Myc or ABI4-152 peptide (ABI4M)-Myc with a 16-amino-acid deletion of the C terminus were designated OE and OEM, respectively. The transgenic lines overexpressing ABI4-GFP or ABI4-152 peptide-GFP were named 35S:ABI4-GFP and 35S:ABI4M-GFP, respectively.

All seeds were surface-sterilized, subjected to cold pretreatment at 4°C for 3 days, and placed on plates containing MS medium (2% sucrose and 0.5% Phytagel). The seeds were then transferred to growth chambers at 21°C under a 16-h white light (50 μmol/m^2s)/8-h dark cycle. The chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Mutant Screening and Map-Based Cloning

EMS-mutagenized M_1 seeds were obtained as follows. Approximately 20 000 seeds were treated with 0.3% EMS for 16 h at room temperature, washed extensively with water, and sown in soil. M_2-generation seeds were separately harvested as independent pools. To isolate the mutants, we sowed approximately 50 M_2 seeds from each parental group on MS plates containing 0.5 μM ABA, which effectively inhibited the cotyledons of the wild-type from expanding and turning green. This method differed from previous reports in which high ABA concentrations were used in

the screening conditions (Giraudat et al., 1992; Meyer et al., 1994; Finkelstein et al., 1998). After a 5- or 6-day incubation the germinated, presumably ABA-resistant seeds were transferred to MS plates without ABA and were subsequently cultured in soil. By observing the phenotypes of the M_2-seed germination, 385 ABA-resistant lines were identified. M_3-generation seeds were further confirmed to have an ABA-resistant phenotype and to produce seedlings with green, expanded cotyledons. In the M_3 generation, 55 of the 385 lines inherited ABA insensitivity. After confirmation of ethylene production, the candidate mutants were backcrossed at least twice before physiological and molecular characterization.

Genetic analysis was conducted using a Col-0-backcrossed population of mutants. The germinated F_1 seeds exhibited resistance to ABA, and the segregation of the F_2 seeds was analyzed using χ^2 in response to ABA. Then a genetic mapping population was obtained by crossing the mutant with Landsberg (Ler). Col-0/Ler InDel markers from the Monsanto/Cereon collection (www.arabidopsis.org/Cereon/index.jsp) were used to determine the linkage of the mutations. The markers used in the fine mapping were chosen from a platform of high-density INDEL/CAPS markers for map-based cloning in *Arabidopsis* (Hou et al., 2010). To conveniently identify *abi4-152*, we designed the derived cleaved amplified polymorphic sequence (dCAPS) near the mutation site to contain the *Bsr*I restriction site. The dCAPS primers are provided in Supplemental Table 1.

Assays of ABA and Ethylene Responses

Sterilized seeds were plated in MS medium containing 2% sucrose and 0.5% Phytagel supplemented with ABA, NaCl, ACC, or AVG. The plates were pretreated for 3 days at 4°C and were then transferred to a 16-h/8-h light/dark cycle at 21°C. Germination, which was identified as radicle emergence, was scored on the fifth day. All images are representative of three independent experiments with similar results. The lengths of the hypocotyls and roots were measured from the obtained digital images for at least 50 approximately 4-day-old seedlings using ImageJ software.

Measurement of Ethylene Emission

The ethylene evolution was measured as previously described, with modifications (Vogel et al., 1998b; Chae et al., 2003). One hundred seeds were sown in 10-ml transparent glass vials containing MS medium supplemented with 2% sucrose. The vials were pretreated for 3 days at 4°C and were then transferred to a 16-h/8-h light/dark cycle at 21°C. Four-day-old *Arabidopsis* seedlings were treated with different concentrations of ABA and the vials were sealed for 24 h. Next, 1 ml of gas from each vial was collected to analyze the ethylene emission using a gas chromatograph (Hitachi, Japan). The levels of ethylene in the vials containing seedlings were compared with those in the control vials containing MS medium only, as previously described (Li et al., 2011).

Transcriptional Activation Assay

Different fragments of ABI4 were amplified and subsequently cloned into pGBKT7 (Clontech, USA). The resulting fusion plasmids and the vectors pGBKT7-53/pGADT7-Rec T and pGBKT7-lam/pGADT7-Rec T (as positive and negative controls, respectively) were transformed into the Gold yeast strain (Clontech, USA) using the lithium-acetate method. The transformants were selected by monitoring their growth on synthetic dextrose/Trp medium. X-α-gal was spread on top of the medium before plating the liquid cultures to test for β-galactosidase activity according to the manufacturer's protocol (Clontech, USA).

RNA Extraction, Reverse Transcription, and qPCR

Total RNA was extracted from 4-day-old seedlings using TRIzol reagent (Transgen, China) and was treated with RNase-free DNase I (Promega, Madison, WI) before the following procedures were performed. Five micrograms of total RNA was reverse-transcribed into cDNA using a TransScript One-Step RT–PCR SuperMix kit (Transgen, China) according to the manufacturer's instructions. Gene expression was measured by qPCR with TransStart Top Green qPCR SuperMix (Transgen, China). All amplification reactions were performed in 96-well optical reaction plates with 45 cycles of denaturation for 15 s at 95°C, annealing for 20 s at 60°C, and extension for 45 s at 72°C. The expression levels were normalized to the expression of *TUB4*. The primers used for qPCR are listed in Supplemental Table 1.

Chromatin Immunoprecipitation Assay

Seedlings (approximately 4-day-old plants) were fixed in 1% formaldehyde, after which the ChIP assay was performed as described previously (Saleh et al., 2008) using monoclonal c-myc 9E10 immobilized onto Sepharose Fast Flow beads (9E10 affinity matrix; Covance). After the eluates were digested with proteinase K (Merck, Germany) and RNase, they were extracted with phenol/chloroform, and the DNA was precipitated with ethanol and sodium acetate. The purified DNA was then suspended in 100 μl of water. The identity of the precipitated DNA fragments was determined by qPCR using the primers listed in Supplemental Table 1. For each PCR reaction, 1 μl of recovered DNA from ChIP (experimental or control) or 1 μl of input DNA diluted 20-fold was added as the template. Each reaction was repeated three times. Quantification involved the normalization of the C_t value of each immunoprecipitation (IP) or control sample to the C_t value of the input DNA sample to obtain a ΔC_t (ΔC_t IP or ΔC_t control); then the relative enrichment of each fragment was calculated using the following equation: $2^{-(\Delta Ct\ IP\ -\ \Delta Ct\ control)}$. An unrelated DNA sequence from the *TUB4* gene was used as an internal control (Zhang et al., 2011).

GFP Fluorescence

A general translational inhibitor, 100 μM cycloheximide (CHX; Sigma, USA), was used to pretreat the seedlings to exclude *de novo* protein synthesis. The roots of plants expressing 35S:ABI4-GFP or 35S:ABI4(M)-GFP were imaged using a Zeiss LSM510 confocal laser-scanning microscope. The images are representative of 10 roots per transgenic line with three biological replicates.

Western Blot Analysis

After pretreatment with 100 μM CHX, proteins were extracted from approximately 100 mg of 4-day-old seedlings in 100 μl of denaturing buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% NP-40, 4 M urea, 1 mM phenylmethylsulfonyl fluoride). The total extract was centrifuged at 16 000 *g* and 4°C for 30 min, and 10 μg of the supernatant (approximately 15 μl) was subjected to western blot analysis.

For the immunoblot analysis, the proteins were separated by SDS-PAGE (12% bis-acrylamide gel) and were electroblotted to a nitrocellulose membrane (Hybond-C, Amersham) at 90 V for 90 min. The membrane was blocked with PBST (PBS with Tween 20) containing 5% skim milk powder for 8 h at 4°C. The membrane was incubated first with a primary antibody for 8 h at 4°C and then with a secondary antibody diluted in PBST containing 5% skim milk for 30 min at room temperature. Proteins were detected with a chemiluminescent horseradish peroxidase substrate kit (Transgen, China). The antibodies and the dilutions used in these experiments were anti-c-Myc mouse monoclonal antibody (1:3000; Transgen, China) and goat anti-mouse immunoglobulin G (1:10 000; Transgen, China). An anti-TUB4 antibody was used as a loading control (1:5000; Transgen, China).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

R.H., Z.D., and Y.Y. designed the study. Z.D., Y.Y., S.L., and J.W. performed the experiments. Z.D., R.H., J.W., Y.Y., and S.T. analyzed and discussed the data. R.H., S.T., and Z.D. wrote the manuscript.

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