Auxin Response Factor2 (ARF2) and Its Regulated Homeodomain Gene HB33 Mediate Abscisic Acid Response in Arabidopsis

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Abstract

The phytohormone abscisic acid (ABA) is an important regulator of plant development and response to environmental stresses. In this study, we identified two ABA overly sensitive mutant alleles in a gene encoding Auxin Response Factor2 (ARF2). The expression of ARF2 was induced by ABA treatment. The arf2 mutants showed enhanced ABA sensitivity in seed germination and primary root growth. In contrast, the primary root growth and seed germination of transgenic plants over-expressing ARF2 are less inhibited by ABA than that of the wild type. ARF2 negatively regulates the expression of a homeodomain gene HB33, the expression of which is reduced by ABA. Transgenic plants over-expressing HB33 are more sensitive, while transgenic plants reducing HB33 by RNAi are more resistant to ABA in the seed germination and primary root growth than the wild type. ABA treatment altered auxin distribution in the primary root tips and made the relative, but not absolute, auxin accumulation or auxin signal around quiescent centre cells and their surrounding columnella stem cells to other cells stronger in arf2-101 than in the wild type. These results indicate that ARF2 and HB33 are novel regulators in the ABA signal pathway, which has crosstalk with auxin signal pathway in regulating plant growth.

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Introduction

Abscisic acid regulates many important aspects including seed development, dormancy, germination, vegetative growth, and plant responses to environmental stresses [1]. ABA is required for normal plant growth as ABA-deficient mutants reduce cell vigor and are usually smaller [2]. Different developmental stages of Arabidopsis seedlings exhibit different response to ABA. In the early germination stage for establishing embryonic axis, the seed germination and post-germination growth are more sensitive to ABA (during 48 hr after seed imbibition) than other stages and more than 3 μM ABA will block the germination and post-germination growth [3,4]. Genetic screening during this stage has been performed and identified some specific ABA responsive factors such as ABA INSENSITIVE3 (ABI3) and ABI5, which play critical roles in regulating seedling growth mainly during seed germination and post-germination growth period [5,6]. However, after more than 48 hr of seed imbibition, higher concentrations of ABA can inhibit root growth through inhibiting cell division [17,18]. Some DNA replication related mutants are hypersensitive to ABA in seed germination and seedling growth, suggesting that ABA signal might inhibit cell division through regulating the DNA replication related proteins [18].

In order to find the new genes in ABA response, we performed a genetic screen by using ABA inhibiting root growth phenotype [18–20]. Here we identified two ARF2 mutant alleles that were hypersensitive to ABA in both seed germination and primary root growth. ARF2 directly regulates the expression of a homeodomain gene HB33. Our data indicate that ARF2 is a negative, and HB33 is a positive regulator in ABA mediating seed germination and primary root growth.

Results

arf2 mutants are more sensitive to ABA than the wild type in both seed germination and root growth

The sensitivity of seed germination on ABA has been used to identify some classic ABA sensitive and ABA insensitive mutants [4]. In order to find more new ABA responsive mutants, we take
Author Summary

Abscisic acid is a phytohormone that regulates many aspects in plant growth and development and response to different biotic and abiotic stresses. Research on ABA inhibiting seed germination, controlling stomatal movement, and regulating gene expression has been widely performed. However, the molecular mechanism for ABA regulating root growth is not well known. We have set up a genetic screen by using ABA inhibiting root growth to identify ABA related mutants and to dissect the molecular mechanism of ABA regulating root growth. In this study, we identified two new mutant alleles that are defective in ARF2 gene. ARF2 is a transcriptional suppressor that has been found to be involved in ethylene, auxin, and brassinosteroid pathway to control plant growth and development. Our study indicates that ARF2 is an ABA responsive regulator that functions in both seed germination and primary root growth. ARF2 directly regulates the expression of a homeodomain gene HB33. We demonstrate that ABA treatment reduces the cell division and alters auxin distribution more in arf2 mutant than in the wild type, suggesting an important mechanism in ABA inhibiting the primary root growth through mediating cell division in root tips.

Advantage of root growth sensitivity to ABA as a selection standard. 5-day seedlings grown on MS were transferred to MS medium supplemented with 30 μM ABA, and the mutants whose root growth is slower than the wild type were selected after growing for 7 days. From an ethyl methyl sulfonate-mutagenized Arabidopsis Columbia M2 population [18,20], we identified two mutants whose primary root growth is hypersensitive to ABA. Genetic analysis indicates that the two mutants were caused by different recessive mutations in the same gene. Because the two mutants showed similar ABA sensitivity and growth phenotypes, we selected one mutant, named arf2-101, for further analysis. This mutant was backcrossed four times with the wild type to remove other possible mutations. For map-based cloning of the ARF2 gene, we used an F2 population obtained from a cross of arf2-101 with the Arabidopsis Landsberg mutant alleles such as arf2-7 (a T-DNA insertion mutant from Arabidopsis stock center) [21–23].

As primary root growth is much easier to compare than shoot growth, here we mainly focus on the primary root growth inhibition by ABA. We compared the primary root growth of arf2-101 and the wild type with and without ABA treatment; arf2-7 was included for comparison. Seedlings grown for 5 days on MS medium without ABA were transferred to MS medium supplemented with different concentrations of ABA. On MS medium without ABA, primary root growth was similar for arf2-101, arf2-7, and the wild type (Figure 1A). Inhibition of primary root growth by ABA, however, was greater for arf2-101 and arf2-7 than for the wild type. At 5 μM ABA, the relative primary root growth was about 20% in arf2-101 and arf2-7, but more than 65% in the wild type (Figure 1A, 1B). ABA at ≥30 μM almost completely arrested primary root growth of arf2-101 and arf2-7 but only inhibited 60% the wild-type (Figure 1A, 1B). In contrast, primary root growth did not differ between arf2-101 and the wild type when the MS medium contained NaCl (from 50 mM to 150 mM, for salt stress), mannitol (200 to 350 mM, for osmotic stress), LiCl (15 to 30 mM, for ionic toxic stress), or the plant hormone jasmonate (JA), ACC (a precursor for ethylene), brassinosteroid (BR), or gibberellic (GA3) (Figure S1). We also compared the sensitivity of arf2-101 mutant to ABA with the wild type during seed germination and post-germination stage (Figure 1C, 1D). At 0.3 μM ABA, about 40% of arf2-101 and 22% of arf2-7 showed seed germination greening (green cotyledon), while about 85% of wild type showed seed germination greening, indicating that arf2 mutants are more sensitive to ABA than the wild type in the seed germination and post-germination.

Combined together, the results indicate that ARF2 is involved in two different ABA responsive stages, i.e. both the earlier stage during seed germination and later developmental stage of root growth.

Expression of ARF2 is induced by ABA, and transgenic plants over-expressing ARF2 are more resistant than the wild type to ABA

To investigate the role of ARF2 in ABA responses, we first measured the effect of ABA on expression of ARF2. In the ARF2 promoter region from −750 to −744, there is a reverse ABRE binding cis-element (GCCACCTG) [24], suggesting that ARF2 expression might be regulated by ABA response factor(s). Two-week-old seedlings were treated with 30 μM ABA for 0–30 h, and total RNAs were extracted and used for qRT-PCR. As shown in Figure 2A, ABA treatment increased the expression of ARF2 (relative to the control without ABA, from about 5 folds at 12 hr to 2 folds at 30 hr). The ABA inducible expression further supports that ARF2 is involved in ABA response.

We next determined whether increasing the transcripts of ARF2 influences the ABA sensitivity of the wild type plants. We constructed a super promoter-driven ARF2 that is fused with a flag tag and transferred it to wild-type plants by Agrobacterium-mediated flower dip transformation. Although most transgenic plants overexpressing ARF2-flag showed the arf2 mutant phenotype because of co-suppression as reported previously [23], several independent transgenic lines with high expression of ARF2-flag were obtained. We selected two of these independent high expression transgenic lines for further study. qRT-PCR analysis indicated that ARF2 transcripts were more abundant in the two over-expressing lines than in the wild type or arf2-101 (Figure 2B).

As reported previously, two ARF2-flag over-expressing lines flowered earlier and showed leaf senescence earlier than the wild type [23], suggesting that the flag tag did not affect ARF2 function. We analyzed seed germination greening of the ARF2-flag over-expressing lines in response to ABA. Without addition of ABA (Figure 2C and 2D), seed germination greening ratio was similar for the wild type, arf2-101, and the two ARF2 over-expressing lines. With increasing ABA concentration in the medium, however, seed germination greening ratio was much greater in the two ARF2 over-expressing lines than in the wild type or arf2-101; arf2-101 was the most sensitive to ABA at 0.5 μM ABA, seed germination greening ratio was about 40% in the wild type, about 95% in two overexpressing lines and 10% in arf2-101 (Figure 2D).

ARF2-flag over-expressing plants had shorter primary roots, and more lateral roots than the wild type. However, the primary root growth of ARF2 over-expressing lines was more resistant to ABA than the wild type (Figure 2E, 2F). These results indicate that

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**Plants Over-Expressing ARF2 are More Resistant to ABA**

Plants expressing ARF2 were more resistant to ABA than wild-type plants. The results indicate that ARF2 plays a role in ABA responses.

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**Seed Germination Greening in Response to ABA**

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ARF2 negatively regulates ABA inhibition of seed germination and primary root growth.

**ARF2 binds to the HB33 promoter and regulates HB33 expression**

*arf2* mutants decrease transcriptional levels of three ethylene biosynthesis genes (*ACS*) in flowers [21] and a senescence-related gene *SAG12* (*SENSENCE ASSOCIATED GENE 12*) [22]. In both in vitro and in vivo assays, ARF2 negatively regulates the reporter genes under the control of a synthetic promoter with AuxREs (auxin-responsive elements) [23,25]. In searching for genes regulated by ARF2 from microarray data [21], we found that *AT1G75240* encoding HOMEOBOX PROTEIN 33 (HB33) was up-regulated in the *arf2* mutant (the average signal was 153 in the wild type and 276 in *arf2-6* from three independent repeats) [21]. We confirmed the microarray results by qRT-PCR, which showed that the expression of HB33 was about 1.5 times greater in *arf2-101* than in the wild type (Figure 3A). In order to know whether HB33 is also regulated by other ARFs, we compared the HB33 expression of the wild type with *arf1*, *arf6* and *arf21* mutant, and did not find the expression difference of HB33 between these mutants and the wild type (Figure 4B), suggesting that HB33 is regulated specially by ARF2. In contrast to the expression of *ARF2*, the expression of HB33 was inhibited by ABA treatment in the wild type but not in the *arf2-101* mutant (Figure 3A). The reduction of HB33 expression by ABA was further confirmed by a time-course experiment (Figure 3C). We also determined that the expression of HB33 was lower in two *ARF2-flag* overexpressing lines than in the wild type and was not further reduced by ABA treatment (Figure 3A), suggesting that ARF2 represses HB33 expression. It appears that auxin treatment weakly induced the expression of HB33 in the early times in both the wild type and *arf2* mutant (Figure 3D). It is likely that ARF2 does not regulate HB33 expression by auxin.

**AUXIN RESPONSE FACTORS** (ARFs) are transcription factors with a conserved N-terminal DNA-binding domain that binds to TGTCTC *cis*-elements in promoters of auxin-responsive genes [25–27]. In the HB33 promoter region, we found two AuxREs (TGTCTC), one in the position −202−−197 and the other in the reverse direction (GAGACA) in the position −157−−152. ARF2 contains the N-terminal DNA-binding domain that targets the AuxREs without the help of a middle or C-terminal part (the middle region for transcriptional activation or repression, and the C-terminal dimerization domain) [25]. We expressed the GST fused with the DNA-binding domain in N-terminus (ARF2-N1-470) in *Escherichia coli* and purified the fused protein with the help of the GST tag. Gel-shifting was performed to test whether the recombinant protein could bind to the AuxREs. As shown in Figure 3E, a shifted DNA-binding band was detected with addition of GST-ARF2-N1-470 and labeled DNA probes, but no band was detected in the GST control. When unlabeled DNA probe was increased gradually in the reaction mixture, the DNA-binding band was abolished. The GST-ARF2 protein, however, did not bind the mutated DNA probes (mP), and the mP did not compete with labeled DNA probes. These results suggest that the ARF2 N-terminal DNA binding domain binds to AuxREs in the promoter of HB33.

We then used the chromatin immunoprecipitation (ChIP) assay to test whether ARF2 could bind to the *HB33* promoter in vivo. In this experiment, we used one transgenic line over-expressing ARF2-Flag (OE1) and the wild type as a negative control. Flag antibody was used for ChIP analysis. As shown in Figure 4B, ARF2-Flag bound to the HB33 promoter region, which contains two *cis*-elements as used in the Gel-shift assay (F2/R2), but could not bind to the gene encoding region (primer pair F3/R3) or to the promoter region that does not contain the AuxRE *cis*-element (primer pair F1/R1). qRT-PCR results were shown in Figure 4C for each pair of primers (F1R1, F2R2 and F3R3). In theory, all ARF proteins should have ability to bind the AuxREs. However, in vivo, each of ARFs only binds to specific AuxRE in the promoter regions of limited genes. In order to exclude no specific binding of ARF2 to the promoter region, we also included two genes, *SAUR-15* and...
AT4G33680, both of which contain the AuxRE cis-element in their promoter regions, but their expressions are not regulated by ARF2, and the gene GH3.1 which contains the AuxRE cis-element in their promoter region and is regulated by ARF2 [28], as controls. ChiP assay indicated that ARF2 did not bind to the promoter regions of SAUR-15 and AT4G33680, but bound to the promoter region of GH3.1 (Figure 4B, 4C). The results indicate that ARF2 does not bind to all of the AuxRE cis-element regions. ARF2 binding to the promoter of HB33 and GH3.1 in vivo might need the help of other components for its binding specificity [29].

Transgenic plants overexpressing HB33 have an increased sensitivity to ABA in primary root growth inhibition

HB33 in Arabidopsis belongs to a zinc finger-homeodomain (ZF-HD) subfamily containing 14 members that can dimerize with each other in a yeast two-hybrid assay [30]. Most proteins in this family do not have an intrinsic activation domain and might need to interact with other factors for transcriptional activation [30]. We made transgenic plants that over-expressed HB33 under control of a super promoter [31], and qRT-PCR analysis confirmed the higher expression of HB33 in these independent transgenic lines (Figure 3A). We selected two transgenic lines, HB33-OE10 and HB33-OE16, for further study. We tested the seed germination sensitivity of two transgenic lines on MS medium without or with 0.3 or 0.5 μM ABA. As shown in Figure 5B and 5C, no difference in seed germination greening was found among wild type, arf2-101 and two overexpression lines on MS medium without ABA. However, two overexpression lines were more sensitive to ABA than wild type, but less sensitive to ABA than arf2-101 in seed germination. We further compared the effect of ABA on primary root growth of HB33-OE10 and HB33-OE16 lines. Five-day-old seedlings grown on MS medium were transferred to MS medium supplemented with different concentrations of ABA. With all of the tested ABA concentrations (10–50 μM), primary root growth of HB33-OE10 and HB33-OE16 was more sensitive than that of the wild type to ABA, although the sensitivity was still less than that of arf2-101 (Figure 5D, 5E). These results indicate that HB33 overexpression exhibits similar ABA sensitive phenotypes as arf2-101 in seed germination and primary root growth.

HB33 RNAi transgenic plants are more resistant to ABA than the wild type

It appears that ARF2 is a negative regulator, while HB33 is a positive regulator which is controlled by ARF2 in ABA signal pathway. We hypothesize that reducing HB33 would result in ABA resistance. We used a fragment of HB33 which has low homologous sequence with other HB genes and made HB33 RNAi transgenic plants. We took two (hb33-1, hb33-2) from several

Figure 2. Overexpression of ARF2 increases resistant to ABA. (A) Expression of ARF2 was induced by ABA treatment. Total RNAs extracted from 14-day-old seedlings treated with 30 μM ABA for different times were reversely transcripted and used for qRT-PCR. ACTIN was used as a control. Three independently experiments were done with similar results. The results shown were from one experiment with triple technical repeats. Values are means ±SD. (B) The relative expression of ARF2 in transgenic plants over-expressing ARF2. The relative ARF2 levels in two over-expressing lines, ARF2-Flag OE1 (OE1) and ARF2-Flag OE2 (OE2) with earlier flowering phenotype, were measured by qRT-PCR. ARF2 from the wild type (WT) or arf2-101 were used as controls. The arf2-101 mutation did not affect itself expression. (C,D) Seed germination greening of WT, arf2-101, OE1, and OE2 on MS medium containing different concentrations of ABA. Seeds of the different accessions were imbibed on MS medium plates with or without ABA at 4°C for 2 days before the plates were transferred to 20°C. Three independent experiments were done. At least 30 seeds were accounted in each of three different plates for each time. Values are means ±SD. **p<0.01. (E,F) Relative root growth of WT, arf2-101, and arf2-7 on MS medium containing different concentrations of ABA. Five-day-old seedlings grown on MS medium were transferred to MS medium containing 0, 10 and 30 μM ABA and were grown for 7 days before being photographed and measured. The dot line indicates the places where the root tips were after just transferring. 30 seedlings were measured in each experiment. Relative root growth represents the root growth of seedlings after treatment with ABA comparing with that without ABA treatment. Three independent experiments were done. Values are means ±SD, **p<0.01.
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Figure 3. ARF2 binds to the HB33 promoter and regulates HB33 expression. (A) Expression of HB33 was negatively regulated by ABA and by ARF2. Two-week-old seedlings of the wild type (WT), arf2-101, ARF2-OE1, and ARF2-OE2 were treated with 0 or 30 μM ABA for 12 h. Total RNAs were extracted, reversely transcripted, and used for qRT-PCR. HB33 expression in WT without ABA treatment was used as a standard for normalizing the relative expression level. Three biologically independent experiments were done with the similar results. The shown results are from one experiment with three technical replicates. Values are means ± SD, n = 3. (B) HB33 expression was not affected by ARF1, ARF6 and ARF21. The similar experiments were done as in (A) by using arf1, arf6 and arf21 mutants. Three biologically independent experiments were done with the similar results. The shown results are from one experiment with three technical replicates. Values are means ± SD, n = 3. (C) Time course of HB33 expression in WT. Two-week-old seedlings were treated with 30 μM ABA for different times. The relative expression of HB33 was determined by qRT-PCR. Three biologically independent experiments were done with similar results. The results are from one experiment with three technical replicates. Values are means ± SD, n = 3. (D) The expression of HB33 under IAA treatment. Two-week-old seedlings were treated with 5 μM IAA for different times. The relative expression of HB33 was determined by qRT-PCR. Three biologically independent experiments were done with similar results. The results are from one experiment with three technical replicates. Values are means ± SD, n = 3. (E) Gel-shift analysis of ARF2 N-terminus binding to cis-elements in the promoter of HB33. a, The oligonucleotide sequences of probe and mutated form probe (mProbe) within the HB33 promoter used in the EMSA. Underlined letters indicate the sequences of ARF2-recognition motifs (TGTCCT). mProbe: ARF2-recognition motifs in probe were mutated as indicated by small letters. b, Interaction between GST-N-ARF2 protein and biotin-labeled Probe and mProbe by SDS-PAGE analysis of purified GST-N-terminus ARF2 fusion protein. Purified protein (6 μg) was incubated with 25 fmol biotin-labeled probe or mProbe (mP). For the competition test, non-labeled probe with different concentrations (from 10 to 1000 times) or labeled mP (1000 times) was added in the above experiment.
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independent transgenic lines and checked HB33 expression by qRT-PCR. Two RNAi lines had only about 25% HB33 expression of the wild type (Figure 5F). We then compared the ABA sensitivity of hb33-1 and -2 in seed germination and primary root growth with that of the wild type and arf2-101, hb33-1 and hb33-2 were more resistant to ABA than the wild type or arf2-101 in both seed germination and primary root growth (Figure 5G–5J). The results indicate that plants with reducing HB33 show similar ABA phenotypes as plants over-expressing ARF2. However, arf2-101 hb33-1 or arf2-101 hb33-2 double mutants showed ABA sensitive phenotype as arf2-101 mutant (data not shown), indicating that reduced HB33 is required, but not sufficient for suppressing the arf2 mutant phenotype in ABA signaling.

ABA treatment reduces the expression of CYCB1;1 promoter-GUS more in the arf2-101 mutant than in the wild type

Previous studies indicate that ethylene inhibits root growth by retarding cell elongation but not by affecting cell division [32], while ABA inhibits root growth by inhibiting cell division [17,18]. CYCB1;1 is a G2/M marker protein that might be regulated by PLETHORAS2 that is essential for root quiescent center (QC) establishment and stem cell maintenance [33,34]. We compared CYCB1;1 promoter-GUS expression between the wild type and arf2-101. Under normal growth conditions (i.e., when ABA was not added), GUS expression in the root tips did not clearly differ (Figure 6A, wild type; Figure 6B, arf2-101). Addition of different concentrations of ABA decreased GUS expression in root tips of both the wild type and arf2-101. However, the GUS expression level was decreased more in arf2-101 than in the wild type.

As plants over-expressing HB33 exhibit a similar ABA sensitive phenotype as arf2 mutant in root growth, we further checked the CYCB1;1::GUS expression pattern in the HB33 over-expressing lines. As shown in Figure 6C and 6D, without ABA treatment, GUS expression was a little less in HB33 OE10 and OE16 than in the wild type or arf2-101. 30 μM ABA treatment for 36 h significantly reduced the expression of CYCB1;1::GUS in two HB33 over-expressing lines (OE10 and OE16). The results suggest that similar to arf2 mutants, HB33 overexpressors are more sensitive to ABA inhibition of cell division than the wild type.

Genetic interaction between arf2 and the classic abi mutations

Many ABA-insensitive mutants have been identified by different screens in Arabidopsis. We crossed arf2-2 (carrying a T-DNA insertion that can be easily used to identify a double mutant) with abi1-1 and abi2-1, two dominant negative mutants, [4,13], and

Figure 3. ARF2 binds to the HB33 promoter and regulates HB33 expression. (A) Expression of HB33 was negatively regulated by ABA and by ARF2. Two-week-old seedlings of the wild type (WT), arf2-101, ARF2-OE1, and ARF2-OE2 were treated with 0 or 30 μM ABA for 12 h. Total RNAs were extracted, reversely transcripted, and used for qRT-PCR. HB33 expression in WT without ABA treatment was used as a standard for normalizing the relative expression level. Three biologically independent experiments were done with the similar results. The shown results are from one experiment with three technical replicates. Values are means ± SD, n = 3. (B) HB33 expression was not affected by ARF1, ARF6 and ARF21. The similar experiments were done as in (A) by using arf1, arf6 and arf21 mutants. Three biologically independent experiments were done with the similar results. The shown results are from one experiment with three technical replicates. Values are means ± SD, n = 3. (C) Time course of HB33 expression in WT. Two-week-old seedlings were treated with 30 μM ABA for different times. The relative expression of HB33 was determined by qRT-PCR. Three biologically independent experiments were done with similar results. The results are from one experiment with three technical replicates. Values are means ± SD, n = 3. (D) The expression of HB33 under IAA treatment. Two-week-old seedlings were treated with 5 μM IAA for different times. The relative expression of HB33 was determined by qRT-PCR. Three biologically independent experiments were done with similar results. The results are from one experiment with three technical replicates. Values are means ± SD, n = 3. (E) Gel-shift analysis of ARF2 N-terminus binding to cis-elements in the promoter of HB33. a, The oligonucleotide sequences of probe and mutated form probe (mProbe) within the HB33 promoter used in the EMSA. Underlined letters indicate the sequences of ARF2-recognition motifs (TGTCCT). mProbe: ARF2-recognition motifs in probe were mutated as indicated by small letters. b, Interaction between GST-N-ARF2 protein and biotin-labeled Probe and mProbe by SDS-PAGE analysis of purified GST-N-terminus ARF2 fusion protein. Purified protein (6 μg) was incubated with 25 fmol biotin-labeled probe or mProbe (mP). For the competition test, non-labeled probe with different concentrations (from 10 to 1000 times) or labeled mP (1000 times) was added in the above experiment.
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under the HB33 promoter. We then compared the expression of HB33 in two RNAi lines and the wild type. As shown in Figure 6B, the expression of HB33 was decreased more in arf2-101 than in the wild type. At 10 μM ABA, more GUS staining spots were observed in the wild type than in the arf2-101. ABA at ≥30 μM completely inhibited the GUS expression in the arf2-101, but not in the wild type (Figure 6A and 6B). The results indicate that arf2-101 mutants are more sensitive to ABA inhibition of cell division than the wild type.

As plants over-expressing HB33 exhibit a similar ABA sensitive phenotype as arf2 mutant in root growth, we further checked the CYCB1;1::GUS expression pattern in the HB33 over-expressing lines. As shown in Figure 6C and 6D, without ABA treatment, GUS expression was a little less in HB33 OE10 and OE16 than in the wild type or arf2-101. 30 μM ABA treatment for 36 h significantly reduced the expression of CYCB1;1::GUS in two HB33 over-expressing lines (OE10 and OE16). The results suggest that similar to arf2 mutants, HB33 overexpressors are more sensitive to ABA inhibition of cell division than the wild type.

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were from one experiment with three technical replicates. Values are means shown. (C) qRT-PCR of ChIP assay in (B). Three independent experiments were done with similar results, each with triple biological repeats. Data were included in this experiment as controls. Three independent experiments were done with the similar results. Results from one experiment GH3.1 ARF2-Flag and flag antibody were used for the ChIP assay. The wild-type seedlings were used as the negative control.

Figure 4. Chromatin immunoprecipitation (ChIP) assay on the promoter of HB33. (A) Three pairs of primers were used. Primer pair F2 and R2 covered the promoter region containing AuxRE cis-element. F1 and R1 pair of primers are upstream of AuxRE cis-element, and F3/R3 pair are in the coding region of HB33. (B) Chromatin immunoprecipitation (ChIP) assay on the promoter of HB33. One transgenic line (ARF2-OE1) overexpressing ARF2-Flag and flag antibody were used for the ChIP assay. The wild-type seedlings were used as the negative control. ACTIN, AT4G33680, SAUR-15 and GH3.1 were included in this experiment as controls. Three independent experiments were done with the similar results. Results from one experiment are shown. (C) qRT-PCR of ChIP assay in (B). Three independent experiments were done with similar results, each with triple biological repeats. Data were from one experiment with three technical replicates. Values are means ± SD, n = 3.

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abi3-1, abi4-1 and abi5-1 mutants [5,6,35], and obtained af2-7 abi1-1, af2-7 abi2-1, af2-7 abi3-1, af2-7 abi4-1 and af2-7 abi5-1 double mutants. The homozygous double mutants were confirmed by both sequencing of the mutation sites and genetic analysis of F3 and F4 seeds for no segregation. With respect to ABA inhibition of root growth, af2-7 abi1-1 and af2-7 abi2-1 had an ABA-insensitive phenotype similar to that of the abi1-1 and abi2-1 single mutants (Figure 7), while af2-7 abi3-1, af2-7 abi4-1 and af2-7 abi5-1 had an ABA-sensitive phenotype similar to af2-7 mutant, suggesting that the af2 effect on root growth inhibition by ABA requires the canonical ABA signaling pathway that can be blocked by the dominant abi1 or abi2 mutation, but not by abi3-1, abi4-1 or abi5-1 mutation. Gene expression analyses in these abi mutants indicated that the induction of ARF2 transcripts by ABA treatment was impaired by abi1-1 mutation, partially reduced by abi2-1 mutation, but not affected by abi3-1, abi4-1 or abi5-1 mutation (Figure 7B). Furthermore, ARF2 induction by ABA was not changed in auxin receptor quadruple mutant tio1 ghi1 ghi2 ghi3 [36] (Figure 7C), indicating that ARF2 expression is specially regulated by ABA signaling pathway.

ABA treatment altered the auxin distribution or auxin signal in the primary root tips

As ARF2 is an auxin response factor, and auxin and ABA have cross talk, we want to know whether the auxin components are changed under ABA treatment in af2 mutants. We first used the auxin responsive marker LLA2::GUS whose expression is closely related to endogenous auxin [37]. As shown in Figure 8A, under normal condition, there was no much difference of GUS staining (in cells of the QC, columella and the provascular tissue) between af2-101 and the wild type. ABA treatment reduced LLA2::GUS expression in both af2-101 and the wild type, but LLA2::GUS expression was reduced more in af2-101 than in the wild type at different times. LLA2 transcripts by quantitative RT-PCR and the GUS intensity quantified using Adobe Photoshop CS (Adobe Systems Inc.; San Jose, CA, USA) software were consistent with LLA2::GUS expression pattern (Figure 8B, 8C). It appears that relative higher GUS staining was observed around QC and columella stem cells than other cells in af2-101 (staining for 1 and 3 hr), but not in the wild type. We further checked the expression of DR5, a synthetic promoter with AuxREs that reports auxin response [38]. We introduced DR5::GUS into af2-101 by crossing a DR5::GUS transgenic plant with af2-101. Under normal condition, af2-101 exhibited more GUS staining than the wild type in root tips (in QC cells, columella stem cells, differentiated columella and weak expression in some vascular cells (Figure 8D, 8E, −ABA), suggesting that ARF2 negatively regulates DR5::GUS [23]. ABA treatment reduced GUS expression in both af2-101 and the wild type. However, we always observed that after ABA treatment, DR5::GUS expression was highly accumulated around QC center, columella stem cells, differentiated columella cells and weak expression in some vascular cells adjacent to the QC in the wild type (Figure 8D, +ABA), but GUS staining in the af2-101 was not so widely. In af2-101, GUS staining was highly accumulated around QC and columella stem cells, but neither in vascular cells adjacent to the QC nor in differentiated columella cells (Figure 8D, +ABA). The results suggest that ABA treatment reduces the whole auxin accumulation or auxin response in the root tips of af2-101 and the wild type, but the relative auxin distribution or auxin signal in the QC and columella stem cells was higher or stronger than other cells in af2-101, but not in the wild type.
We also introduced DR5::GUS or proIAA2::GUS into two HB33 overexpressing lines, OE10 and OE16. In HB33 over-expressing plants, DR5::GUS expression was lower than that in the wild type or arf2-101 under normal condition (Figure 5D and 5E). ABA treatment did not apparently reduce the expression of DR5::GUS in HB33 over-expressing plants (Figure 5D and 5E), but made DR5::GUS accumulated around QC and columella stem cells similar with the GUS distribution in arf2-101. ABA treatment reduced the expression of proIAA2::GUS in HB33 over-expressing plants to a level that was higher than that in arf2-101, lower than that in the wild type, which is consistent with IAA2 transcripts quantified by qRT-PCR (Figure 5C). These results indicate that HB33 over-expressing plants exhibit similar regulation on the expression pattern of DR5::GUS and IAA2 as arf2 mutants.

As ABA treatment reduces auxin accumulation or auxin signal, we want to know whether the arf2-101 sensitivity is due to reduced...
auxin. We tested the sensitivity of arf2-101 mutant to supplied auxin 1-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D). NAA can diffuse through the plasma membrane without the help of auxin carriers, while 2,4-D needs auxin carriers to penetrate the plasma membrane [39]. A low concentration of ABA combined with a low concentration of either 2,4-D or NAA inhibited the primary root growth of both the wild type and arf2-101 more than the same concentration of only ABA, 2,4-D or NAA [Figure 8F]. Root growth inhibition by ABA plus auxin was greater in arf2-101 than in the wild type. The results indicate that ABA and auxin have a synergistic effect on inhibiting root growth, and the root ABA sensitivity in arf2-101 is not due to the reduced auxin.

Auxin facilitators are involved in ABA response

Because auxin facilitators affect auxin distribution and thereby mediate root meristem patterning [34], we examined whether PIN2, and AUX1 are involved in ABA mediating root growth. PIN2 is an auxin efflux carrier, and AUX1 is an auxin influx carrier [40–42]. First, we checked expression levels in the arf2-101 mutant by using PIN2 or AUX1 promoter derived PIN2-GFP or AUX1-YFP transgenic plants. Interestingly, ABA treatment did not change the PIN2-GFP level so much in the wild type (Figure 9A, 9B), but greatly decreased the PIN2-GFP protein in the arf2-101 mutant as well as HB33- OE10 and -OE16 (Figure 9A, 9B). PIN2 is a major regulator of the basipetal auxin transport that controls root meristem cell division [40]. We hypothesize that if the reduced PIN2 expression is the reason for ABA sensitivity, then pin2 mutants should be sensitive to ABA treatment as arf2-101. However, pin2 mutants exhibited similar ABA sensitive phenotypes as wild type in root growth, while arf2-101 pin2 double mutants showed similar ABA sensitive phenotype as arf2-101 in root growth (Figure 9C), indicating that the decreased PIN2 observed in arf2-101 should be resulted from the ABA hyper-response, but is not the reason for ABA sensitivity.

PIN2 and AUX1 overlap for the basipetal auxin transport through the outer root cell layers. Besides, AUX1 is also responsible for the phloem-based auxin transport from source leaves to the root basal meristem [43]. For AUX1 expression, we also included HB33 overexpressing lines. ABA treatment decreased the AUX1 to the similar level in the wild type, arf2-101 and two HB33 overexpressing lines (Figure 9D, 9E). In order to know whether auxin transported from phloem affects ABA sensitivity in root tips or not, we examined aux1 mutant. aux1 mutant showed more resistant to ABA in root growth than the wild type (Figure 9F). arf2-101 aux1 double mutant showed phenotype similar to aux1 that is more resistant to ABA than the wild type in root growth (Figure 9F).

Several auxin efflux carrier PINs (PIN3, PIN4 and PIN7) are expressed in QC and columella stem cells to regulate cell division and cell expansion in the primary root [34]. We used PIN3 or PIN7 promoter derived PIN3-GFP or PIN7-GFP to check their expression. Without ABA treatment, arf2-101 and the wild type exhibited the similar expression level of PIN3-GFP or PIN7-GFP. ABA treatment reduced the expression of PIN3-GFP or PIN7-GFP in both the wild type and arf2-101, but the expression of PIN3-GFP or PIN7-GFP was reduced more in arf2-101 than in the wild type (Figure 9G, 9H). These results indicate that the reduced expression of PIN3 and PIN7 might lead to high accumulation of auxin in QC and columella stem cells and inhibit cell division.

NOA is an inhibitor of auxin influx carrier that phenocopies aux1 [44]. NOA concentrations from 30 to 50 μM did not differently affect root growth between arf2-101 and the wild type (Figure 9I, 9J). When different concentrations of NOA were added to the medium, the root growth inhibition by ABA was...
differentially released, i.e., the inhibition of arf2-101 became similar to that of the wild type (Figure 9K, 9L). These results indicate that inhibition of root growth by ABA in arf2-101 is rescued or alleviated by blocking AUX1 mediating auxin influx, suggesting that ABA mediating root growth is involved in auxin transport.

Discussion

It has long been known that root growth is inhibited by high levels of ABA, but the molecular mechanism is poorly understood [1,17]. In our studies, we have used the Columbia accession to study the molecular mechanism of how ABA regulates root growth, and we have identified several new root-sensitive mutants to ABA [18–20], suggesting that our screening is very powerful in identifying new components in ABA response pathway. In the current study, we provide several lines of evidence to show that ARF2 and HB33 are important players in ABA response which has cross talk with auxin response pathway in regulating plant growth.

ARF2 was originally identified as an ARF1 binding protein (ARF1-BP) [26,38], and could bind to AuxRE target site [45,46]. Later, ARF2 was isolated as a suppressor that regulates hypocotyl bending of the hookless1 mutant (an ethylene-response mutant), which suggested that ARF2 has an important role in linking ethylene and auxin signaling in the apical hook [23]. ARF2 is a pleiotropic regulator that represses the expression of targeted genes to regulate plant development [21,22,47]. Furthermore, brassinosteroid-regulated BIN2 kinase can phosphorylate ARF2, which releases the ARF2 repression activity and thereby increases expression of auxin-induced genes [28]. Here, we found that the expression of ARF2 is induced by ABA, and that ARF2 is a negative regulator in the ABA response pathway controlling seed germination and primary root growth. In contrast, no single ARF gene among the 23 ARF members is a positive regulator in the control of embryonic axis growth by ABA, probably because of functional redundancy among the genes [3]. These results suggest that ARF2 is a central integrator which connects auxin, ethylene, brassinosteroid and ABA signal pathway in controlling the growth and development of different organs and tissues [3,17,32,48,49].

In the auxin signaling pathway, Aux/IAA proteins dimerize with and inhibit the activities of ARF proteins at low auxin concentrations [50,51]. At high auxin concentrations, Aux/IAA is degraded by the F-box protein TRANSPORT INHIBITOR RESPONSE1 (TIR1)/AUXIN SIGNALING F-BOX (AFB)-mediated ubiquitination, which releases the inhibition on the ARFs [51]. The released ARFs then target downstream genes. In Arabidopsis, the ARF family has 23 members exhibiting either positive or negative regulatory roles [52]. Study of regulation of ARF10 mRNA stability by miR160 suggested that ARF10...
mediates the link between ABA and auxin responsiveness during and after seed germination [53]. Although ARFs are important in plant growth and development, only some of the direct targets of ARFs have been identified [54]. Previous studies have indicated that ARF2 expression is also regulated by miRNAs and/or ta siRNAs [55,56]. Our data indicate that ARF2 is a transcriptional repressor that directly targets the promoter of HB33 and suppresses the expression of HB33. Consistent with the induced expression of ARF2 by ABA is the suppressed expression of HB33 by ABA. Although many AuxREs exist in the genomic sequence, our ChIP analyses indicate that ARF2 can only bind to some specific promoters including HB33 with AuxREs, suggesting that the targeted sites are determined by both ARF2 and its partner(s). HB33, however, is only one of the genes targeted by ARF2. Our data indicate that ARF2 is a negative regulator in ABA mediating seed germination and root growth as arf2 mutants are sensitive to, and ARF2 over-expressors are resistant to ABA in both seed germination and root growth. In contrast, HB33 is a positive regulator in ABA mediating seed germination and root growth as showed by both HB33 over-expressing and HB33 RNAi study. Our results suggest that ARF2 regulates seed germination and root growth partially through direct repression of HB33 in Arabidopsis. However, reducing the expression of HB33 by RNAi could not rescue the ABA sensitive phenotype of arf2 mutant, suggesting that other components besides HB33 are needed for controlling the ABA response in arf2.

Previous study indicates that ABA has cross talk with ethylene in regulating plant growth [23,57,58]. Ethylene insensitive mutant ein2 is recovered as a mutant with enhanced response to ABA in seed germination (named era3) [57], and also from screening the suppressors and enhancers of abi1-1 in seed germination [58]. Interestingly, EIN2 is a negative regulator in seed germination, but a positive regulator in root growth as the root growth of ein2 is more resistant to ABA than that of the wild type [57,58]. As
mentioned above, ARF2 was isolated as a suppressor of the *hokkaido1* mutant [23]. *ABI3* encoding a transcriptional factor is induced by auxin in lateral root primordia [39]. The ABA-insensitive *ab3* mutant reduces while the ABA-hypersensitive *eat1* mutant increases the number of lateral roots when exogenous auxin is applied [59,60]. *ABI4* encodes another transcriptional factor that is up-regulated by cytokinin and ABA, but repressed by auxin in roots [61]. These results indicate that ABA and ethylene signal have a cross-talk with auxin signal.

However, the molecular mechanism of ABA inhibition of primary root growth is different from that of ethylene. Addition of ethylene reduces cell elongation, but does not affect cell division, and does not change *CycB1;1* expression [32]. But, addition of ABA reduces cell division and greatly decreases *CycB1;1* expression. Ethylene inhibits root growth by increasing auxin biosynthesis and enhancing the expression of PIN2 and AUX1 [32,49,62]. Nevertheless, ABA inhibiting root growth seems mainly through interfering with the distribution of auxin in root tips. ABI4 negatively regulates PIN1 and interferes with auxin distribution to control lateral root growth [61]. Here, we used two marker genes, *DR5:GUS* and *proIAA2:GUS*, to examine the gene expression by ABA treatment. Under normal condition, *DR5:GUS* was expressed at a higher level in *arf2-101* than in the wild type, indicating that *arf2* mutation results in a stronger auxin response. Although ABA treatment greatly reduced *DR5::GUS* expression in both wild type and *arf2-101* mutant, interestingly, ABA treatment made *DR5:GUS* accumulated to a relative, but not absolute, higher level around QC center and columella stem cells than other cells comparing with that in normal condition in both wild type and *arf2-101*. Different from wild type, GUS staining was not observed in differentiated columella cells and some vascular cells in *arf2-101*, but could be observed in the wild type. ABA treatment also decreased the expression of endogenous auxin responsive marker *proIAA2:GUS* in both wild type and *arf2-101* mutant, but more in *arf2-101* than wild type. Again, the relative GUS staining became stronger around QC center and columella stem cells in *arf2-101* by ABA treatment comparing with no ABA treatment. Nevertheless, the relative stronger GUS staining was not so apparent in the wild type. These results suggest that ARF2 mutation interferes with auxin distribution and the relative high auxin accumulation or auxin signal around QC and columella stem cells inhibits the cell division in the root tips. *HB33* overexpressing plants show the similar ABA sensitive phenotypes as *arf2-101*. Interestingly, ABA treatment leads to the similar expression patterns of both *DR5:GUS* and *proIAA2:GUS* in *HB33* overexpressing plants as in *arf2* mutant. However, the expression of both *DR5:GUS* and *proIAA2:GUS* is lower in *HB33* overexpressing plants than in *arf2-101*, suggesting that *arf2* mutation may regulate the expression of other genes besides *HB33*. Previous study indicate that mutations in some DNA replication related proteins such as DNA polymerase ε (ABO4) and DNA replication A2A (RO1) or FAS1 lead to ABA hypersensitivity in root growth, suggesting that ABA signal might target DNA replication related proteins for inhibiting DNA replication and cell division [18]. A recent study also shows that ABA treatment reduces the phosphorylation level of DNA replication factor C, suggesting the importance of ABA signal transduction in modifying DNA replication related proteins [63].

Auxin distribution is determined by auxin transporters [39,64]. Although ABA treatment reduced the expression of the auxin basipetal efflux transporter PIN2 to a lower level in *arf2-101* than wild type, our genetic analysis of *arf2-101 pin2* double mutant excludes the possibility of PIN2 involving in ABA inhibition of root growth of *arf2* mutant, which is different from its roles in mediating ABA repression of embryonic axis elongation under ABA treatment [3]. The reduced PIN2 might be caused by the low auxin or low auxin signal after ABA treatment as PIN2 expression is regulated by auxin homeostasis [34]. AUX1 is a basipetal auxin influx transporter pairing with PIN2, but at the same time it functions in transporting auxin via phloem from source leaves to the root basal meristem [43]. AUX1 expression in roots was decreased by ABA treatment, but AUX1 levels did not differ between the wild type and *arf2-101* mutant. Our genetic analysis indicates that AUX1 mutation was able to suppress the ABA sensitivity of root growth of *arf2-101*, suggesting that auxin transporting from leaves to root tips is important in ABA inhibition of root growth. Interestingly, auxin efflux carriers PIN3 and PIN7 are reduced more in *arf2* than in the wild type by ABA treatment. PIN3 and PIN7 are key transporters that direct the flow of auxin in root tip [65]. The reduced expression of PIN3 or PIN7 might lead to relative high accumulation of auxin in QC and columella stem cells, which might result in the inhibition of cell division. Although ABA treatment appears to reduce auxin biosynthesis or reduce the whole auxin signal (judged by reduced *IAA2:GUS* and *DR5:GUS*), our auxin feeding experiment indicates that auxin and ABA have a synergistic effect on inhibiting root growth, suggesting that the possible reducing whole auxin amount is not a factor for ABA sensitivity. These results further point out the importance of auxin distribution in ABA inhibiting root growth. Previous studies also indicate that ABA inhibits seedling growth through enhancing auxin signaling [3]. Mutations in some auxin components such as AXR2/IAA7 and AUX1 lead plants to be resistant to both ABA and auxin [3,42,66,67]. This synergistic effect requires the canonical ABA signaling pathway, which is blocked by the dominant *abi1* or *abi2* mutation, but not by *ab3*, *abi5* or *abi6* mutation, indicating that the importance of early ABA signaling components in ABA inhibiting root growth.

**Materials and Methods**

*Arabidopsis thaliana* (Columbia accession) was used unless noted. The plant materials used in this study were: *abi1-1* (Landsberg accession) [4], *abi2-1* (Landsberg accession) [68], *abi3-1* (Landsberg accession) [69], *abi4-1* (Columbia accession) [19], *abi5-1* (Columbia accession) [19], *tir1* *abi1* *afb2* *afb3* quadruple mutant [36], *proCycB1;1:GUS* [18], *pin2-151A5.1/CS89461* [70], *aux1-2* [71], *proDR5:GUS* [38], *proIAA2:GUS* [62], *proAUX1-AUX1:FP* [43], and *proPIN2-PIN2:GFP* [72], *proPIN3-PIN3:GFP* and *proPIN7-PIN7:GFP* [34]. The T-DNA insertion mutant *arf2-7* (CS24601, *AT5G62000*, *arf1* (CS24600, *AT1G30330*, *arf6* (CS24601, *AT1G30330*, *arf21* (CS24621, *AT1G34410*) were obtained from the Arabidopsis Stock Center. *arf2-101* and *arf2-102* mutants were isolated from a screen as described previously [18]. *arf2-101* was crossed with Landsberg accession, and mutants from the F2 population were used for mapping (Table S2).

Seeds were sown onto plates containing MS medium supplemented with 3% (w/v) sucrose and 0.8% agar. After 2 d at 4°C, the plates were transferred to a growth chamber at 20°C with a 16-h light/8-h dark cycle. After 7 days, the seedlings were transplanted into soil and were grown in a greenhouse at 20°C under long-day (16-h light/8-h dark) condition.

**Transgenic plants**

For construction of super promoter, *ARP2* flag (in a modified pCAMBIA 1300 vector, superP3101), *ARP2* cDNA fragment was obtained using the following primers: 5’- CGCGGGGCCCGCTA TGCGGAGTCGGAGGTCTTCAATG (containing the *ApaI* site) and 5’- CGGACTAGTAGGTTCCCAGGCCGCTGAC-3’.
(containing the SpeI site). ARF2 cDNA amplified from total RNAs isolated from seedlings was fused with a flag tag and constructed into a binary vector superP1300. For super P1300-ATHB33: MYC, ATHB33 cDNA fragment was amplified by the specific primer: 5'- GCCGGGCCCAGATATGAGAA-GCCATGAAATAGATAAGGAG-3' (containing the SpeI site) and 5'-GGACTAGTAGAGTACAGGATGTGCTTTGTTGTTG-3' (containing the SpeI site). The amplified cDNAs were verified by sequencing and were cloned into the binary vector super P1300. The Agrobacterium strain GV3101 carrying the constructs was transformed to Arabidopsis thaliana (Columbia) by the floral dip method. More than 10 independent transgenic lines were selected based on hygromycin B resistance in the T2 and T3 generations. The expression level of the gene in each line was analyzed by real-time PCR using specific primer pairs. Two independent lines were generally used for further study.

We constructed pGreen104-HY104 : HB33 vector to silence HB33 gene expression by double-stranded RNAi. A HB33 cDNA fragment which has low homologous sequence with other HB genes was chose and amplified using two primer pairs: RNAi-75240-F:5'- GCTGCA (PstI) GAATTC (EcoRI) GGAGCCATCAACTCTCCATTGAAACTC-3', RNAi-75240-R:5'- CGGATC (BamHI) CTCGAG (XhoI) CACCGAGCTCTTCTCCGCCTTG-3'. In bacteria, the plasmid was selected by Kanamycin. The transgenic plants were selected by Basta. Homozygous lines were identified by real-time PCR using the chemiluminescent method according to the manufacture's protocol.

Phenotypic analyses

The root-bending assay was previously described [18]. Briefly, Seeds sown on MS plates were first kept at 4°C for 2 days and then transferred to a growth chamber at 22°C for 5 days. Seedlings were transferred to various media containing different plant hormones or chemicals; the seedlings were photographed after 7 days unless a different time period is indicated. Relative root growth represents the root growth of seedlings after treatment with ABA or other chemicals comparing with that without ABA or other chemical treatment.

Purification of recombinant protein and electrophoretic mobility shift assay (EMSA)

The N-terminal of ARF2 which contains DNA binding domain was fused in frame with GST and expressed in E. coli BL21 cell line. The fused protein was induced by 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG), and incubated under 28°C for 6 h. The recombinant protein was purified by GST-agarose affinity. The electrophoresis mobility shift assay (EMSA) was carried out using the LightShift Chemiluminescent EMSA Kit (Pierce, 20148) according to the manufacturer’s instructions. The biotin-labeled DNA fragments (5'-CCAAATTGTCCTTTATATAACCGATCTGTCAAAAGGGTTGGGATAGCAGTACGTACAACG-3', 5'-GTTGTTATCTGTCACTGTGTCACCTCAGAAAGAACCCACCTTTTGGATACGATTATAAGAGAACAATTGTG-3') and mutated DNA fragments (5'-CCAAATTGTCCTTTATATAACCGATCTGTCAAAAGGGTTGGGATAGCAGTACGTACAACG-3', 5'-GTTGTTATCTGTCACTGTGTCACCTCAGAAAGAACCCACCTTTTGGATACGATTATAAGAGAACAATTGTG-3') were synthesized, annealed and used as probes, and the biotin-unlabeled same DNA fragments as competitors in this assay. The probes were incubated with the N-ARF2-GST fused protein at room temperature for 20 mins in a binding buffer (5× concentration: 50 mM HEPES-KOH [pH 7.5], 375 mM KCl, 6.25 mM MgCl₂, 1 mM DTT, 0.5 mM BSA, Glycerol 25%). Each 20 μl binding reaction containing 25 fmol Bioitin-probe, 6 μg protein, and 1 μg Poly (dI-dC) was supplemented to the reaction to minimize nonspecific interactions. The reaction products were analyzed by 6.5% native polyacrylamide gel electrophoresis. Electrophoresis was performed at 120 V for about 1 h in TGE buffer (containing 12.5 mM Tris, 95 mM glycine, 0.5 mM EDTA, pH 8.3, precooled at −10°C). The DNA fragments on gel were transferred onto nitrocellulose membrane with 0.5XTBE at 100 V (~380 mA) for 40 mins at 4°C. After cross-linking the transferred DNA to membrane, the membrane was incubated in the blocking buffer for 15 mins with gently shaking, then transferred to conjugate/blocking buffer by mixing 33.3 μl stabilized Streptavidin-Horseradish Peroxidase Conjugate with 10 nl blocking buffer according to manufacture’s protocol (no detail information for the blocking buffer is provided in the kit). The membrane was washed 6 times, each for 5 mins with a washing buffer. Biotin-labeled DNA was detected by the chemiluminescent method according to the manufacture’s protocol.

Chromatin immuno-precipitation (ChIP) analysis

The transgenic lines over-expressing ARF2-Flag were used in this assay. ChIP was performed on 2-week-old seedlings growing on MS plates as described previously [73]. Flag tag-specific monoclonal antibody was used for ChIP analysis. Wild-type plants were treated in the same way and served as the control. The ChIP DNA products were analyzed by PCR using three pairs of primers that were synthesized to amplify about 200-bp DNA fragments in the promoter region or coding region of HB33 or other genes used in ChIP analysis. The primer sequences were listed in Figure S3 (Table S1). The experiment was repeated three times, and similar results were obtained each time.

RNA extraction and quantitative RT–PCR

Two-week-old seedlings on MS plates were used for extraction of total RNAs. Real-time RT-PCR was performed as described previously to determine the relative expression levels of ARF2 or HB33 [18]. The gene-specific primers used for real-time PCR were: RT-ARF2-F: 5'- CCTGATCCGAAGGATGCTCAACG-3', RT-ARF2-R: 5'- GGAGCCATCAACTCTCCATTG AACTC-3'; RT-HB33-F: 5'-GGAACATTACAACCGGAGAGGAGC-3'; RT-HB33-R: 5'-CTCCGATCTCGCCGAGAAACTC-3'. All experiments were independently repeated at least three times, each with triplicates.

Histochemical GUS analysis

The transgenic plants carrying proARF5::GUS, proARL2::GUS, proCYP701::GUS HB33-OE10 or HB33-OE16 were crossed with the af2-101 mutant, and F2 seedlings were selected on kanamycin and transferred to soil after 1 week. Plants that showed the af2-101 growth phenotype were harvested and rechecked for other markers. Expression analysis of the GUS reporter gene was performed as described previously [18].

Confocal microscopy

Five-day-old seedlings carrying proAUX1-AUX1::YFP, proPIN2- PIN2::GFP, proPIN3-PIN3::GFP or proPIN7-PIN7::GFP were transferred to MS medium supplemented with 30 μM ABA for another 36 h. The roots were examined by confocal microscopy and photographed with the same settings to enable comparison of image strength.

Supporting Information

Figure S1  af2-101 shows the similar phenotypes as the wild type on MS medium containing different chemicals. 5-day seedlings grown on MS medium were transferred to the MS medium
containing different concentrations of NaCl, mannitol, LiCl, 2,4-D, IAA, NAA, ACC, brassinosteroid (BR), coronaline, methyljasmonate (MeJA), gibberellic (GA3), or salicylic acid (SA), and cultured for 7 days before taking pictures.

**Table S1**

| The primers used for ChIP. |
|---------------------------|

**Table S2**

| The primers used for map-based cloning. |
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**Author Contributions**

Conceived and designed the experiments: LDH JZ. Performed the experiments: LDH JZ YD ZC XH. Analyzed the data: LW DH JH. Contributed reagents/materials/analysis tools: LDH JZ. Wrote the paper: LW ZG.
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