A Role for Auxin Response Factor 19 in Auxin and Ethylene Signaling in Arabidopsis

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Although auxin response factors (ARFs) are the first well-characterized proteins that bind to the auxin response elements, elucidation of the roles of each ARF gene in auxin responses and plant development has been challenging. Here we show that ARF19 and ARF7 not only participate in auxin signaling, but also play a critical role in ethylene responses in Arabidopsis (Arabidopsis thaliana) roots, indicating that the ARFs serve as a cross talk point between the two hormones. Both arf19 and arf7 mutants isolated from our forward genetic screens are auxin resistant and the arf19arf7 double mutant had stronger auxin resistance than the single mutants and displayed phenotypes not seen in the single mutants. Furthermore, we show that a genomic fragment of ARF19 not only complements arf19, but also rescues arf7. We conclude that ARF19 complements ARF7 at the protein level and that the ARF7 target sequences are also recognized by ARF19. Therefore, it is the differences in expression level/pattern and not the differences in protein sequences between the two ARFs that determines the relative contribution of the two ARFs in auxin signaling and plant development. In addition to being auxin resistant, arf19 has also ethylene-insensitive roots and ARF19 expression is induced by ethylene treatment. This work provides a sensitive genetic screen for uncovering auxin-resistant mutants including the described arf mutants. This study also provides a likely mechanism for coordination and integration of hormonal signals to regulate plant growth and development.

Many aspects of plant growth and development are regulated by the plant hormone auxin. Auxin exerts its biological functions by activating signal transduction pathways that ultimately regulate the expression of downstream target genes that control particular developmental processes (for review, see Dharmasiri and Estelle, 2004; Kepinski and Leyser, 2005a; Woodward and Bartel, 2005). The current model of auxin signal transduction suggests that binding of auxin to the F-box protein Transport Inhibitor Response 1 (TIR1) promotes the interaction between TIR1 and the negative regulators auxin/indole-3-acetic acid proteins (AUX/IAA), thereby facilitating the degradation of AUX/IAA proteins through the ubiquitin-related protein degradation machinery (Dharmasiri et al., 2005; Kepinski and Leyser, 2005b). Degradation of AUX/IAAs in response to an auxin signal is believed to allow auxin response factors (ARFs) to form ARF/ARF homo- or heterodimers that then bind to the cis auxin response elements to activate or repress gene expression (Ballas et al., 1993; Abel et al., 1994; Ulmasov et al., 1995, 1997, 1999a, 1999b; Hardtke et al., 2004). Therefore ARFs and AUX/IAAs are the key transcription factors in regulating the expression of auxin-responsive genes, and elucidation of the roles of the ARFs and AUX/IAAs in auxin signaling is crucial for our ultimate understanding of the mechanisms governing various auxin-regulated developmental processes.

The AUX/IAAs are the first well-characterized early auxin-inducible genes, and the AUX/IAAs are found to be short-lived transcription factors (Hagen and Guilfoyle, 1985; Theologis et al., 1985; Abel et al., 1994). The AUX/IAAs belong to a family with 29 members in the Arabidopsis (Arabidopsis thaliana) genome, and a subset of the AUX/IAAs appears to have redundant functions (Reed, 2001; Liscum and Reed, 2002; Remington et al., 2004). Most of the AUX/IAA proteins contain four highly conserved domains (domains I to IV; Abel et al., 1994). While domains I and II are important for AUX/IAA activities and stability (Worley et al., 2000; Oulett et al., 2001; Tiwari et al., 2004), domains III and IV are proposed to serve as a dimerization domain for forming homo- or heterodimers with other AUX/IAAs or ARFs (Ulmasov et al., 1999a, 1999b; Oulett et al., 2001; Reed, 2001). So far, all of the loss-of-function aux/iaa mutants do not display obvious developmental defects. However, many gain-of-function AUX/IAA mutants, including axr2/iaa7 (Nagpal et al., 2000), axr3/iaa17 (Leyser et al., 1996), axr5/iaa1 (Yang et al., 2004), shy2/iaa3 (Kim et al., 1998), slr/iaa14 (Fukaki et al., 2002), msg2/iaa19 (Tatematsu et al., 2004), and bdl/iaa12 (Hamann et al., 1999) have pleiotropic developmental defects. The gain-of-function aux/iaa mutants have been isolated from previous auxin-resistant mutant screens or screens for mutants with developmental defects. All of the gain-of-function

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iaa mutants contain mutations in the conserved domain II, and it has been shown that such mutations disrupt the interaction between TIR1 and the AUX/IAA proteins, thus preventing degradation of AUX/IAA proteins in an auxin-dependent manner (Gray et al., 2001).

Unlike AUX/IAAs that lack a DNA-binding domain (DBD), the ARFs that belong to a 23-member family in Arabidopsis have a B3-like DBD in the N-terminal region and an AUX/IAA domain in the C-terminal region (Ulmasov et al., 1997). The first ARF (ARF1) was initially identified biochemically by its ability to bind the auxin response elements and later other ARFs were identified on the basis of sequence homology to ARF1 (Ulmasov et al., 1997). Although ARF1 has been isolated for almost 2 decades, it is still not clear what roles ARF1 plays in auxin signaling and ARF1 was initially identified biochemically by its C-terminal region (Ulmasov et al., 1997). The first ARF N-terminal region and an AUX/IAA domain in the family in Arabidopsis have a B3-like DBD in the 2001).

Analysis of T-DNA insertion mutants of arf19 and arf7 mutants by screening for mutants resistant to sirtinol, a synthetic drug that was shown to activate auxin signaling pathways without interfering with the auxin polar transport (Zhao et al., 2003; Dai et al., 2005). In addition to the auxin resistance phenotypes, both arf19 and arf7 also displayed root-specific ethylene insensitivity, and the arf7arf19 double mutant had stronger resistance to both ethylene and auxin than the single mutants, suggesting that ARF19 and ARF7 have overlapping functions. Furthermore we found that the expression of ARF19 is induced by either IAA or ethylene treatments, providing a mechanism for ARF19 participating in auxin and ethylene signaling. Finally, we found that an ARF19 genomic fragment not only complemented arf19 mutations, it also rescued arf7 in terms of auxin resistance, non-phototropic responses, and adult plant phenotypes. These results suggest that ARF19 can be substituted for ARF7 at the protein level and the different phenotypes observed for arf7 and arf19 are probably caused by the differential expression patterns and levels of the two genes.

RESULTS

Isolation of arf19 Mutants

We previously carried out a genetic screen for mutants resistant to sirtinol, a small organic molecule that activates auxin signal transduction pathways (Zhao et al., 2003; Dai et al., 2005). In addition to the known auxin-resistant mutants such as axr1 through axr6, new auxin-resistant mutant loci including Atcand1 were also identified from the sirtinol screen (Cheng et al., 2004). One of the sirtinol-resistant loci named arf19-101 is shown in Figure 1A and characterized in this paper. When germinated and grown on Murashige and Skoog (MS) media containing sirtinol in total darkness, the mutant had long hypocotyls and elongated primary roots whereas the wild-type control had stunted hypocotyls with essentially no elongation of the primary roots (Fig. 1A). Interestingly, apical hook development in both arf19-101 and wild type was disrupted by sirtinol (Fig. 1A), indicating that the mutant is not totally insensitive to the sirtinol effects. When the mutant was backcrossed to wild type, the resulting F1 plants were sensitive to sirtinol and approximately 25% of the F1 population displayed sirtinol resistance (data not shown), indicating that the sirtinol resistance was caused by a recessive mutation in a single gene. The mutant also displayed resistance to both IAA (data not shown) and a synthetic auxin, 2,4-D (Fig. 1B). In the dark, exogenous IAA or 2,4-D inhibited hypocotyl and root elongation and apical hook formation in wild-type seedlings (Fig. 1B). In...
contrast, the mutant arf19-101 had long hypocotyls and visible primary roots when grown on media containing 250 nM 2,4-D (Fig. 1B). Although arf19-101 appeared to be recessive in terms of sirtinol resistance, the heterozygous arf19-101 was less sensitive to 2,4-D in hypocotyl elongation (Fig. 1B). The light-grown arf19-101 seedlings were also auxin resistant and had elongated primary roots when grown on media containing 100 nM 2,4-D (Fig. 1C).

We mapped the arf19-101 mutation to a 150 kb interval on chromosome I between the markers F14D16A and F18O4B (Fig. 1D). We sequenced candidate genes in the mapping interval and found out that arf19-101 had a C to T change in the open reading frame At1g19220, which was annotated as ARF19 (Fig. 1E). ARF19 belongs to a family of transcription factors, and some of the ARFs, including ARF19, contain a conserved DBD, an AUX/IAA domain, and a Q-rich activation domain (Fig. 1E). The identified mutation in arf19-101 converted a Trp codon to a stop codon, which likely led to the expression of a truncated ARF19 with the intact DBD (Fig. 1E). Sequencing another allele of arf19 (arf19-102) revealed a mutation in the fourth exon of ARF19 and the mutation also created a premature stop codon (Fig. 1E).

Identification of mutations in the ARF19 gene in both arf19 alleles indicated that the arf19 mutations were responsible for the observed auxin-resistant phenotypes. To further confirm that the auxin-resistant phenotypes were caused by the identified arf19 mutations, we transformed arf19-101 with a 7 kb ARF19 genomic fragment that contains the entire ARF19 open reading frame and its regulatory sequences. As shown in Figure 1F, the ARF19 genomic fragment restored auxin sensitivity of arf19-101 in a root elongation assay, providing proof that the mutation in arf19-101 caused the auxin-resistant phenotypes (Fig. 1F). Although a wild-type copy of ARF19 genomic fragment largely
restored the auxin sensitivity of arf19-101 (Fig. 1F), interestingly, the transgenic seedlings were still slightly less sensitive to auxin than wild type (Fig. 1F), which is consistent with our observations that arf19 mutants were weakly semidominant in terms of auxin resistance.

The Mutant arf19 Had Ethylene-Insensitive Roots

We tested whether arf19 mutants had altered sensitivities to other hormones and found out that arf19-101 was as sensitive to the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) as the wild type in the aerial parts of the seedlings (Fig. 2, A and B). However, arf19-101 roots were less sensitive to ACC than wild-type roots. (Fig. 2, B and C). The arf19-102 allele displayed a very similar response to ACC as arf19-101 (data not shown), indicating that the ethylene resistance was caused by the mutations in the ARF19 gene.

Isolation of arf7 Mutants

From our sirtinol-resistant mutant screen, multiple alleles of arf7 mutant were also isolated. When grown on sirtinol in the dark, the arf7-201 seedlings had long hypocotyls, but did not have an apical hook (Fig. 3A). The arf7-201 seedlings grown on sirtinol in the dark also had primary roots (Fig. 3A), but the roots were much shorter than those of arf19-101 (Figs. 1A and 2A). Dark-grown seedlings of arf7-201 were also resistant to IAA (data not shown) and 2,4-D (Fig. 3B). Unlike the arf19 mutants that were resistant to 2,4-D in both the primary roots and hypocotyls, arf7-201 seedlings did not show resistance in roots when grown on 250 nM 2,4-D in the dark. However, arf7-201 had hypocotyls clearly resistant to 2,4-D (Fig. 3B). When grown on 100 nM 2,4-D in light, there is no obvious difference between wild type and arf7-201 primary roots (Fig. 3C), which may explain why arf7 mutants were missed from previous auxin-resistant mutant screens. The arf7-201 mutant was mapped to the middle of chromosome 5 and mutations were identified in the gene At5g20730 in arf7-201 (a splice junction mutation) and arf7-202 (Arg-371 to stop codon; Fig. 3D). At5g20730 is annotated as ARF7, which was previously isolated from screens for nonphototropic mutants (Stowe-Evans et al., 1998; Harper et al., 2000).

Mutant arf7 Enhances arf19 Phenotypes

Dark-grown seedlings of arf19-101 were strongly resistant to sirtinol in primary root and displayed relatively weaker resistance in the hypocotyls (Fig. 1A) whereas arf7-201 was very resistant to sirtinol in hypocotyls and only slightly resistant in the roots (Fig. 3A). Dark-grown seedlings of the arf7-201arf19-101 double mutant had both long primary roots and long hypocotyls when grown on sirtinol (Fig. 3E). The double mutant was also clearly more resistant to 2,4-D (Fig. 3, F and G) and IAA (Fig. 3F) in both dark-grown and light-grown seedlings than the single mutants. When grown on 100 nM 2,4-D in light, the double mutant (root length = 9.2 ± 2.1 mm at day 6, n = 21) had even longer roots than arf19-101 (5.7 ± 1.0 mm, n = 30, see Figs. 3G and 1C), although arf7-201 (2.3 ± 0.5 mm, n = 30) itself did not show much auxin resistance in light (wild-type root length = 2.1 ± 0.5 mm, n = 22, see Fig. 3C). After careful examination of root growth on IAA-containing media, we found that arf7-201 had weak resistance to auxin in the root (Fig. 3F). The ethylene-resistant root phenotype of arf19-101 was also enhanced by arf7-201 (Figs. 3, H and K, and 2). In addition to the synergistic effects on auxin and ethylene sensitivity by arf19 and arf7, the arf7arf19 double mutant displayed many developmental defects including disruption of gravity responses and lateral root development (Fig. 3I).

Regulation of ARF19 Expression by Auxin and Ethylene

We introduced a β-glucuronidase (GUS) reporter under the control of ARF19 promoter into wild type

![Figure 2. Ethylene-insensitive phenotypes of arf19-101. A, Triple response displayed by wild-type Ler seedlings. Left two seedlings were grown on MS media and the right two seedlings were grown on 10 μM ACC for 3 d in dark. B, The same as A except that the seedlings are arf19-101. C, Effects of ACC on root elongation: a dose response curve. Error bars refer to se.](image)
and analyzed the expression patterns of the ARF19 promoter. As shown in Figure 4A, ARF19 is expressed throughout the etiolated seedling with the strongest staining in the primary roots and cotyledons. In both the root and cotyledon, the GUS staining is mainly located along the vascular tissue. Weaker staining is also visible in the hypocotyl vascular tissue (Fig. 4A). In response to IAA treatment, expression of ARF19 is induced (Fig. 4A). In addition to the increased staining in the vascular tissues, auxin treatment also led to increased GUS staining in other tissues (Fig. 4, B and C). In primary roots, ARF19 expression induced by
IAA treatment appeared mainly in the elongation zone (Fig. 4B). When treated with ethylene gas, ARF19 expression was also induced in primary roots (Fig. 4D). Interestingly, the most staining induced by ethylene also appeared mainly in the elongation zone and root meristem (Fig. 4D) and the expression pattern of ARF19 in roots induced by ethylene is very similar to that induced by IAA treatment (Fig. 4, B and D). The inducible expression of ARF19 in roots by ethylene was further confirmed by quantitative reverse transcription (RT)-PCR analysis (Fig. 4E).

Effects of the arf Mutations on the Expression of Ethylene Response Factor 1

Ethylene response factor 1 (ERF1) is one of the early genes induced upon ethylene treatment (Solano et al., 1998). Because the arf mutants had ethylene-insensitive roots and ARF19 was induced by ethylene treatment, we tested whether mutations in the ARF genes had any effects on ethylene-inducible gene expression. When treated with ethylene gas, the single and double arf mutants had ERF1 levels similar to those induced in wild type (Fig. 4F). However, the induction fold was reduced as a result of elevated expression of ERF1 in the mutant arf19 and the double mutant (Fig. 4F), indicating that the ARF genes may participate in desensitizing ethylene responses.

Complementation of arf7 Phenotypes by an ARF19 Genomic Fragment

The phenotypic differences between arf7 and arf19 could be caused by the different expression patterns of the two genes or difference between the two proteins,
or both. We transformed arabidopsis transform.7-201 with the ARF19 genomic construct that was shown to be able to complement arf19-101. The ARF19 genomic fragment contains the ARF19 open reading frame and the ARF19 regulatory elements. To our surprise, the ARF19 genomic fragment complemented the arf7-201 mutant in several aspects. Loss-of-function mutations of arf7 were previously shown to cause disruption of phototropic growth in response to directional blue light. The new arf7 alleles also displayed nonphototropic hypocotyl growth (Fig. 5, A and B). In contrast, arf19-101 still retains the phototropic hypocotyl bending to light source (Fig. 5C). When arf7-201 was transformed with the ARF19 genomic fragment, the nonphototropic response phenotype of arf7-201 was rescued (Fig. 5D). We then checked whether the ARF19 genomic fragment could also rescue the auxin-resistant phenotype of arf7-201. Indeed, the ARF19 transgene also restored the auxin sensitivity of arf7-201, although the dark-grown seedlings of arf7 transformed with ARF19 were still slightly less sensitive to auxin than wild type (Fig. 5, E–H). In addition to restoring the seedling phenotypes, the ARF19 transgene also rescued the hyponastic leaf phenotype of arf7-201 (Fig. 5, I–J).

**DISCUSSION**

In this report, we provide evidence showing that the transcription factors ARF7 and ARF19 are not only important for auxin signaling, but also play critical roles for Arabidopsis to respond to ethylene. In response to auxin or ethylene treatments, ARF19 expression is induced. Furthermore, we show that ARF19 genomic fragment complements arf7 mutations, indicating that it is the different expression patterns/levels of ARF19 and ARF7, not the differences between the two proteins, that determines the relative roles of the two genes in auxin signaling and plant development.

Analysis of auxin-resistant mutants has been very informative in dissecting auxin signaling mechanisms. Previous auxin-resistant mutants isolated by Mark Estelle and colleagues were identified by screening for mutants with long primary roots in the presence of 2,4-D in light (Hobbie and Estelle, 1994). Such a screen would miss those auxin-resistant mutants with very weak auxin resistance in roots such as the arf7 mutants or mutants without a primary root such as arf5/mp or bdl/iaa12 (Hardtke and Berleth, 1998; Hamann et al., 1999). For reasons not totally understood yet, the screen for sirtinol resistance mutants appeared to provide a more sensitive screen for auxin-resistant mutants. For example, we have isolated several novel auxin-resistant loci and multiple alleles of all the previously known auxin-resistant mutants, including multiple interesting alleles of axr6. Previous work on arf19 and arf7 mutants indicated that ARF7 mainly regulates auxin-regulated hypocotyl growth and bending whereas ARF19 mainly participates in auxin signaling in roots (Okushima et al., 2005a; Wilmoth et al., 2005). We also observed that arf19-101 displayed strong auxin resistance in the root (Fig. 1, A–C) while the arf7-201 hypocotyl was very auxin resistant (Fig. 3, A–C). From previous analysis of arf7arf19 double mutants and the analysis of the arf7-201arf19-101 double mutant here (Fig. 3, E–H), it is clear that arf7 and arf19 function redundantly in auxin responses in both hypocotyl and root, although the auxin resistance of arf7-201 roots is too weak to be identified in a screen for root phenotypes (Fig. 5, A–C). However, the resistant root phenotype is evident when arf7-201 was grown on sirtinol-containing media, suggesting that sirtinol can be used to carry out sensitive auxin-resistant mutant screens.

**Figure 5.** Complementation of arf7-201 with an ARF19 genomic fragment. A to D, Phototropic response of wild type and the mutants. Light came from the left to right. A, Ler, B, arf7-201, C, arf19-101, D, arf7-201 transformed with an ARF19 genomic fragment. E to H, The auxin-resistant phenotype of arf7-201 is rescued by an ARF19 fragment. E, Ler, F, Homozygous arf7-201, G, Heterozygous arf7-201, H, arf7-201 transformed with an ARF19 genomic fragment. I, Complementation of the upward leaf curling phenotype of arf7-201 by ARF19. Left, arf7-201, right, arf7-201 transformed with an ARF19 genomic fragment. J, A side view of the leaves. Left, arf7-201, right, arf7-201 transformed with an ARF19 genomic fragment.
The interactions between ethylene pathways and auxin pathways are intriguing and are believed to occur at various levels and junctions. Auxin is known to regulate ethylene biosynthesis by up-regulating the expression of several ACC synthase genes and some of the auxin-induced phenotypes may be directly caused by the increased ethylene levels induced by auxin (Theologis, 1989; Abel et al., 1995). On the other hand, ethylene has also been shown recently to stimulate the accumulation of IAA in roots through the activation of two tissue-specific anthranilate synthase genes, and the accumulation of IAA levels is required for normal ethylene responses in roots (Stepanova et al., 2005). Moreover, ethylene treatment led to elevated expression of a UDP-Glcnidole-3-acetate β-d-glucosyltransferase that was proposed to participate in forming IAA-sugar conjugates (De Paepe et al., 2004). Many auxin signaling or transport mutants, including axr1, tir1, and ckr1/pin2 were significantly less sensitive to ethylene (Swarup et al., 2002; Alonso et al., 2003), suggesting that either the auxin signaling/transport components are also used in ethylene signaling or normal auxin responses may be required for normal ethylene responses. Some components of protein degradation machinery participate in regulated degradation of AUX/IAA proteins as well as EIN3 family transcription factors (Leyser et al., 1993; Guo and Ecker, 2003; Hellmann et al., 2003; Potuschak et al., 2003). Loss-of-function mutants of those components therefore may affect both auxin and ethylene pathways. It was reported that ethylene biosynthesis genes were down-regulated in some auxin mutants whereas some mutants including ein2 and dst1 had altered responses to both hormones (Vandenbussche et al., 2003; Okushima et al., 2005b), suggesting complex interactions between the two hormones. It is intriguing that ARF19 and ARF7 also participate in ethylene responses because EIN3 family transcription factors have been shown to be responsible for the expression of almost all of the ethylene-inducible genes (Guo and Ecker, 2003). The fact that ARF19 is induced by both auxin and ethylene suggests two plausible mechanisms that may explain how ARF19 plays a role in both auxin and ethylene signaling. Upon auxin treatment, the anthranilate synthase genes are induced. Accumulation of IAA in the roots caused by increased expression of anthranilate synthase then activates the expression of ARF19 that regulates the transcription of downstream targets. Alternatively, auxin treatment activates the expression of ACC synthase genes, which lead to the increased ethylene levels that induce the expression of ARF19 to regulate downstream gene expression. The difference between the two models is which hormone is the direct activator of ARF19 expression. In either case, inactivation of ARF19 will lead to decreased sensitivity to both auxin and ethylene.

The ARF protein family consists of 23 members in Arabidopsis and it is a challenge to determine the physiological functions of each ARF gene. Sequence analysis revealed that ARF7 is the closest homolog of ARF19, and the double mutant analysis indicated that ARF19 and ARF7 have unique and overlapping functions in auxin signaling and plant development (Okushima et al., 2005a; Wilmoth et al., 2005). The fact that arf19 displayed developmental phenotypes different from arf7, yet the ARF7 genomic fragment can complement arf7 phenotypes, indicates that ARF19 can complement ARF7 at the protein level and the ARF7 target sequences are also recognized by ARF19. These results also suggest that the overlapping of the expression patterns between the two genes may be larger than the promoter-GUS data indicated. Because the ARF19 gene under the control of its own promoter (not ARF7 promoter or 35S promoter) can rescue arf7, it is likely that ARF19 is also expressed in most of the cells where ARF7 is expressed, but with very low levels that were not detected by the GUS staining. Once an extra copy of ARF19 is introduced, the expression levels of ARF19 in those cells may increase to complement arf7. We noticed that the degree of auxin sensitivity in Arabidopsis hypocotyls depends on the gene dosage of ARF19 and ARF7. Heterozygous arf7-201 is less sensitive to auxin than the wild-type controls, but more sensitive than the homozygous arf7-201 (Fig. 3B). Consistent with our observation, the nonphototropic phenotype of arf7/ nph4 is also reported to be semidominant (Stowe-Evans et al., 1998; Harper et al., 2000). The arf19 mutants were also semidominant in terms of auxin resistance in the hypocotyls (Fig. 1B). Therefore ARF7 and ARF19 provide a sensitive and buffered system to mediate auxin signaling in response to developmental and environmental changes.

**MATERIALS AND METHODS**

**Mutant Screen and Plant Growth Conditions**

All the mutants described in this study are in the ecotype Landsberg (Ler) background. Ethylmethane sulfate-mutagenized M2 Arabidopsis (Arabi-
dopsis thaliana) Ler seeds were purchased from Leibie Seeds. The mutagenized seeds were surface sterilized and plated on 0.5 MX MS containing 3.5 µM sirtinol. After the seeds were cold treated at 4°C for 2 d, the plates were exposed to light at room temperature for 3 h before they were wrapped in foil. After germination and growth in total darkness at 23°C for 72 h, the etiolated seedlings were scored for mutants with long root, long hypocotyl, or both. The putative mutants were transferred to 0.5 MX MS containing 1.5% Suc to recover. The mutants eventually were transferred to soil to set seeds.

**Cloning of arf19 and arf7 by Positional Cloning**

For the arf19 mutant, we put the mapping population generated by crossing arf7-101 with Arabidopsis Columbia onto 0.5 MX MS plates containing 100 mg 2,4-D. Six-day seedlings grown on 2,4-D in light were scored for long root phenotypes. The homozygous arf19-101 seedlings were transferred to soil and the auxin-resistant phenotypes were tested in F3. Genomic DNA samples from the individual F2 homozygous arf19-101 were used to find linkage and to determine relative chromosome locations of the mutation to molecular markers. Fine mapping was performed according to the methods described in Lukowitz et al. (2000). New PCR-based simple sequence length polymorphic and cleaved-amplified polymorphic sequence markers were designed based on the polymorphisms between Columbia and Ler (www. Arabidopsis.org).

To complement arf19, a 6.8 kb SacI fragment of ARF19 was cloned into the SacI site of pZIP211 and the resulting construct was transformed into arf19-101 and arf7-201 mutants (Clough and Bent, 1998). Transgenic plants were selected on kanamycin-containing media.
For the arf7 mutant, we scored homozygous arf7-201 from the F2 mapping population on media containing 3.5 μm sirtolin in the dark. The mutation was mapped with PCR-based molecular markers as described above.

**Hormone Assays**

For auxin and ethylene responses, Arabidopsis seeds were surface sterilized and put on 0.5 × MS plates containing various concentrations of either IAA or ethylene precursor ACC. After 72 h growth in total darkness at 23°C, seedlings were scanned and photographed. To analyze mutant response to auxin in light, seeds were germinated on 0.5 × MS containing 100 nM 2,4-D and incubated in growth chamber at 23°C with 16-h-light/8-h-dark cycle. The length of the seedling roots or hypocotyls were measured with the NIH Image software (http://rsb.info.nih.gov/nih-image/Default.html). All data points were derived from at least 15 seedlings.

**Genotyping and Double Mutant Analysis**

The arf7-201 and arf19-101 were genotyped with the following derived cleaved amplified polymorphic sequence markers. For arf7-201, the genotyping primers are 5′-caagctagccagtagaagttggtg-3′ and 5′-aggatcctgatagctactc-3′. The PCR fragment is digested with restriction enzyme HindIII. The arf19-101 genotyping primers are 5′-agggattagatgtttagctgtc-3′ and 5′-aggatcctgatagctactc-3′. The PCR fragment is cut with enzyme BstXI. The arf19arf7 double mutant was confirmed by genotyping with the above molecular markers. For the transgenic plants of arf7-201 transformed with ARF19 genomic fragment, we also genotyped the lines to ensure that they still contain the homozygous mutations of arf7-201.

**GUS Staining and Promoter Activity Analysis**

The ARF19 promoter was amplified with the following primers: 5′-aaaa-agtgcagtatggtctgcaggttc-3′ and 5′-aaaaagctttcattcagttagagcgtc-3′. The PCR fragment was then cloned into pBI110.3 with restriction enzymes BamHI and SalI. The construct was introduced into wild type using Agrobacterium-mediated transformation. The transgenic lines were selected on kanamycin plates. GUS staining was carried out according to protocols described previously (Zhao et al., 2001).

To test the effects of hormones on ARF19 expression, we treated seedlings as follows. For auxin treatment, the 3-d-old etiolated seedlings were immersed in 0.5 × MS liquid media containing 10 μM IAA for 2 h, and 0.5 × MS treated seedlings served as controls. The experiments were carried out in a green room to minimize the interference of lights. For ethylene treatment, 3-d-old etiolated seedlings were treated with ethylene gas for 6 h as described previously (Guo and Ecker, 2003).

**Quantitative RT-PCR**

Quantitative real time RT-PCR was performed using a LightCycler (Roche). cDNA was extracted from whole seedlings or roots using the QIAGEN RNeasy plant kit. cDNA was synthesized with Superscript First-Strand kit (Invitrogen) according to manufacturer’s manual. Two microliters of a 10× cDNA reaction dilution was added to each PCR reaction containing 4 μl MgCl2, 1× FastStart DNA Master SYBR green reaction mix (Roche), and 0.5 μM of the each of the primer for ERFI mRNAs, ERFI-F (5′-gggagggaggtcagagctgct-3′) and ERFI-R (5′-tagctctgcattgccatgctc-3′). Samples were denatured initially for 10 min followed by 45 cycles of 95°C for 1 s, 65°C for 5 s, and 72°C for 10 s. All experiments were repeated at least twice and the data were analyzed with RelQuant software (Roche).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AY669794.

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