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Repression of *AUXIN RESPONSE FACTOR10* by microRNA160 is critical for seed germination and post-germination stages

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Summary

AUXIN RESPONSE FACTORS (ARFs) are transcription factors involved in auxin signal transduction during many stages of plant growth development. *ARF10*, *ARF16* and *ARF17* are targeted by microRNA160 (miR160) in *Arabidopsis thaliana*. Here, we show that negative regulation of *ARF10* by miR160 plays important roles in seed germination and post-germination. Transgenic plants expressing an miR160-resistant form of *ARF10*, which has silent mutations in the miRNA target site (termed *mARF10*), exhibited developmental defects such as serrated leaves, curled stems, contorted flowers and twisted siliques. These phenotypes were not observed in wild-type plants or plants transformed with the targeted *ARF10* gene. During *sensu stricto* germination and post-germination, *mARF10* mutant seeds and plants were hypersensitive to ABA in a dose-dependent manner. ABA hypersensitivity was mimicked in wild-type plants by exogenous auxin. In contrast, overexpression of *MIR160* (*35S:MIR160*) resulted in reduced sensitivity to ABA during germination. Transcriptome analysis of germinating *ARF10* and *mARF10* seeds indicated that typical ABA-responsive genes expressed during seed maturation were overexpressed in germinating *mARF10* seeds. These results indicate that negative regulation of *ARF10* by miR160 plays a critical role in seed germination and post-embryonic developmental programs, at least in part by mechanisms involving interactions between ARF10-dependent auxin and ABA pathways.

Keywords: microRNA, seed germination, ARF10, miR160, ABA, auxin.

Introduction

microRNAs (miRNAs) are small (approximately 21–24 nucleotides), single-stranded RNAs that downregulate target genes at the post-transcriptional level (Bartel, 2004). In plants, most miRNAs regulate target genes by guiding mRNA cleavage (Jones-Rhoades *et al.*, 2006), although non-degradative suppression mechanisms may also exist (Aukerman and Sakai, 2003; Chen, 2004). miRNAs play crucial roles in a broad range of processes, including plant development, hormone signaling (Palatnik *et al.*, 2003; Juarez *et al.*, 2004; Baker *et al.*, 2005), maintenance of homeostasis, and responses to environmental and nutritional signals (Jones-Rhoades *et al.*, 2006).

Several hormonal signal transduction pathways in plants are controlled by miRNAs. miR159 targets mRNAs encoding GA (gibberellin)-MYB transcription factors that interact with GA-response elements and are involved in short-day floral initiation and anther development (Achard *et al.*, 2004; Millar and Gubler, 2005). Interestingly, the expression of miR159 is repressed in the absence of GA, possibly through the action of DELLA proteins (named due to conserved pentapeptide motif) that are in turn targeted for GA-induced proteolysis (Achard *et al.*, 2004). The auxin signal transduction pathways are also regulated by miRNAs. Auxin binds the auxin receptor TIR1, an F-box protein of the SCF ubiquitin ligase complex (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005), which mediates ubiquitination of the AUX/IAA protein and subsequent proteolysis through the 26S proteasome pathway (Gray *et al.*, 2001; Rogg and Bartel,

2001; Kepinski and Leyser, 2002). miR393 targets *TIR1* mRNA and the three most closely related F-box proteins (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004). miR164 targets the mRNA encoding NAC1 (Rhoades *et al.*, 2002; Mallory *et al.*, 2004), a putative transcription factor that acts downstream of TIR1 to promote lateral root development (Xie *et al.*, 2000, 2002).

AUX/IAA proteins heterodimerize with AUXIN RESPONSE FACTORS (ARFs), which bind auxin-response elements and either activate or repress gene expression (Ulmasov *et al.*, 1997a,b, 1999a,b). The interaction of AUX/IAA with ARF proteins represses ARF function (Ulmasov *et al.*, 1997b; Tiwari *et al.*, 2001). Several clades within the ARF family are negatively regulated by small RNAs. *ARF10*, *ARF16* and *ARF17* mRNAs are targeted by miR160, while *ARF6* and *ARF8* mRNAs are targeted by miR167 (Rhoades *et al.*, 2002; Kasschau *et al.*, 2003; Jones-Rhoades and Bartel, 2004; Vazquez *et al.*, 2004). Additionally, miR390 guides in-phase processing of *TRANS-ACTING siRNA3* (*TAS3*) primary transcripts that generate tasiRNAs, which in turn target *ARF2*, *ARF3* and *ARF4* (Allen *et al.*, 2005; Williams *et al.*, 2005).

ARF10, ARF16 and ARF17 share high amino acid sequence similarity and form a subgroup within the ARF family (Guilfoyle and Hagen, 2001; Remington *et al.*, 2004; Okushima *et al.*, 2005). Expression of miR160-resistant target genes is known to cause over-accumulation of their gene product and phenotypic changes (Chen, 2004; Laufs *et al.*, 2004; Mallory *et al.*, 2004; Guo *et al.*, 2005; Millar and Gubler, 2005). miR160-resistant mutants have been used successfully to characterize the biological functions of *ARF16* (Wang *et al.*, 2005) and *ARF17* (Mallory *et al.*, 2005), revealing that downregulation of *ARF16* and *ARF17* is essential to maintain the normal developmental programs of root, leaf and flower organs. The focus of this study was to determine the function of miR160-mediated regulation of *ARF10*, especially in seed germination. Transgenic plants expressing the miR160-resistant *mARF10* driven by its own 5′ upstream regulatory sequence were analyzed. The phenotypes of mutant seeds and seedlings showed the importance of auxin signaling in the early stages of plant growth. A key role of miR160 and ARF10 in interaction between auxin and ABA during germinative and post-germinative stages was identified.

Results

Expression of miR160-resistant ARF10 causes developmental defects in leaves, flowers and siliques

The ARF10 protein contains a conserved family domain and a B3 domain, which occurs in multiple transcription factors including LEC2 (Stone *et al.*, 2001) and FUS3 (Luerssen *et al.*, 1998; Figure 1a). The miR160 target site (5′-AGGAAUACAGGGAGCCAGGCA-3′) in *ARF10* mRNA is located

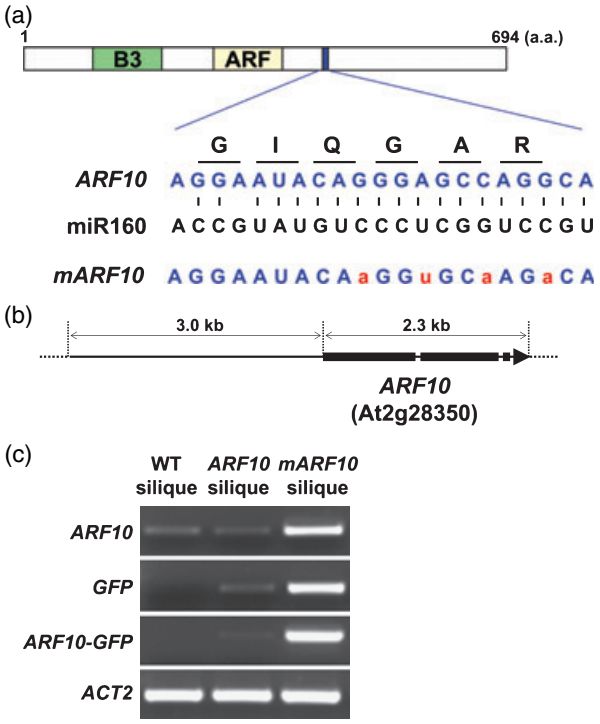

Figure 1. De-repression of miR160-resistant *mARF10* with silent mutations.
(a) Domain structure of the ARF10 protein. The B3 DNA binding domain (B3) and the conserved ARF domain (ARF) are highlighted in green and yellow, respectively. The miR160 complementary sequence in the *ARF10* mRNA (*ARF10*) and the corresponding region of amino acid sequence (GIQGAR) are shown. The silent mutations introduced in the miR160-resistant form of *ARF10* (*mARF10*) are highlighted in red.
(b) Genomic DNA region used for transformation of the miR160-resistant *mARF10* gene (*ARF10:mARF10*). Four silent mutations were introduced to the native *ARF10* gene (At2g28350) containing the 5′ upstream regulatory sequence (3.0 kb) using mutagenized PCR primers (see Experimental procedures), and the mutated sequence was subcloned into the vector, which was used for plant transformation.
(c) Semi-quantitative RT-PCR of *ARF10*, *ARF10*-GFP and *mARF10*-GFP fusion genes. RNA was extracted from wild-type, *ARF10* or *mARF10* siliques. PCR was performed using *ARF10* forward and reverse primers (*ARF10*), GFP forward and reverse primers (GFP) or *ARF10* forward and GFP reverse primers (*ARF10*-GFP). Quantification of the band intensities using two independent biological replicates is shown in Figure S1.

at a position corresponding to the amino acid sequence GIQGAR. The miR160 sequence shows high complementarity to the *ARF10* sequence, with only two mis-matched bases. The *ARF10* gene, including the coding region (2.3 kb) and the 5′ upstream region (3 kb) (*ARF10:ARF10*) (Figure 1b), was amplified by PCR, and then four mutations were introduced into the miR160 target site by PCR to generate *ARF10:mARF10* (Figure 1a). The *ARF10* and *mARF10* sequences were fused to the green fluorescent protein sequence (*ARF10:ARF10*-GFP or *ARF10:mARF10*-GFP), and plants were transformed with each construct. Twelve transformants were selected on hygromycin-containing agar plates, transferred to soil and grown to produce seeds.

The mutant and control plants are hereafter termed *mARF10* and *ARF10* plants, respectively.

To examine the accumulation of transcripts in wild-type, *ARF10* and *mARF10* plants (both T_3 generation), RT-PCR was performed using RNA extracted from siliques with *ARF10*- and GFP-specific primers. Experiments with three different sets of primers (*ARF10* forward/*ARF10* reverse, GFP forward/GFP reverse and *ARF10* forward/GFP reverse) showed that transcript levels were higher in *mARF10* than in *ARF10* transgenic plants or wild-type (Figure 1c and Figure S1). These results suggest that release of *mARF10* transcripts from cleavage guided by miR160 results in over-accumulation of the transgene mRNA, and further indicate that *ARF10* mRNA is under negative regulation by miR160.

Phenotypic analysis was performed to determine the effects of *ARF10* and *mARF10* in transgenic plants. Seeds harvested from *mARF10* plants produced seedlings with a range of rosette phenotypes, including normal rosettes (Figure 2a), relatively small rosettes with serrated leaves (Figure 2b, termed type B) and extremely small rosettes with narrow leaves (Figure 2c, termed type C). Seeds from plants transformed with non-mutagenized *ARF10* did not produce type B and C rosettes (data not shown). Genotyping experiments showed that the serrated- and narrow-leaf mutant phenotypes were associated with the transgene insert, whereas the normal plants lacked a transgene (Figure 2d). These results suggested that the mutant phenotypes were due to the expression of mutagenized, miR160-resistant *mARF10*.

The type C, narrow-leaf seedlings (Figure 2c) ceased to grow in the juvenile stage and died. Lethal effects were also observed in the transgenic plants expressing miR160-resistant *ARF16* (Wang *et al.*, 2005) and *ARF17* (Mallory *et al.*, 2005). The type B, serrated-leaf seedlings were 32% smaller

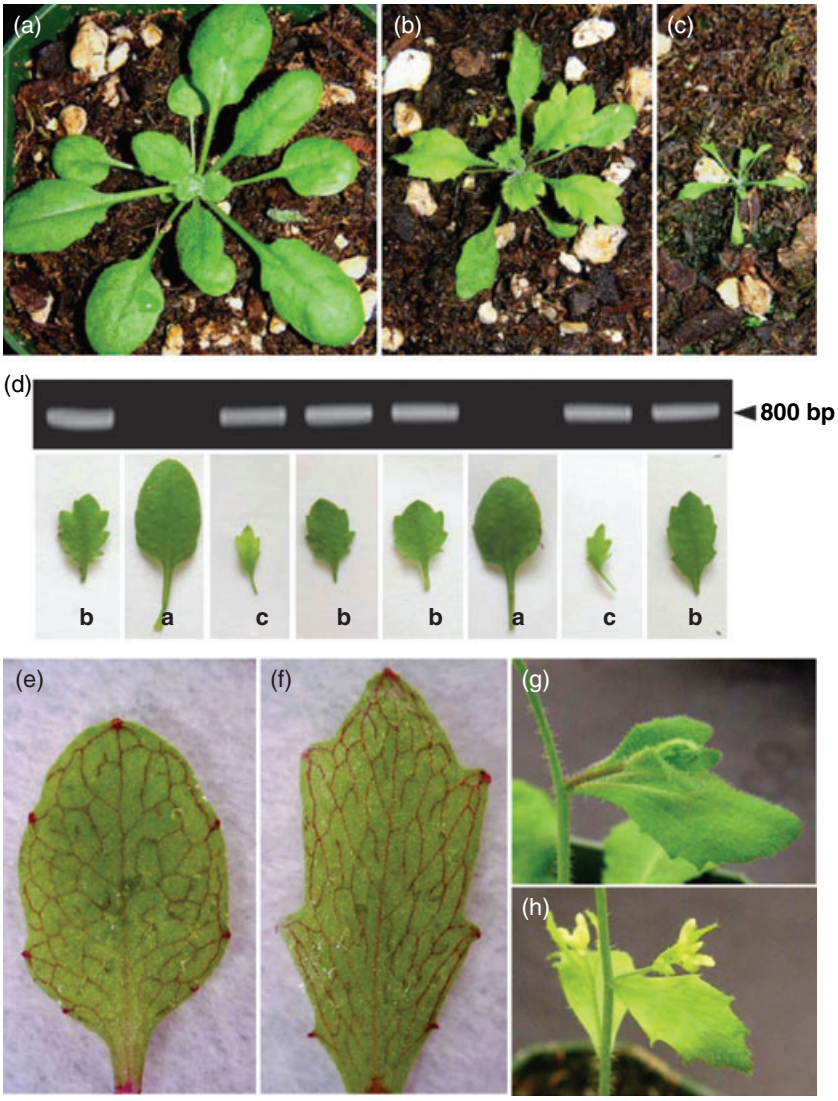

Figure 2. Leaf phenotypes and genotyping of the miR160-resistant *mARF10* plants.
(a–c) Progeny of plants expressing the miRNA-resistant *mARF10*, showing wild-type rosettes (a), relatively small rosettes with serrated leaves (b), and extremely small rosettes with narrow leaves (c).
(d) Genotyping of plants shown in (a–c) by PCR using specific primers designed for the hygromycin phosphotransferase gene (*HPT*).
(e, f) Venation patterns in wild-type and *mARF10* leaves, respectively.
(g, h) Cauline leaves and flowers of wild-type and *mARF10* plants, respectively.

than wild-type seedlings, but were able to produce viable seeds. The segregation of T_3 progeny from the type B plants was 23.3% wild-type (70/300), 49.7% type B phenotype (149/300) and 27.0% type C phenotype (81/300), indicating that type B seedlings were dominant, heterozygous mutant lines. No homozygous transgenic *mARF10* plants were recovered. The type C lethal, narrow-leaf plants probably contained the *mARF10* transgene in a homozygous state.

It is well-known that auxin is critical for venation patterning (Sachs, 1989). *ARF5* (*MONOPTEROS*) and *ARF7* play key roles in vascular differentiation (Hardtke *et al.*, 2004). To determine whether the serrated-leaf phenotype of *mARF10* was a result of defective vascular differentiation, we examined venation patterns in wild-type and *mARF10* rosette leaves. No defects were observed in the venation of *mARF10* leaves, suggesting that the serrated-leaf phenotype was not associated with vascular patterning (Figure 2e,f).

mARF10 plants displayed yellowish cauline leaves and sepals (Figure 2g,h). Flowers had contorted and elongated petals (Figure 3a) and produced twisted siliques (Figure 3b). The silique wall was wavy, such that the shape of individual seeds was apparent from the outside of the silique (Figure 3c). No apparent defects were detected in mature seeds. Flowers and seeds from *ARF10* transgenic plants were normal (data not shown).

Involvement of auxin and ARF10 in sensu stricto *germination and post-germination*

The involvement of auxin in seed germination and post-germination is understood poorly. To obtain evidence of free auxin location during germination, seeds were produced from transgenic plants expressing a GUS reporter gene driven by the auxin-responsive promoter *DR5* (Ulmasov *et al.*, 1997b). As it is difficult to detect GUS activity in Arabidopsis seeds before testa rupture due to limited permeability of the GUS substrate, GUS assays were performed during the lag phase between testa rupture and endosperm penetration (Liu *et al.*, 2005a,b). Activation of *DR5* promoter was detected only in the micropylar region of germinating seeds (Figure 4a). Excised embryos showed highly localized GUS expression in the radicle tip (Figure 4b), which became more apparent after radicle emergence (post-germination) (Figure 4a). These results indicated that free auxin accumulates in the radicle tip of germinating and germinated seeds. Scanning electron microscopic imaging of seeds exhibiting a GUS signal during early imbibitional stages confirmed that the radicle tip was still enclosed by the endosperm layer (Figure 4c). This indicates that the content of, or the sensitivity to, free auxin increases in the radicle tip during *sensu stricto* germination. To determine whether *ARF10* promoter expression co-localizes with the zone of active *DR5:*GUS expression, Arabidopsis plants were transformed with a vector containing the 5′ upstream regulatory sequence of the *ARF10* gene fused to the GUS coding sequence. The *ARF10* promoter was activated in the radicle tip of germinating Arabidopsis seeds before testa and endosperm rupture (Figure 4d), which is coincident with the localization of *DR5* expression. *ARF10* promoter activity was also high in the root at the post-germinative stages, and was eventually detected through whole seedlings (data not shown). Radicle-specific GUS expression detected in the *DR5:*GUS and *ARF10:*GUS seeds was not due to selective absorption of the GUS substrate in the radicle, as shown using Arabidopsis enhancer-trap lines exhibiting diverse tissue-specific GUS expression in endosperm-, axis- and cotyledon-specific

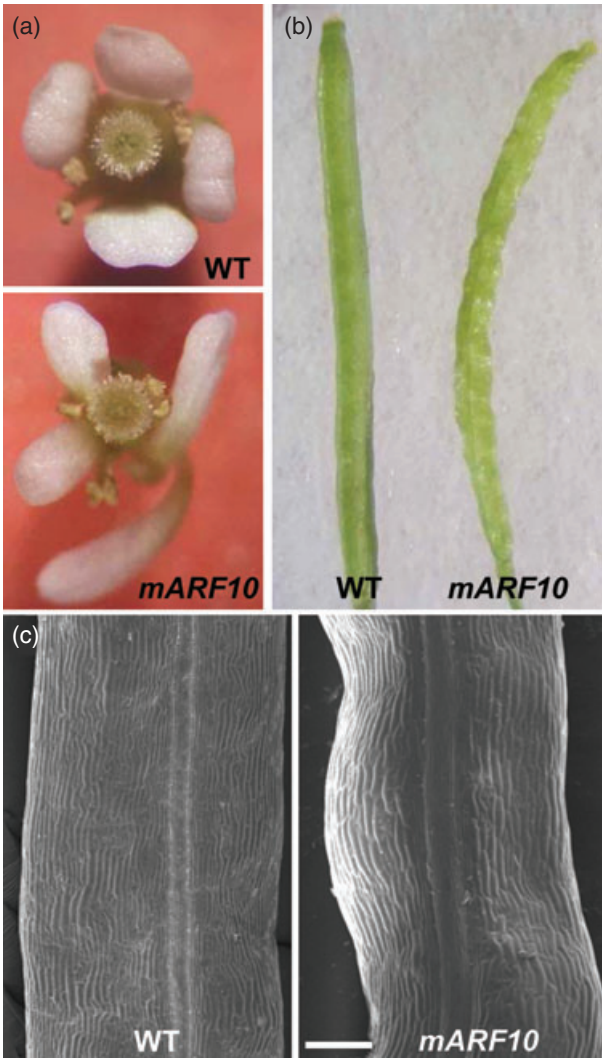

Figure 3. Flower and silique development in wild-type and the *mARF10* plants.
(a) Morphology of wild-type (upper) and the *mARF10* (lower) flowers.
(b) Morphology of wild-type (left) and *mARF10* (right) siliques. Note that the *mARF10* silique is twisted.
(c) Close-up view of wild-type (left) and *mARF10* (right) siliques by a scanning electron microscope.

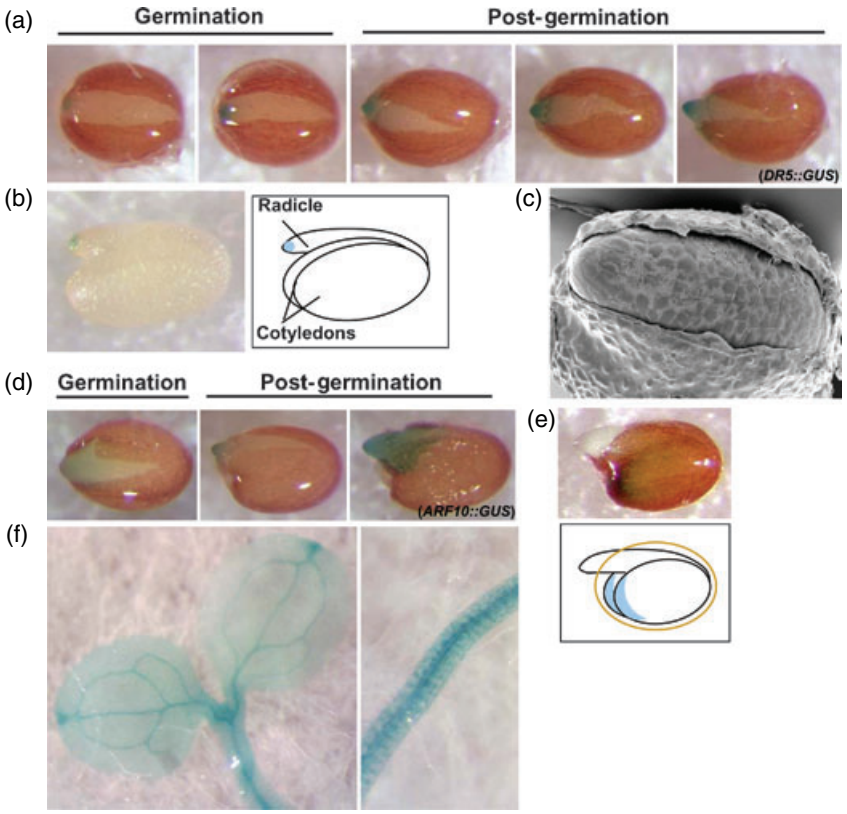

Figure 4. Activation of *DR5* and *ARF10* promoters during and following Arabidopsis seed germination.
(a) *DR5:*GUS expression in germinating and germinated seeds.
(b) *DR5:*GUS expression in the radicle tip of the embryo excised from germinating seed. A schematic representation of the seed is shown to the right of the panel.
(c) Scanning electron microscopy of one of the GUS-expressing seeds at the germinative stage. Note that the radicle tip is still enclosed by the endosperm.
(d) *ARF10:*GUS expression in germinating and germinated seeds.
(e) An example of cotyledon-specific GUS expression in germinating seed of an enhancer-trap line to show the specificity of *DR5:*GUS and *ARF10:*GUS expression in the radicle. A schematic representation of the seed is shown below.
(f) *ARF10:*GUS expression in the vascular tissues in the cotyledons and hypocotyls (left) and the root (right).

expression within a whole seed (Liu *et al.*, 2005a). An example of cotyledon-specific GUS expression in seeds is shown in Figure 4(e). *ARF10* promoter activity gradually decreased as seedlings grew, and was detected mainly in the vascular tissues during later seedling development (Figure 4f).

miR160-resistant mARF10 *mutants exhibit impaired seedling establishment*

The presence of free indole-3-acetic acid (IAA), as indicated by the *DR5:*GUS reporter, and *ARF10* promoter expression during seed germination and post-germination prompted a detailed characterization of the miR160-resistant *mARF10* mutant. No significant difference in germination frequency or speed was detected between control and *mARF10* seeds (data not shown). Defects were observed in seedling morphology when they were grown on Murashige and Skoog (MS) agar medium (Murashige and Skoog, 1962) containing 1% w/v sucrose. While 29% of seedlings from *mARF10* seeds showed wild-type morphology (transgene nulls), the rest exhibited bent cotyledons (Figure 5a,b). A few seedlings in this population (4/100) had three cotyledons and three true leaves emerging at one time (Figure 5c). These phenotypes were not observed in plants transformed with the non-mutated *ARF10* transgene (Figure 5d).

Although cotyledon development was altered in the mutant seedlings, the percentage seedling establishment on agar medium did not differ between wild-type and *mARF10* plants during the early stages of development (data not shown). When *mARF10* seedlings were incubated on agar medium for prolonged periods (approximately 3 weeks), 26% (13/50) of the plants exhibited white true leaves (Figure 5e). These were most likely homozygous plants, which could be grown further by transferring to soil before eventually dying as described above (Figure 2c). The developmental defects in the *mARF10* seedlings were more drastic when they were directly grown on soil (Figure 5f,g). Twenty-three per cent of the seedlings that were probably transgene nulls showed normal growth (Figure 5h). The smaller seedlings (Figure 5i) that eventually exhibited serrated leaves grew slowly, while most of the extremely small seedlings (Figure 5j) did not survive. Slower growth of the young *mARF10* seedlings explains the reduction in size observed in *mARF10* plants at later stages in development (Figure 2b,c). Thus, the *mARF10* transgene caused defects in post-germinative seedling establishment, suggesting the importance of auxin regulation immediately after seed germination.

Potential cross-talk between auxin and ABA signal transduction during seed germination and post-germination

Detailed analysis of *mARF10* seedling establishment indicated that the post-germinative defects were more

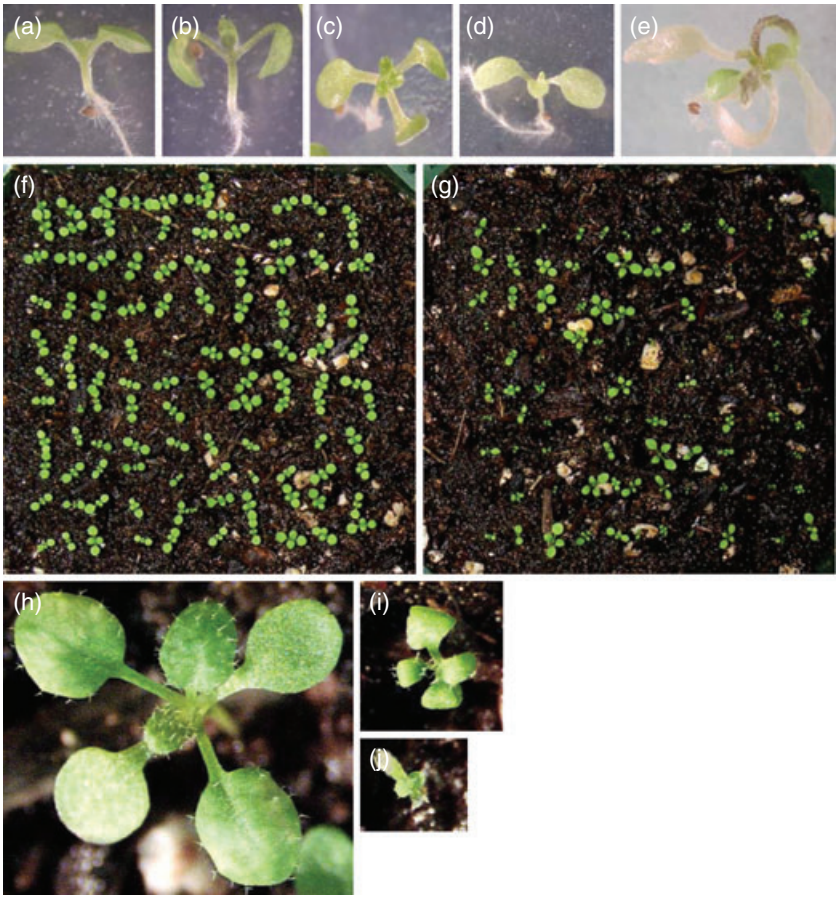

Figure 5. Post-germinative growth of wild-type and the *mARF10* seedlings.
(a–e) Seedlings grown on MS agar medium containing 1% sucrose. Wild-type (a), bent cotyledon (b) and tricotyledon (c) seedlings were observed in the progeny of the *mARF10* transformants, while plants transformed with the non-mutated *ARF10* produced all wild-type seedlings (d). Putative homozygous seedlings had white leaves and eventually died (e).
(f,g) Seedling establishment of wild-type and the *mARF10* plants, respectively, on soil. Note that the percentages of seedling emergence do not differ between wild-type and *mARF10*.
(h–j) Representative seedlings in the *mARF10* population. Wild-type (h), relatively smaller but healthy (i) and extremely small and lethal (j) seedlings were observed.

exaggerated on soil, which may reflect an increased sensitivity to stressful conditions encountered in soil compared to conditions on agar plates. Abscisic acid (ABA) is known to play important roles in stress responses and to inhibit seed germination and post-germinative growth. Therefore, we examined the responses of *mARF10* seeds and seedlings to ABA. First, dose-dependent responses to ABA of *sensu stricto* germination, i.e. the control of radicle emergence, were measured for wild-type, *ARF10* and *mARF10* seeds. Radicle emergence from *mARF10* seeds was inhibited at lower concentrations of ABA than the concentration inhibitory to *ARF10* control seeds (Figure 6a) and wild-type seeds (Figure 6b), indicating that *mARF10* seeds are hypersensitive to ABA. By contrast, transgenic seeds overexpressing *miR160* (*35S:MIR160a*) exhibited reduced sensitivity to ABA (Figure 6a). The effect of exogenous IAA (10 μM) plus ABA on germination of wild-type seeds was also tested. Although exogenous IAA did not significantly affect germination in the absence of ABA (data not shown), IAA suppressed germination at relatively low concentrations of ABA (Figure 6b), which is consistent with earlier studies (Brady *et al.*, 2003). Exogenous IAA also reversed the reduced sensitivity of the *35S:MIR160* seeds to ABA (Figure S2). These results suggest that *ARF10* and its repression by miR160 play a critical role in cross-talk between auxin and ABA during germination.

To examine potential cross-talk between auxin and ABA at post-germinative stages, wild-type and *mARF10* seedlings were grown on agar medium in the presence and absence of an ABA concentration series. Although there were morphological differences between wild-type and *mARF10* seedlings as described above, the percentage seedling establishment did not differ between wild-type and *mARF10* plants in the absence of exogenous ABA (Figure 7a). In contrast, hypersensitivity of *mARF10* seedlings to ABA was detected at concentrations as low as 0.05–0.2 μM (Figure 7b–d). Twenty-two per cent (80/371) of seedlings in the *mARF10* population that were probably transgene nulls grew well in the presence of 0.2 μM ABA, whereas the remainder of the *mARF10* seedlings arrested after cotyledon emergence. The cotyledons of the arrested seedlings were dark green and unopened. Accumulation of anthocyanin was observed in the upper hypocotyls of arrested seedlings (Figure 7e). ABA hypersensitivity was not observed in *ARF10* seedlings (data not shown). These results indicate that release of *ARF10* from repression by miR160 also affects ABA sensitivity during post-germination stages.

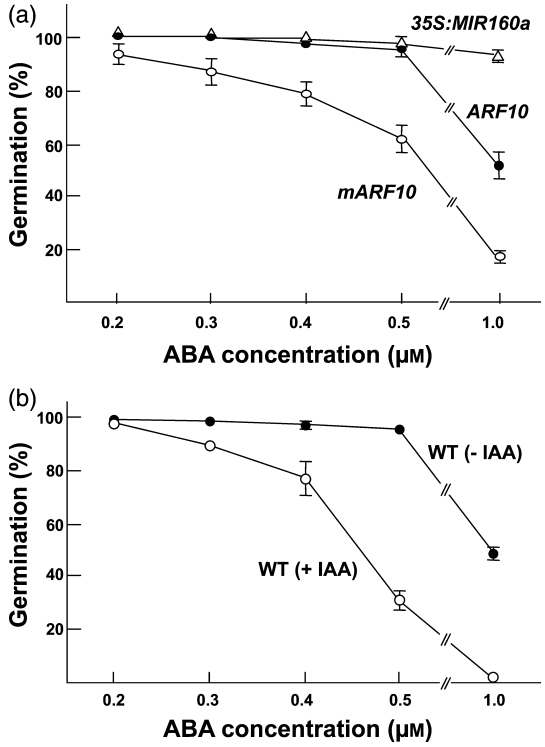

Figure 6. ABA dose–response curves for wild-type, *ARF10*, *mARF10* and *35S:MIR160a* seed germination.
(a) ABA dose–response curve for *ARF10* control (*ARF10*, closed circle), *mARF10* (open circle) and *35S:MIR160a* (open triangle) seeds. Seed germination was performed in triplicate samples, each of which contained *ARF10*, *mARF10* and *35S:MIR160a* seeds in the same solution for side-by-side comparison.
(b) ABA dose–response curve for of wild-type seeds in the presence (+IAA, open circle) and absence (–IAA, closed circle) of 10 μM IAA. Completion of germination was defined as radicle protrusion through the endosperm layer (*sensu stricto* germination).

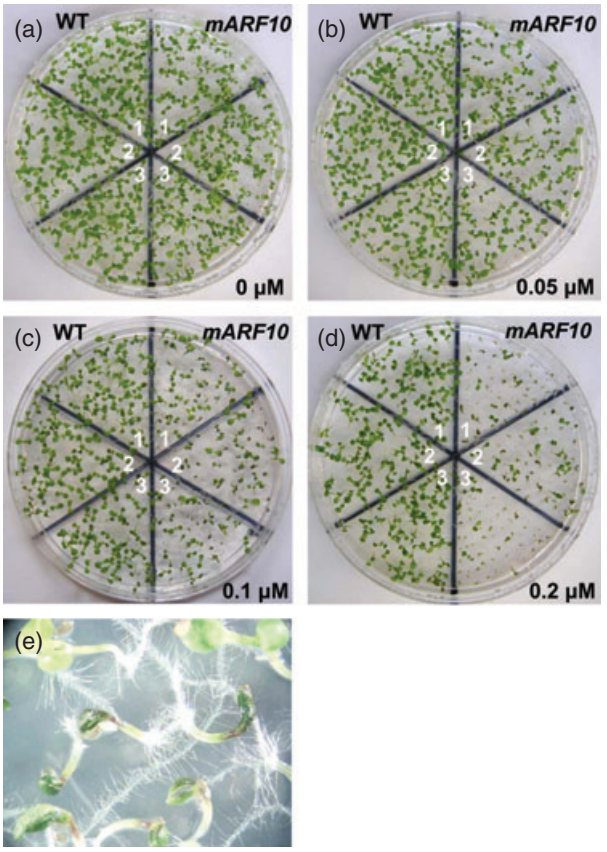

Figure 7. ABA response of wild-type and *mARF10* seedlings.
(a–d) Growth of wild-type (WT) and *mARF10* seedlings in the absence (a) or presence of ABA (b–d, 0.05, 0.1 and 0.2 μM, respectively). Seeds harvested from three (1, 2 and 3) individual *mARF10* or wild-type plants were examined.
(e) Close-up view of the *mARF10* seedlings in the presence of 0.2 μM ABA. The majority of the mutant seedlings had dark unopened cotyledons with anthocyanin accumulation at the upper hypocotyls; however, 22% of the *mARF10* population exhibited a wild-type phenotype in this respect (upper two seedlings), and did not accumulate anthocyanin in the presence of ABA.

The results above indicate that over-accumulation of ARF10, a component of auxin signal transduction, alters ABA sensitivity in seeds. To further examine the auxin–ABA cross-talk at the molecular level, the gene expression profile in *mARF10* seeds was compared with that in wild-type or *ARF10* control seeds using microarray analysis. Comparison of gene expression between *mARF10* and wild-type seeds identified 31 genes that were overexpressed more than twofold ($P < 0.05$) in the *mARF10* mutant seeds (Table 1). These genes included typical ABA-inducible genes that are known to be expressed during seed development and maturation, such as *LATE EMBRYOGENESIS ABUNDANT* (*LEA*), *EARLY METHIONINE-LABELED* (*EM*), *2S SEED STORAGE PROTEINS*, and *OLEOSIN* (seed oil body surface protein). Lectins and enzyme inhibitors that are often expressed during seed development were also upregulated. In addition, *KIN1*, a typical ABA/stress response-associated gene was upregulated in *mARF10* mutant seeds. To statistically analyze whether ABA-associated genes are over-represented in the group of 31 genes, the promoter regions (1000 bp upstream sequences) of these genes were subjected to motif analysis using the *Athena* program (O'Connor *et al.*, 2005). Table S1 shows the top 20 motifs detected among these genes. The highly significant, top four over-represented motifs were the ABA-responsive element (ABRE) binding site motif, the ABRE-like binding site motif, the ACGTABREMOTIFA2OSEM motif and GBOXLERBCS, all of which are known to mediate gene regulation by ABA.

It is well-known that many ABRE-containing genes are regulated by ABA during seed development and maturation. We also experimentally examined the ABA responsiveness of the identified genes during seed germination. For these experiments, we used the 14 genes that were specifically upregulated in *mARF10* but not in *ARF10* control seeds (shown in bold in Table 1). Figure 8 shows the distribution of the over-represented ABRE-related motifs in the promoter regions of these 14 genes and their response to ABA during

Table 1 Genes upregulated in *mARF10* mutant seeds (comparison of *mARF10* and Col wild-type)[a]

AGI code[b]	Annotation[c]	Function/biological process	Fold induction	ABA references[d]
AT4G33560	Expressed protein	Unknown	11.4	
AT4G27140	**2S seed storage protein 1**	Seed storage/maturation	9.0	(1), (4)
AT5G46960	**Invertase/pectin methylesterase inhibitor family protein**	Enzyme inhibitor/defense	8.2	
AT5G49360	β-xylosidase	Hydrolase	7.9	
AT5G15960	*Stress-responsive protein (KIN1)*	Stress response	6.9	(3), (5), (9)
AT1G43800	Acyl-(acyl-carrier-protein) desaturase, putative	Seed storage/maturation	6.8	
AT2G34430	Chlorophyll *a,b* binding protein	Photosynthesis	6.1	
AT4G27150	**2S seed storage protein 2**	Seed storage/maturation	6.1	(1), (4)
AT3G16450	Jacalin lectin family protein	Seed storage/maturation	5.5	
AT1G14940	Major latex protein (MLP)-related	Unknown	5.0	
AT3G56980	Basic helix-loop-helix (bHLH) family protein	Transcription factor	5.0	
AT1G47540	**Trypsin inhibitor, putative**	Enzyme inhibitor	4.9	(2)
AT3G17520	*Late-embryogenesis-abundant domain-containing protein*	Seed storage/maturation	4.8	(1), (8)
AT2G40170	**Em-like protein GEA6 (EM6)**	Seed storage/maturation	4.5	(1), (6), (8)
AT5G14780	Formate dehydrogenase (FDH)	Stress response	4.4	
AT1G67330	Expressed protein	Unknown	4.3	
AT1G55260	**Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein**	Seed storage/maturation	4.2	
AT1G29910	Chlorophyll *a,b* binding protein 2	Photosynthesis	4.2	
AT5G62340	Invertase/pectin methylesterase inhibitor family protein	Enzyme inhibitor	3.8	
AT4G25140	**Glycine-rich protein/oleosin**	Seed storage/maturation	3.8	(7)
AT5G67370	**Expressed protein**	Unknown	3.7	
AT5G07330	Expressed protein	Unknown	3.5	
AT3G04290	GDSL-motif lipase/hydrolase family protein	Hydrolase	3.3	
AT1G14930	Major latex protein (MLP)-related	Unknown	3.3	
AT4G09600	**Gibberellin-regulated protein 3 (GASA3)**	Unknown	3.3	(2)
AT2G45490	**Protein kinase, putative**	Kinase	2.9	
AT1G75830	**Plant defensin-fusion protein, putative**	Defense	2.8	
AT2G23630	Multi-copper oxidase type I family protein	Oxidase	2.3	
AT3G63040	**Expressed protein**	Unknown	2.2	
AT2G18340	*Late-embryogenesis-abundant domain-containing protein*	Seed storage/maturation	2.0	(1), (8)
AT4G20230	Terpene synthase/cyclase family protein	Metabolism	2.0	

[a]Genes with greater than twofold changes are shown.
[b]Genes specifically expressed in the *mARF10* mutant but not in the control *ARF10* transgenic seeds are shown in bold.
[c]Genes known to be regulated or potentially regulated by ABA are shown in italic.
[d]The numbers of the references indicating ABA regulation of gene expression (below) are shown: (1) Devic *et al.*, 1996; (2) Genevestigator (http://www.genevestigator.ethz.ch/); (3) Knight *et al.*, 1999; (4) Koornneef *et al.*, 1989; (5) Kurkela and Franck, 1990; (6) Nakamura *et al.*, 2001; (7) Plant *et al.*, 1994; (8) Vicient *et al.*, 2000; (9) Wang *et al.*, 1995.

seed germination (22 h imbibition). The results indicate that the majority of the ABRE-related motif-containing genes are responsive to ABA during seed germination, although some of genes containing the motifs did not respond to exogenous ABA (Figure 8). Overexpression of ABA-responsive genes in *mARF10* seeds was consistent with their hypersensitivity to ABA during seed germination and seedling establishment. The effects of exogenous IAA on these ABA-responsive genes were smaller than the ABA effects (Figure S3).

Only five genes were found to be significantly downregulated in *mARF10* seeds. These include *SUCROSE-H+ SYMPORTER GENE SUC1* (At1g71880), *SULFITE REDUCTASE* (At5g04590), expressed proteins (At3g30720 and At2g40000) and *PIN3* (At1g70940), which is known to be involved in lateral relocation of auxin efflux in tropism responses (Friml *et al.*, 2002).

Discussion

Repression of ARF10 *by miR160 is essential for normal plant development*

Functional redundancy in the *ARF* gene family is known. Single knockout mutants of *arf7* and *arf19* do not show apparent phenotypes, while *arf7 arf19* double mutants exhibit a strong auxin-related phenotype (Okushima *et al.*, 2005). Attempts to overexpress *ARF* genes are frequently uninformative. For example, Mallory *et al.* (2005) examined 23 *ARF17*-overexpressing plants under the control of the CaMV 35S promoter and found that 21 primary transformants displayed no obvious developmental defects and two displayed a slight reduction in rosette leaf size and overall stature. This is probably because the transgene-derived *ARF17* mRNAs are still repressed by relatively abundant

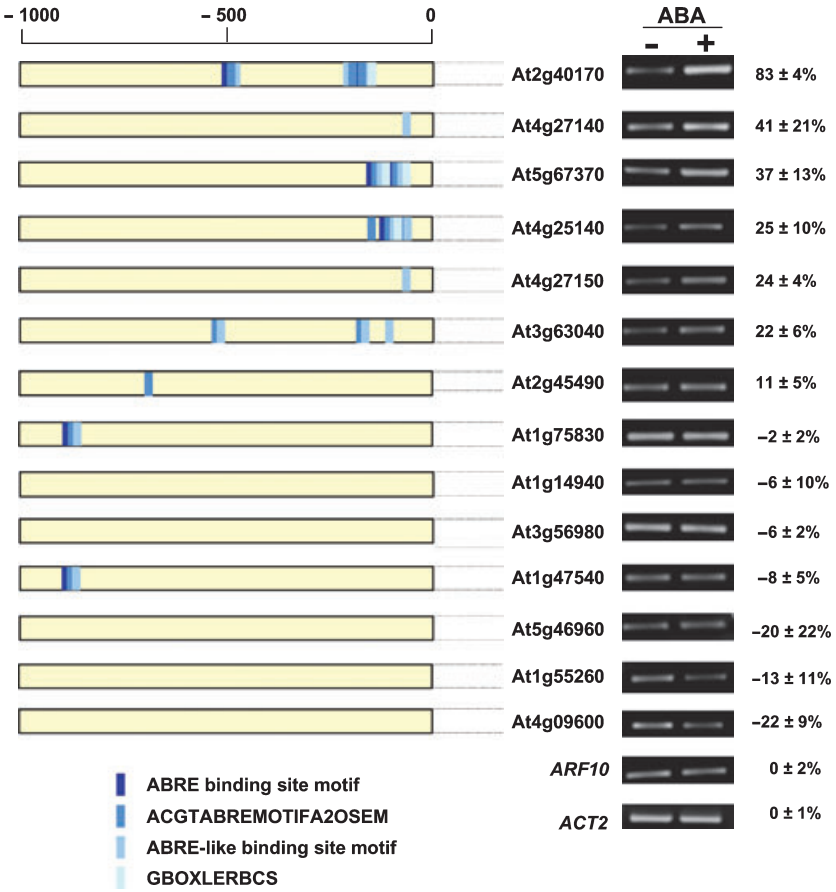

Figure 8. Distribution of ABA-responsive element (ABRE)-related motifs in the promoter regions of *mARF10*-upregulated genes and their responses to exogenous ABA during seed germination.
The approximate positions of the ABRE binding site, ACGTABREMOTIFA2OSEM, ABRE-like binding site and GBOXLERBCS motifs are shown in the 1000 bp upstream sequences. Gene expression in wild-type imbibed seeds (22 h) in the absence (–, left lanes) or presence (+, right lanes) of 1 μMABA was analyzed by semi-quantitative RT-PCR. Values next to the panels indicate the percentage increase or decrease in gene expression as a result of ABA treatment. The experiments were performed using three biological replicates, and the means ± SD are shown.

miR160. Therefore, introducing silent mutations in the miRNA target sequence provides a unique tool to analyze function of miRNA-targeted genes. Multiple morphological defects were observed during development of the *mARF10* plants, which is consistent with the previous observation that *ARF10* is expressed in many tissues and developmental stages, such as seedling, rosette leaf, stem, cauline leaf and flower (Mallory *et al.*, 2005). Some defects detected in *mARF10* were similar to those observed in the miR160-resistant *5mARF17* mutant (Mallory *et al.*, 2005), while other *mARF10* phenotypes were distinct. Leaf margin serration (Figure 2b) and curling (data not shown) were observed in both *mARF10* and *5mARF17* (Mallory *et al.*, 2005). Seedlings with three cotyledons, which produced three simultaneously emerging true leaves (Figure 5c), were observed in *mARF10* and *5mARF17* plants (Mallory *et al.*, 2005). While *5mARF17* exhibited reduced petal size and produced sterile flowers, *mARF10* flowers had elongated and contorted petals (Figure 3a) and were fertile. Embryonic defects found in *5mARF17* were not observed in *mARF10*. We did not detect any apparent defects in root development in *mARF10* plants. While neither of *arf10* nor *arf16* single loss-of-function mutants show root phenotypes, *arf10 arf16* double mutants exhibit impaired root gravitropism, suggesting that both genes play important but redundant roles in root development (Wang *et al.*, 2005). *ARF5* (*MONOPTEORS*) is also known to mediate root meristem formation (Berleth and Jurgens, 1993).

ARF10, *ARF16* and *ARF17* have both specific and overlapping functions in plant development. However, it is clear that miR160-directed repression is essential for normal regulation and restriction of activity. When miR160-resistant *mARF16* (Wang *et al.*, 2005) and *5mARF17* were overexpressed under the control of 35S promoter, most of the *35S:*m*ARF16* plants and about 67% of the *35S:5mARF17* plants died without flowering (Mallory *et al.*, 2005; Wang *et al.*, 2005). In the present study, the putative homozygous *mARF10* plants also exhibited seedling lethality. Therefore, *ARF* mRNA downregulation by miRNAs appears to be a key dampening mechanism, presumably to restrict auxin-initiated signaling or to maintain low transcript levels in non-responsive cells.

A critical role of auxin signal transduction in seed germination and post-germination

The major plant hormones regulating seed germination in diverse plant species are GA and ABA, which promote and

inhibit germination, respectively. Brassinosteroids and ethylene are also known to have promotive effects on seed germination. Auxin alone is not generally considered to be important in the control of seed germination, although recent studies suggest that cross-talk between auxin and GA or ethylene might affect germination and seedling establishment (Fu and Harberd, 2003; Ogawa *et al.*, 2003; Chiwocha *et al.*, 2005).

Our data suggest that alteration of auxin signaling, by de-repression of *ARF10*, dramatically increases the sensitivity of seeds and seedlings to exogenous ABA during *sensu stricto* germination and post-germinative shoot formation (Figures 6a and 7). *DR5:*GUS expression analysis revealed that free IAA is present during and following seed germination (Figure 4a). Auxin is known to affect seed germination in the presence of ABA (Brady *et al.*, 2003) (Figure 6b). Importantly, the ABA dose–response curve of *mARF10* seed germination mimics the ABA dose–response curve of wild-type seeds in the presence of IAA (Figure 6a,b). This strongly suggests that de-repression of *ARF10 in vivo* increased sensitivity to ABA. Consistently, overexpression of *MIR160a* exerted the opposite effects and reduced the sensitivity of seeds to ABA (Figure 6a), which was reversed by exogenous auxin (Figure S2). Taken together, our results indicate that miRNA is involved in the regulation of auxin and ABA cross-talk in seeds.

Potential cross-talk between auxin and ABA was suggested by a number of studies in multiple species. ABA suppresses expression of the auxin-responsive reporter gene *DR5:*GUS in lateral roots (De Smet *et al.*, 2003). Conversely, the *ABI3* promoter is induced by auxin (Brady *et al.*, 2003). Cross-talk was implicated by the reduced sensitivity to ABA during root elongation in the auxin signaling-defective mutants *axr1* and *axr2* (Wilson *et al.*, 1990; Tiryaki and Staswick, 2002). Mutations affecting RCN1 protein phosphatase 2A, which influences auxin transport and gravity responses (Garbers *et al.*, 1996; Rashotte *et al.*, 2001), also decrease ABA sensitivity (Kwak *et al.*, 2002). The concentrations of IAA and NPA (*N*-1-naphthylphthalamic acid) required to promote or suppress, respectively, lateral root formation in ABA-defective *abi3-6* mutant plants were higher than in wild-type plants (Brady *et al.*, 2003). In maize, overexpression of the *ABI3* ortholog, *VIVIPOROUS1* (*VP1*), confers insensitivity to auxin in lateral root formation assays (Suzuki *et al.*, 2001). Additionally, the carrot (*Daucus carota*) *Dc3*:GUS reporter gene, which is ABA-responsive, is affected by exogenous auxin (Rock and Sun, 2005).

Interaction between auxin and ABA during seed germination and post-germination stages is also supported by analysis of *ibr5* (*indole-3-butyric acid-response 5*) mutants. *IBR5* encodes a 257 amino acid protein with approximately 35% identity to known dual-specificity mitogen-activated protein kinase (MAPK) phosphatases. Mutations in this gene cause reduced sensitivity to auxin, faster seed germination in the presence of ABA, and decreased sensitivity of root growth to ABA (Monroe-Augustus *et al.*, 2003). Interestingly, *ibr5* mutants also exhibit serrated rosette leaves that resemble those in *mARF10* plants. IBR5 may modulate auxin–ABA cross-talk by dephosphorylation of key signaling components (Monroe-Augustus *et al.*, 2003).

Although cross-talk between auxin and ABA has been suggested from the observations above, the molecular mechanism of the hormonal cross-talk is unknown. A potential involvement of ARF transcription factors in auxin–ABA cross-talk was predicted by the study of *abi3* mutants (Brady *et al.*, 2003). Our study provides novel evidence in support of this hypothesis. Moreover, microarray data suggest that ARF10 and the repression of *ARF10* by miR160 may be involved in the regulation of ABA-responsive genes. The detailed molecular mechanism of ARF10 involvement in ABA signal transduction is not known at this time. It has been shown that the B3 domain of *PvAlf*, the bean (*Phaseolus vulgaris*) *ABI3* ortholog, can bind the auxin-responsive element of the *GH3* and *Em* genes, the latter of which also contains an ABA-responsive element (Nag *et al.*, 2005). Finally, other phenotypes of *mARF10* mutant plants, such as pale cauline leaves (Figure 2h) and white seedlings (Figure 5e), may also relate to auxin–ABA cross-talk, as *ABI3* is known to play an important role in chloroplast differentiation (Rohde *et al.*, 2000). Identification of downstream targets of *ARF10* and *ABI3* will provide a much clearer picture of cross-talk mechanisms during germination and post-germination.

Experimental procedures

Vector construction and plant transformation

The sequence for *ARF10* (At2 g28350) between the translation start and stop sites, but excluding the stop codon, was amplified from genomic DNA of *A. thaliana* ecotype Col-0 by PfuUltra polymerase (Stratagene; http://www.stratagene.com/) using ARF10 + 1 forward (5′-CACCATGGAGCAAGAGAAAAGCTT-3′)/ARF10 + 2333 reverse (5′-AGCGAAGATGCTGAGCGGAC-3′) primers. Mutations at the miR160 target site were generated by site overlap extension PCR mutagenesis (Ho *et al.*, 1989) using the primers ARF10 miRNA-target mut forward (5′-CTGCAGGAATACAAGGTGCAAGACAAGCTC -3′)/ ARF10 miRNA-target mut reverse (5′-GAGCTTGTCTTGCACCTTGT-ATTCCTGCAG -3′). PCR products were cloned into pENTR/D-TOPO (Invitrogen; http://www.invitrogen.com/), generating *pENTR-ARF10* and *pENTR-mARF10*. Additionally, the putative 5′ regulatory sequence (3 kb) was amplified using the primers attB1 ARF10-3000 forward (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGC-GGCCGCAATGCAAGACAACCCACCAA-3′)/attB2 ARF10-1 reverse (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTGGCGGCCGCCTA-GACGAAGTTGTGTAACC-3′), and cloned into pDONR (Invitrogen). The putative 5′ regulatory sequence was digested from the pDONR vector using *Not*I and ligated into *pENTR-ARF10* and *pENTR-mARF10* constructs generating *pENTR-ARF10:ARF10* and *pENTR-ARF10:mARF10*, which were subsequently recombined into the binary vector pMDC107 (GFP N-terminal fusion vector; Curtis and Grossniklaus, 2003). The putative 5′ regulatory sequence was also

recombined into pMDC162 (GUS N-terminal fusion vector). All constructs were sequenced for accuracy. *Arabidopsis thaliana* (Col-0 accession) plants were transformed by the vacuum-infiltration method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* GV3101 carrying *ARF10*, *mARF10* or empty vector constructs.

RT-PCR

Total RNA was extracted from siliques using standard phenol–SDS extraction (Sambrook *et al.*, 1989). DNase-treated total RNA (2 μg) was used for reverse transcription with a RETROscript kit (Ambion; http://www.ambion.com). The RT product was subjected to semi-quantitative PCR using *ARF10* forward (5′-CAACTCTTCGGATCACCATC-3′)/*ARF10* reverse (5′-CAGTCTTCGTAGAAGCGTCT-3′) primers, GFP forward (5′-CACTGGAGTTGTCCCAATTC-3′)/GFP reverse (5′-AAGGACCATGTGGTCTCTCT-3′) primers, or *ARF10* forward/GFP reverse primers. The conditions for PCR to amplify *ARF10*, GFP and *ARF10*-GFP were: one cycle at 94°C (4 min), one cycle at 80°C (2 min), touchdown cycles (94°C for 15 sec, 72°C → 66°C for 15 sec, and 72°C for 30 sec) (one cycle for each temperature) and 20 cycles at 94°C (15 sec), 65°C (15 sec) and 72°C (30 sec), followed by extension at 72°C (7 min). An actin gene *ACT2* (An *et al.*, 1996) was used as a control in the semi-quantitative PCR with specific primers (5′-GCCATCCAAGCTGTTCTCTC-3′ and 5′-GAACCACCGATCCAGACACT -3′). The conditions for PCR to amplify *ACT2* were essentially the same as above except for the temperatures for touchdown cycles (67°C → 61°C for 15 sec) and the 20-cycle amplification (94°C for 15 sec, 60°C for 15 sec and 72°C for 30 sec).

Genotyping

Genomic DNA was extracted from rosette leaves from three different types of seedlings (wild-type, serrated and narrow leaves) found in the *mARF10* mutant population (Figure 2a–c) using phenol extraction according to the QUICK-PREP method described at http://www.biotech.wisc.edu/NewServicesandResearch/Arabidopsis/, and used in PCR for genotyping. The primers were 5′-CAACCAAGCTCTGATAGAGT-3′ and 5′-CTGTCGAGAAGTTTCTGATC-3′. The following conditions were used for PCR: initial denaturation at 94°C (4 min), touchdown cycles (94°C for 15 sec, 69°C → 63°C for 15 sec, and 72°C for 30 sec) (one cycle for each temperature) and 25 cycles at 94°C (15 sec), 62°C (15 sec) and 72°C (30 sec), followed by extension at 72°C (7 min).

Venation staining

Although the *mARF10* rosette leaves were always smaller than the rosette leaves in wild-type seedlings, similar sizes of wild-type and *mARF10* rosette leaves were used to compare venation patterns. The petioles of rosette leaves were cut by a sharp razor blade, and 0.2% w/v Reactive Red solution (Sigma; http://www.sigmaaldrich.com/) was applied to the cut surface of the petioles. The staining pattern of the venation was examined and photographed after 1 h or later.

Scanning electron microscopy

Germinating Arabidopsis seeds or siliques were fixed in 10 mM potassium phosphate buffer, pH 7.0, containing 4% w/v paraformaldehyde overnight, and then dehydrated in an increasing ethanol series (30–100% v/v). Specimens in 100% ethanol were critical point-dried with carbon dioxide in a Balzer CPD-020 dryer (Balzers Union Ltd) according to the method described by Anderson (1951). The dried specimens were mounted on an aluminum planchette, and coated with approximately 10 nm of 60/40% Au/Pd using an Edwards S150B sputter coater (Edwards High Vacuum Ltd; http://www.chell.co.uk/edwards) operating at 1 x 10^{-2} Torr, 5 mbar argon pressure, 1.5 kV, 20 mA plasma current, for 60 sec. Examination was performed using the AmRAY 3300FE scanning electron microscope (AmRay) in the Electron Microscope Facility, Department of Botany and Plant Pathology, Oregon State University.

GUS staining

GUS staining of seeds and seedlings was performed as previously described (Weigel and Glazebrook, 2002) using 100 mM sodium phosphate buffer (pH 7.0) containing 0.1% v/v Triton X-100 and 2 mM X-Gluc (RPI Co.; http://www.rpicorp.com). Staining was examined with a dissection microscope after overnight incubation at room temperature.

Germination test

For germination, Arabidopsis seeds were placed in 9 cm plastic Petri dishes on two layers of filter paper (no. 2, Whatman Inc.; http://www.whatman.com) moistened with 3 ml water or test solutions and incubated at 4°C for 3 days in the dark and at 22°C for 5 days under the light. The seeds were examined for radicle protrusion through the endosperm (defined as *sensu stricto* germination) under a dissection microscope. For the post-germinative growth examination, seeds were plated on agar medium containing MS salts and 1% w/v sucrose, in the presence or absence of ABA.

Microarrary analysis

Three independent biological replicates of WT, *ARF10* and *mAR10* samples, containing three independent seed lots each, were analyzed using Arabidopsis ATH1 Genome GeneChips (Affymetrix, Inc.; http://www.affymetrix.com). Total RNA was extracted from germinating seeds (22 h imbibition) using standard phenol–SDS extraction as described previously (Sambrook *et al.*, 1989), and then checked for RNA integrity using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.; http://www.agilent.com). Total RNA (5 μg) from individual pools was used to produce double-strand cDNAs that were transcribed to aRNAs (amplified RNA). aRNAs were labeled with biotin-11-CTP and biotin-16-UTP *in vitro* according to the method described in the MessageAmp™ aRNA Amplification Kit instruction manual (Ambion). aRNAs (25 μg) were fragmented prior to hybridization in the Affymetrix GeneChip® Hybridization Oven 640, and washed in the Affymetrix GeneChip® Fluidics Station 450, and then the arrays were stained with biotinylated anti-streptavidin (Vector Laboratories; http://www.vectorlabs.com) at the Center for Genome Research and Biocomputing Core Laboratories at Oregon State University. The arrays were scanned with an Affymetrix GeneChip® Scanner 3000, and the hybridization signals were quantified using the Affymetrix MAS 5.0 method. Raw intensity values were normalized by using RMAEXPRESS (Bolstad *et al.*, 2003) and imported into GENESPRING GX 7.2 (Agilent Technologies, Inc.). Significantly up- or downregulated genes were identified by ANOVA using a false-discovery rate of 0.05 and a minimum fold change of two. Over-represented motifs relative to the entire collection of promoter motifs were determined by the *Athena* program (O'Connor *et al.*, 2005; http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/

cgi/home.pl.). The microarray data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; GSE7227).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Quantification of the expression of (a) *ARF10*, (b) *GFP* or (c) *ARF10-GFP* in wild-type, *ARF10* and *mARF10* siliques.

Figure S2. The effect of exogenous IAA on the ABA sensitivity of *35S:MIR160a* seeds.

Figure S3. Response of the ABRE-containing genes to exogenous auxin.

This material is available as part of the online article from http://www.blackwell-synergy.com

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