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ABI4 Mediates Abscisic Acid and Cytokinin Inhibition of Lateral Root Formation by Reducing Polar Auxin Transport in *Arabidopsis* [C][W]

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Key steps in a plant's development and adaptation to the environment are the initiation and development of lateral roots (LRs). LR development is regulated by auxin, the major plant hormone promoting LR formation, its counteracting hormones cytokinin, and abscisic acid (ABA). Here, we show that mutating *ABSCISIC ACID INSENSITIVE4* (*ABI4*), which encodes an ABA-regulated AP2 domain transcription factor, results in an increased number of LRs. We show that *ABI4* is expressed in roots and that its overexpression impairs LR development. Root expression of *ABI4* is enhanced by ABA, and cytokinin and is repressed by auxin. Using hormone response promoters, we show that *ABI4* also affects auxin and cytokinin profiles in the root. Furthermore, LR development in *abi4* mutants is not altered or inhibited by cytokinin or ABA. Expression of the auxin-efflux carrier protein PIN1 is reduced in *ABI4* overexpressors, enhanced in *abi4* mutants, and is less sensitive to inhibition by cytokinin and ABA in *abi4* mutants than in wild-type plants. Transport levels of exogenously applied auxin were elevated in *abi4* mutants and reduced in *ABI4* overexpressors. We therefore suggest that *ABI4* mediates ABA and cytokinin inhibition of LR formation via reduction of polar auxin transport and that the resulting decrease in root auxin leads to a reduction in LR development.

INTRODUCTION

Plant development is unique in its ability to form new organs throughout the life of the plant. These newly developed organs include lateral organs, such as roots and shoots, as well as reproductive organs. The root system of many dicot plants has a primary root (PR) that branches to yield lateral roots (LRs), which can then undergo further orders of branching (Fukaki et al., 2007; Osmont et al., 2007; Nibau et al., 2008 and references therein). In *Arabidopsis thaliana*, LRs are developed in a sequence of events initiating from pericycle founder cells located opposite the xylem poles. Although pericycle cells form continuous lines adjacent to the stele, only a limited number of them become founders of LRs.

Auxin has been shown to be the key hormone in LR development (Aloni et al., 2006; Fukaki et al., 2007; Osmont et al., 2007; Nibau et al., 2008). In roots, polar auxin transport is achieved by polar localization of PIN proteins, which transport auxin. Auxin is transported toward the root tip via the stele, laterally at the base of the root tip, and backward into the cortex and epidermis. Spatial oscillation in auxin levels leads to regular spacing of LRs (De Smet et al., 2007). The polarity of PIN auxin-efflux regulatory proteins changes from anticlinal to periclinal cells during the establishment of new LR primordia (LRPs) (Benková et al., 2003). LR spacing is also influenced by root bending (Lucas et al., 2008).

The synthetic auxin-responsive promoter *DR5* (Ulmasov et al., 1997) is widely used for monitoring auxin levels in planta: the auxin-responsive reporter *DR5:β-glucuronidase* (*GUS*) was used to confirm the central role of auxin during LRP development (Benková et al., 2003; Dubrovsky et al., 2008). Moreover, a large number of LR mutants have been studied (summarized in Péret et al., 2009), most of which are impaired in auxin homeostasis, signaling, and transport. In addition, most are affected at the initiation stage, whereas only a few show altered emergence or patterning.

Although auxin is the main hormone in LR development, other hormones can also influence this process (reviewed in Fukaki and Tasaka, 2009). Cytokinin is considered to act antagonistically to auxin in the development of LRs (Aloni et al., 2006; Fukaki and Tasaka, 2009). Exogenous cytokinin treatment inhibits the initiation of LRPs in pericycle founder cells (Li et al., 2006; Laplaze et al., 2007). Accordingly, mutants with reduced cytokinin levels (Werner et al., 2003), or mutated in cytokinin perception or signaling pathways (Mason et al., 2005; Riefler et al., 2006), have increased numbers of LRs.

LR formation can also be affected by the availability of macronutrients. Thus, LR density and elongation are affected by nitrogen availability: limiting nitrogen conditions enhance LR elongation, whereas high external nitrogen reduces elongation of PRs and LRs (Linkohr et al., 2002). High levels of phosphorus decrease both LR density and elongation, whereas the length of the PR is enhanced (Linkohr et al., 2002); conversely, the number of LRs is dramatically increased under severe phosphorus deficiency, and growth of the PR is inhibited (López-Bucio et al.,

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2002). Limiting amounts of iron and sulfate also affect LR development (Moog et al., 1995; López-Bucio et al., 2003).

Although abscisic acid (ABA) is considered the universal plant stress hormone (Verslues and Zhu, 2005; Wasilewska et al., 2008), it can also take part in non-stress-related regulatory processes (for a recent review, see Wasilewska et al., 2008). The role of ABA in root branching has recently been recognized (reviewed in De Smet et al., 2006; Wasilewska et al., 2008), and there is some evidence for ABA involvement in the repression of LR initiation and emergence. Upon prolonged water stress, *Arabidopsis* LRs develop as short roots, which are characterized by stubby tuberized structures (Vartanian et al., 1994). These specialized LRs enter a dormant mode and resume growth upon rehydration. This process is severely affected in ABA-insensitive mutants such as *abi1-1*, suggesting that ABA is involved in the signaling of LR development.

Arabidopsis ABI3 (Brady et al., 2003) and its maize (*Zea mays*) ortholog *VP1* encode transcription factors that have been shown to affect root architecture and to mediate ABA/auxin interactions. Mutations in *ENHANCED RESPONSE TO ABA1* (*ERA1*) resulted in increased numbers of LRs. Similar to ABA, high nitrate in the growth medium also inhibited root growth in *Arabidopsis* (Signora et al., 2001). The inhibitory effect of nitrate was significantly reduced in *abi4* and *abi5* mutants, further supporting the involvement of ABA in LR signaling. Further development of LRPs is arrested in osmotically stressed *Arabidopsis* plants (Deak and Malamy, 2005), a regulatory mechanism that is dependent upon ABA and *LATERAL ROOT DEVELOPMENT2*. These two agents have been shown to modulate root architecture, even in the absence of osmotic stress, suggesting that ABA may be a regulator of intrinsic root system development (Deak and Malamy, 2005).

In *Arabidopsis*, ABA can reversibly arrest LR growth by inhibiting the expression of cell cycle genes necessary for meristem activity (De Smet et al., 2003). ABI8, a plant-specific protein, is involved in signaling that also affects LR formation. In *abi8* mutants, LRP formation is inhibited and the LR meristem loses division competence (Brocard-Gifford et al., 2004). The checkpoint between meristem activation and emergence of LRs can be used to modulate root architecture via ABA signaling (Signora et al., 2001; De Smet et al., 2006).

In this study, we demonstrate that *abi4* mutants develop increased numbers of LRs. In addition, *ABI4*-overexpressing plants have a reduced number of LRs. We also show that *ABI4* plays a role in mediating ABA and cytokinin repression of LR development. It also appears to affect the distributions and levels of auxin and cytokinin in the roots. *ABI4* is expressed in phloem companion cells, and its expression reduces the level of the auxin-efflux carrier PIN1. *ABI4* is therefore a key player in coordinating the effects on LR development determined by the balance between counteracting plant hormones.

RESULTS

abi4 Mutants Exhibit Enhanced LR Density

ABI4 is highly studied in germinating seedlings, and *abi4* mutants have been shown to have a wild-type-like phenotype under normal growth conditions, with no glucose-induced arrest of cotyledon expansion and greening (Söderman et al., 2000; Arroyo et al., 2003; Penfield et al., 2006). Looking at *Arabidopsis* plants at later developmental stages, we noted that the root architecture of *abi4* mutant seedlings differs from that of wild-type plants (Figure 1A). Whereas the length of the PR in seedlings mutated in three *abi4* alleles (*abi4-1*, a deletion mutant in codon 157 [Finkelstein et al., 1998], and *abi4-102* and *abi4-103*, nonsense point mutations at codons 80 and 39, respectively [Laby et al., 2000]) was similar to that in the wild-type plants, all three mutant lines had 125 to 135% more LRs than wild-type plants grown under the same conditions (Figure 1D). In the *abi4* mutants, the first LR emerges as early as 4 d after germination, whereas in the wild type, it emerges on day 5 or 6 (Figures 1B and 1C). Moreover, LRs of *abi4* mutants were longer than those of the wild-type plants (Figure 1A). This increased LR phenotype suggested a role for *ABI4* in LR development at later developmental stages as well.

ABI4 Is Expressed in Roots

To determine whether *ABI4* is indeed expressed at later developmental stages, we cloned a 2-kb promoter DNA fragment upstream of the translation start codon. This promoter sequence was subcloned into pCAMBIA 1391Z, the resulting *ABI4:GUS* construct was introduced into wild-type *Arabidopsis* plants, and the activity of the *GUS* reporter gene was detected histologically. Staining of germinating seedlings in the presence and absence of 7% (w/v) glucose confirmed the previously shown patterns in which *ABI4* is highly expressed in cotelydons and hypocotyls of germinating seedlings (see Supplemental Figure 1 online) (Arroyo et al., 2003). As shown previously, *ABI4* is also expressed in flowers and fruits (see Supplemental Figure 1 online). GUS staining was also detected in the vascular system of rosette leaves and in the petiole base and roots of *Arabidopsis* seedlings that were several weeks old (Figure 2; see Supplemental Figure 1 online).

Relative quantification by quantitative RT-PCR (qRT-PCR) analysis showed that expression in the roots was almost 7 times higher than in the leaves (see Supplemental Figure 2 online). High expression levels were observed in mature regions of the PRs and LRs (Figures 2B and 2C) but not in primordia (LRPs) or young LRs (Figures 2D and 2E). Expression decreased toward the younger regions of the root. An expression gradient was also observed in the root hair zone, with low promoter activity in the upper part of the zone and no expression in the lower (younger) part (Figure 2F). No GUS staining was detected in the elongation or meristematic zones, including the root tips (Figure 2G), thereby correlating *ABI4* expression levels with the root zones in which LRs develop. Root cross sections showed that *ABI4* is expressed in companion cells and to a lesser extent in the metaphloem and stele parenchyma (Figures 2H to 2J). Furthermore, the extent of expression in the stele parenchyma increased with root development toward the upper parts of the root (Figures 2H and 2I).

ABI4 Expression Is Modulated by Auxin, Cytokinin, and ABA

To determine whether *ABI4* expression in the root system is affected by auxin and its antagonistic plant hormones, cytokinin and ABA, we used plants expressing the *ABI4:GUS* construct

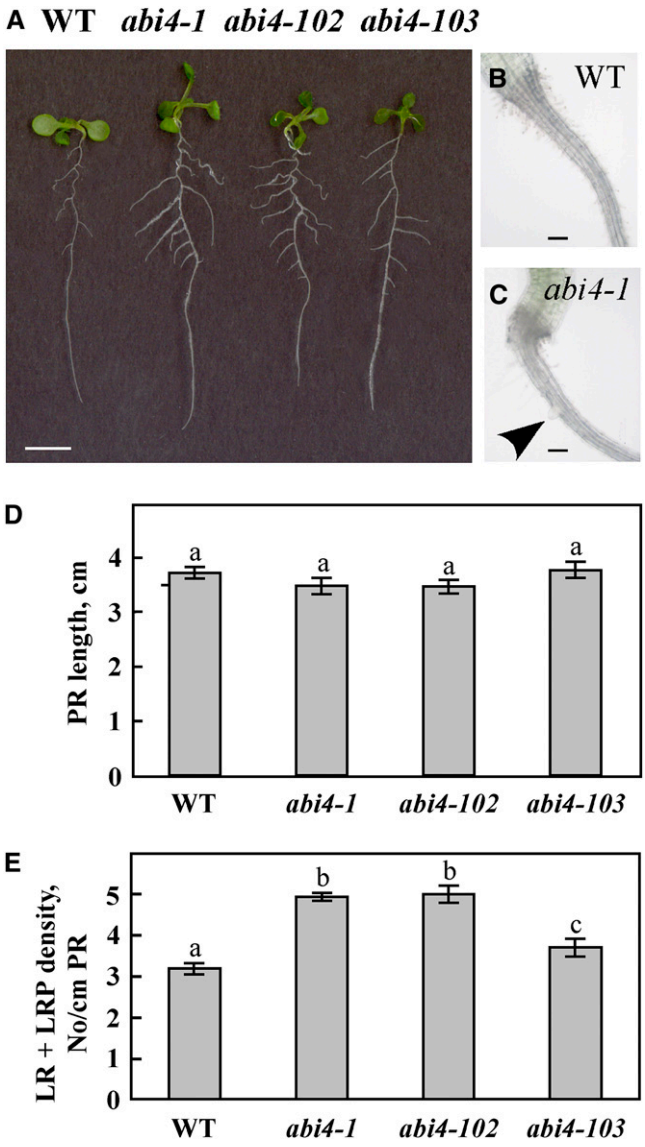

Figure 1. LR Development Is Affected by Expression of *ABI4*: *abi4* Mutants Develop More LRs.

(A) Twelve-day-old seedlings of wild-type (WT) and the specified *abi4* mutants grown on solid 0.5× MS medium. Bar = 5 mm.
(B) and **(C)** Roots of 4-d-old wild-type and *abi4-1* mutant seedlings, respectively. Arrowhead indicates emerging LR. Bars = 100 μm.
(D) Average length of PRs in 12-d-old seedlings. The data represent the mean ± SE of $n = 3$ independent experiments each containing 50 plants per treatment. Bars with different letters represent statistically different values by Tukey's HSD post-hoc test ($P < 0.05$).
(E) Density of LRs at all developmental stages (including LRP) in 12-d-old seedlings. Statistical analysis performed as in **(D)**.
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and explored the effects of these three hormones on steady state levels of *ABI4* in wild-type roots using qRT-PCR analyses (Figure 3). Auxin plays a major role in promoting LR formation (Aloni et al., 2006; Fukaki et al., 2007; Osmont et al., 2007; Nibau et al., 2008). Treatment with the auxin indole-3-acetic acid (IAA) resulted in a 75% decrease in the level of *ABI4* transcript in the roots. On the other hand, application of the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) resulted in increased levels of *ABI4* transcript. LR development is known to be adversely affected by the plant hormones cytokinin and ABA (Bottger, 1974; Wightman et al., 1980), and transcript levels of *ABI4* were elevated 1.8-fold by cytokinin (zeatin) treatment and 2.3-fold by ABA (Figure 3A).

Expression data from qRT-PCR analyses were further supported by histochemical GUS staining of *ABI4:GUS Arabidopsis* plants, treated with the same effectors (Figures 3B to 3K). Zeatin and ABA increased GUS staining in the upper root (Figures 3E and 3F). Furthermore, treatment with the plant hormones ABA and zeatin or the inhibitor TIBA also resulted in expansion of the *ABI4* expression zone into the elongation zone of the root (Figures 3I to 3K), a region where no staining is detected in nontreated plants (Figures 2 and 3G). Thus, *ABI4* expression was induced by ABA and cytokinin, plant hormones that inhibit LR formation, and repressed by auxin, the LR-promoting plant hormone. These results are in accordance with the increased number of LRs in the *abi4* mutants (Figure 1).

Overexpression of *ABI4* Decreases LR Development

We were not able to obtain stable lines of transgenic plants constitutively expressing the *ABI4* gene using *35S:ABI4* constructs. Although seeds from these transgenic lines germinated, the cotyledons senesced and died as soon as they emerged. We therefore used the inducible *pOp6/LhGR* expression system, which can be activated by the addition of dexamethasone (Dex) (Craft et al., 2005). When germinated on Dex-containing medium, the development of plants transformed with the *pOp6:ABI4* construct in the pV-TOP vector was arrested, and the seedlings usually died at ~3 weeks of age. In the absence of Dex, the root architecture of *ABI4*-overexpressing plants was indistinguishable from that of wild-type plants (Figures 4A and 4B). *ABI4* mRNA levels in the roots of Dex-treated *ABI4*-overexpressing plants were ~20,000 times the endogenous *ABI4* levels expressed in the wild type and in uninduced *ABI4*-overexpressing plants (see Supplemental Figure 3 online). When germinated in the presence of 2 μM Dex, *ABI4*-overexpressing plants developed <50% of the LRPs and LRs developed in wild-type plants or plants transformed with empty vector (Figures 4A and 4B). The decrease in LRPs and LRs was similar, indicating that *ABI4* inhibited LR initiation rather than subsequent LR development. The inhibitory effect of *ABI4* overexpression on LR formation (Figures 4A and 4B) was in agreement with the increased number of LRs observed in the *abi4* mutants (Figures 1A and 1D).

ABI4 Affects Formation of LRP and Elongation of Emerged LRs

To determine the developmental checkpoints affected by *ABI4*, we determined the four developmental stages previously

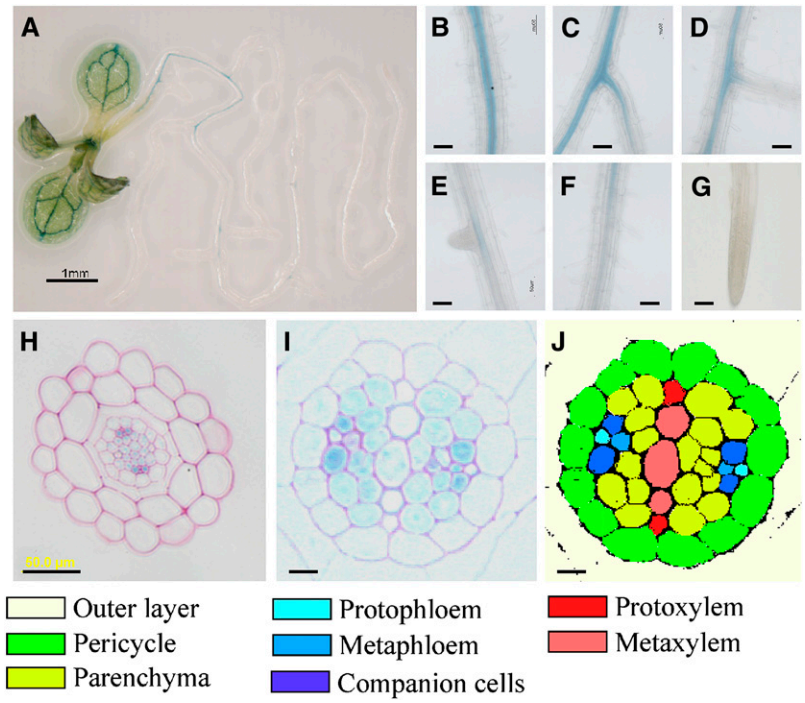

Figure 2. *ABI4* Expression in Roots.

(A) to **(J)** Expression patterns in roots.
(A) Two-week-old *Arabidopsis* seedlings expressing *ABI4:GUS* stained histologically for GUS activity.
(B) to **(G)** Enlarged views of different developmental stages of the PRs and LRs (from top to bottom regions).
(B) Mature PR near the shoot-root junction.
(C) Mature LR.
(D) Newly developed LR.
(E) Emerging LR.
(F) Root hair zone. Note the expression gradient within this zone.
(G) Root tip and meristematic zone.
(H) Section of GUS-stained mid-upper zone (8 mm from the shoot-root junction) of PR counterstained with ruthenium red.
(I) Cross section of PR in the upper zone (3 mm from the shoot-root junction), treated as in **(H)**.
(J) Colored scheme indicating the different cell types in the section depicted in **(I)**.
Bars = 1 mm in **(A)**, 50 μm in **(B)** to **(H)**, and 10 μm in **(I)** and **(J)**.

delineated by De Smet et al. (2003) and described in Methods. Overexpression of *ABI4* resulted in a decrease in the number of stage A LRPs (i.e., the initial LRP developmental stage containing up to two cell layers) and in the number of LRs longer than 0.5 mm (stage D) (Figure 5A). On the other hand, the intermediate stages B and C, representing more developed LRP and short (<0.5 mm) LRs, respectively, were less affected in *ABI4*-overexpressing plants. The results obtained using *ABI4*-overexpressing plants were supported by analysis of the four developmental stages in *abi4* mutants (Figure 5B). Stages A and D were enhanced with all three mutated alleles of *ABI4*, whereas stages B and C were not affected. The results presented in Figures 5A and 5B thus suggest that *ABI4* inhibits both LR initiation and the elongation of emerged LRs.

To further confirm the effect of *ABI4* on the elongation of emerged LRs, we measured the initial growth rates of the first two LRs in the *abi4* mutant and wild-type plants (Figure 5C). The initial growth rates measured in the *abi4* mutants 24 and 48 h postemergence were 41 to 60% and 24 to 41% higher than those determined for the wild-type seedlings at the respective time points. The growth rates, as well as the differences between the wild-type and the *abi4* mutant plants, were gradually decreased with time.

Auxin's Effect on LR Formation Is Not Altered in *abi4* Mutants and Is Reduced in *ABI4* Overexpressors

We used the *abi4* mutant and *ABI4*-overexpressing plants to study the interaction of auxin and cytokinin with *ABI4* gene activity. *abi4* mutant plants were highly affected by exogenous IAA, and their root system was more branched and elongated than that of the wild-type roots when germinated and grown on auxin-containing media (see Supplemental Figure 4 online). The effect of auxin was more pronounced when 1-week-old seedlings lacking developed LRs were treated with IAA (Figures 6E to 6I; see Supplemental Figure 5 online). There was a 40 to 90%

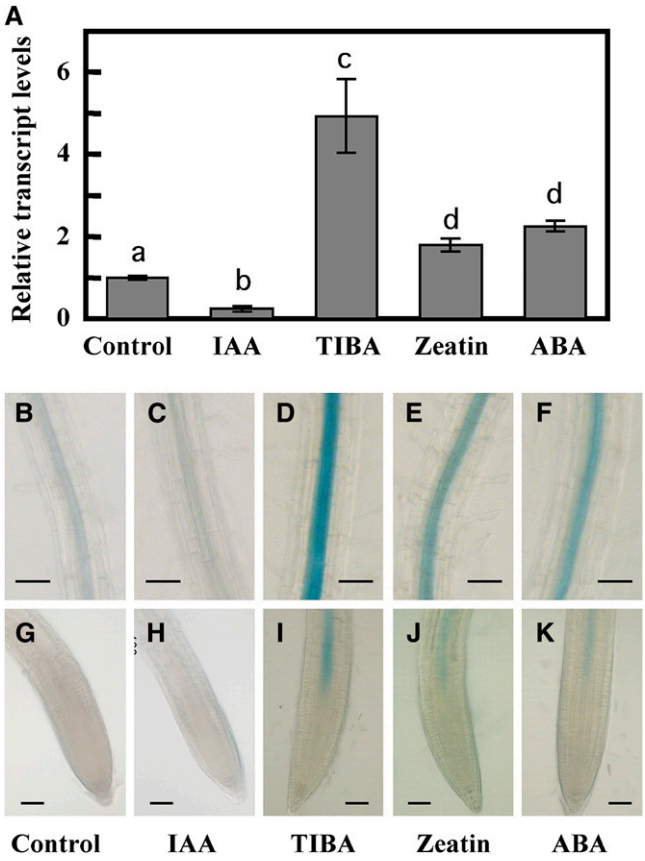

Figure 3. *ABI4* Expression Is Hormone Regulated.

(A) qRT-PCR analysis of *ABI4* transcripts in 2-week-old wild-type plants treated for 4 h with 20 μM of the specified agents. The data represent the mean ± SE of $n = 3$ independent experiments. Bars with different letters represent statistically different values using Tukey's HSD post-hoc test ($P \leq 0.05$).
(B) to **(K)** GUS staining of roots of 2-week-old *ABI4:GUS*/wild-type plants treated as in **(A).**
(B) to **(F)** Upper root zone.
(G) to **(K)** Root tips. Bars = 50 μm.

increase in LR formation in the three IAA-treated *abi4* mutant lines relative to wild-type plants after the same treatment. The extent of the increase in LR number in auxin-treated *abi4* mutants (Figure 6) was similar to that observed without the addition of exogenous hormone (Figure 1), suggesting that the auxin response is not altered in *abi4* mutants.

Two-week-old *pOp6:ABI4*-transformed seedlings were transferred to medium containing Dex alone or Dex + IAA. To avoid analyzing LRs that developed prior to *ABI4* induction, we counted LRs in the distal 5 mm corresponding to the root division and elongation zones. None of the tested lines developed any LRs in this region in the absence of treatment or following Dex treatment (Figures 6J, 6K, 6N, 6O, and 6R). Although IAA treatment resulted in the emergence of a large number of LRs in the distal roots of all lines (Figures 6L, 6M, and 6P to 6R), the number of LRs in Dex-induced *ABI4*-overexpressing plants was 30% of that in the auxin-treated wild-type and empty vector plants (both with and without added Dex) (Figures 6Q and 6R). These results suggest that the number of LR developed is inversely related to the expression levels of *ABI4*.

ABI4 Affects Root Auxin and Cytokinin Localization and Level

The expression activity of the synthetic *DR5* promoter is widely used in studies of endogenous auxin (Ulmasov et al., 1997). This promoter contains multiple binding sites for AUXIN

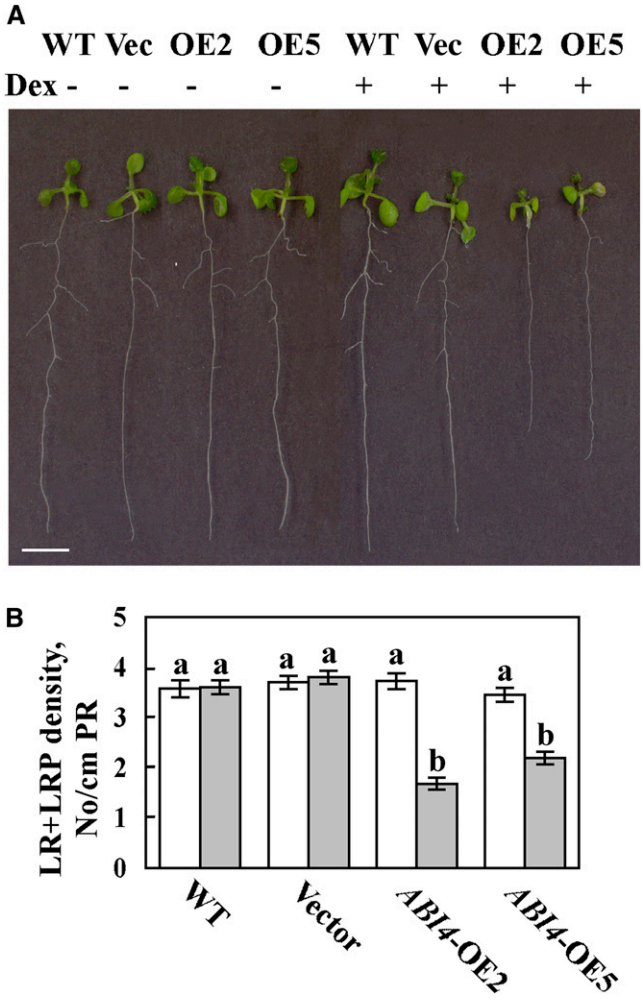

Figure 4. Root Structure of *ABI4*-Overexpressing *Arabidopsis*.

(A) Twelve-day-old seedlings of wild-type plants (WT) , 4C-S5 plants transformed with empty vector (Vec), or two lines of *ABI4* overexpressors (OE2 and OE5) germinated and grown on 0.5× MS plates with (+) or without (−) 2 μM Dex. Bar = 5 mm.
(B) Density of PRs and LRs from the LRP stage of 12-d-old seedlings. The data represent the mean ± SE of $n = 3$ independent experiments each containing 50 plants per treatment. White bars, −Dex; gray bars, +Dex. Bars with different letters represent statistically different values by Tukey's HSD post-hoc test ($P < 0.05$).
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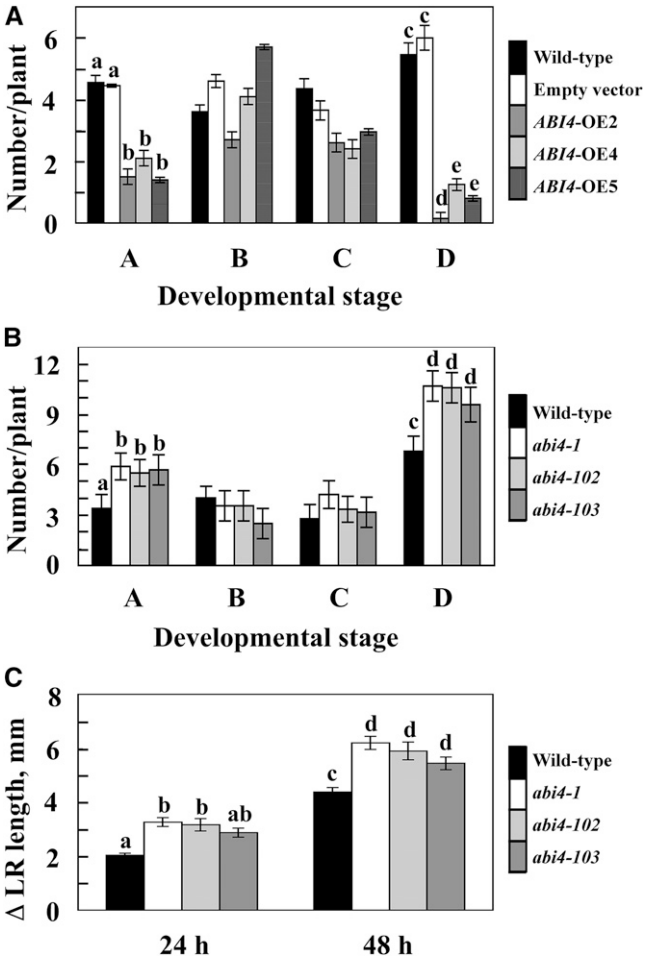

Figure 5. Stage-Specific Inhibitory Effects of *ABI4* on LR Development.

(A) and **(B)** LRs were counted in roots of 12-d-old seedlings in each of four developmental stages (De Smet et al., 2003): Stage A, initials with up to two layers of cells; stage B, three or more layers of cells just prior to emergence; stage C, immediately after emergence but <0.5 mm long; stage D, LRs longer than 0.5 mm.

(A) Effects of *ABI4* overexpression on LR number: wild-type plants (black bars), 4C-S5 plants transformed with empty vector (white bars), or three lines of *ABI4* overexpressors (gray bars) were germinated on 0.5× MS plates with (transgenic plants) or without (wild type) 2 μM Dex.

(B) Effects on LR number in *abi4* mutants: the wild type, *abi4-1*, *abi4-102*, and *abi4-103* mutants were germinated on 0.5× MS plates.

(C) Initial growth rates of LRs of *abi4* mutants. The lengths of the first two visible LRs of 1-week-old vertically grown seedlings were measured. The added lengths at 24 and 48 h are presented. The data represent the mean ± SE of $n = 3$ independent experiments each containing 20 plants per treatment. Bars with different letters represent statistically different values by Tukey's HSD post-hoc test ($P \leq 0.05$).

RESPONSIVE FACTOR transcriptional activators (Ulmasov et al., 1997). *DR5:GUS*-expressing transgenic plants were crossed with *abi4-1*-mutant plants, and the offspring were selfed to yield homozygous plants. Although *DR5:GUS* was expressed in LRPs and in root tips on both wild-type and *abi4-1* mutant genetic backgrounds (Figure 7), the two backgrounds displayed both quantitative and qualitative differences: the number of *GUS*-stained loci on the *abi4-1* mutant background was 350% of that observed on the wild-type background (Figure 7D; see Supplemental Figure 6 online).

In the wild-type background, *DR5:GUS* was expressed in foci in LRPs and in root tips (Figures 7A to 7C). In preemerging wild-type LRs, *DR5:GUS* stained exclusively in the tip (Figure 7B). More intense GUS staining was observed for *DR5:GUS* in the background of the *abi4-1* mutant. Staining is more dispersed in the pericycle layer along the PR (Figure 7E) and in the entire preemerging LRs (Figures 7F and 7G). This pattern might result from the increased level of GUS staining in the *abi4* background. Closely spaced twin foci could be seen in the *abi4-1* mutant background (Figure 7G) but seldom in wild-type plants at this developmental stage. On the other hand, *DR5:GUS* was markedly repressed along the PR in the *ABI4*-overexpressing background (Figures 7I to 7K). In the tips of emerging *ABI4*-overexpressing LRs, *DR5:GUS* expression was either undetectable (Figure 7J) or very low (Figure 7K). *ABI4* overexpression did not alter *DR5:GUS* expression in the PR tips (Figure 7L). These results suggested that *ABI4* results in inhibition of auxin localization in the root by decreasing the number of foci and the apparent concentration of the hormone within these foci.

ARR5:GUS is used as a reporter for cytokinin levels (To et al., 2004). High levels of GUS activity were observed in the stele of the PR of *ARR5:GUS*-expressing plants in a wild-type background (Figure 7M) all the way to the root tip, including the root cap (Figure 7N), with decreasing staining levels toward the younger regions of the root. On the other hand, expression was markedly reduced in the genetic background of the *abi4-1* mutant (Figures 7O and 7P), suggesting that *ABI4* might affect cytokinin levels.

LR Development in *abi4* Mutants Is Insensitive to ABA and Cytokinin

Since *abi4* mutants developed an increased number of LRs (Figure 1) and *ABI4* expression was enhanced by ABA and cytokinin, which inhibited LR development, we tested the effects of these inhibitory hormones on LR formation in *abi4* mutants. In accordance with previous studies (Bottger, 1974; Wightman et al., 1980; De Smet et al., 2003; Benková and Hejátko, 2009), treatment with ABA and zeatin reduced LR number in wild-type seedlings (Figure 8; see Supplemental Figure 7 online). By contrast, LR formation was less affected by these hormones in the three *abi4* mutants (Figure 8; see Supplemental Figure 7 online), suggesting that these hormones act upstream of *ABI4*. Interestingly, zeatin had no effect on the frequency of LRP in *abi4* mutant plants.

Distribution of Auxin-Efflux Transporter PIN1 Is Affected by *ABI4*

PIN1 has been shown to be very important in the polar transport of auxin from the shoot to root apices (Okada et al., 1991; Friml and Palme, 2002). PIN1 is an auxin-efflux carrier localized on the basal side of stele and endodermis cells with occasional weak expression in the quiescent center and up to the four youngest

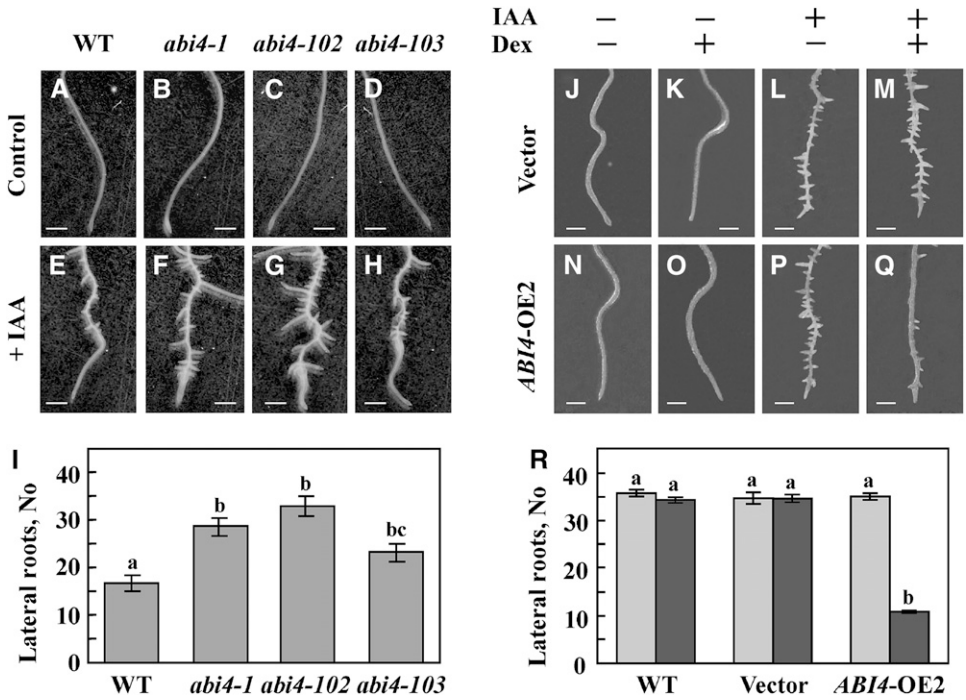

Figure 6. Interaction of *ABI4* with Auxin.

(A) to **(I)** LR formation in control and IAA-treated wild-type (WT) and *abi4* mutants. One-week-old seedlings were transferred to 0.5× MS medium (**[A]** to **[D]**) or to 0.5× MS supplemented with 20 μM IAA (**[E]** to **[H]**). Photographs were taken 1 week later. Bars = 0.5 mm.
(A) and **(E)** The wild type.
(B) and **(F)** *abi4-1* mutant.
(C) and **(G)** *abi4-102* mutant.
(D) and **(H)** *abi4-103* mutant.
(I) Number of LRs was determined in the lower 5-mm section of the roots shown in **(E)** to **(H)**.
(J) to **(Q)** One-week-old seedlings transformed with vector only (**[J]** to **[M]**) or *pOp6:ABI4* (**[N]** to **[Q]**) were transferred to 0.5× MS containing, where indicated, 10 μM Dex and/or 20 μM IAA. Bottom sections of the roots were photographed 1 week later. Bars = 0.5 mm.
(J) and **(N)** Untreated.
(K) and **(O)** +Dex.
(L) and **(P)** +IAA.
(M) and **(Q)** +Dex and IAA.
(R) Number of LRs was determined in the lower 5-mm section of IAA treated roots. Gray bars, +IAA no Dex; black bars, +IAA +Dex. The data represent the mean ± SE of $n = 3$ independent experiments each containing 50 plants per treatment.

epidermis and cortex daughter cells (Gälweiler et al., 1998; Friml et al., 2002). To determine whether ABI4-mediated inhibition of LR formation involves changes in PIN1-mediated auxin transport, we crossed *PIN1:PIN1-GFP*–expressing (for green fluorescent protein) plants with both the *abi4-1* mutants and *ABI4*-overexpressing *Arabidopsis*. In the *abi4-1*-mutant plants, PIN1 protein was expressed at higher levels than in the wild-type background (Figures 9A, 9E, and 9K). Moreover, in the genetic background of the *abi4* mutation, PIN1 expression extended to more distal regions of the root elongation zone. In agreement with this, in the *ABI4* overexpression background, PIN1 levels were reduced and confined to the stele (Figures 9D and 9K). Similarly, higher PIN1 protein levels were observed in emerging LRs of *abi4* mutants, whereas low levels were seen in *ABI4*-overexpressing plants (Figures 9H to 9J). Application of exogenous ABA and the cytokinin zeatin markedly reduced PIN1 protein levels in the roots of wild-type plants and to a lesser extent in *abi4-1* mutant plants (Figures 9B, 9C, 9F, 9G, and 9L), suggesting that these hormones affect PIN1 expression in both *ABI4*-dependent and *ABI4*-independent pathways.

ABI4 Affects Auxin-Polar Transport

To determine if the observed effects of *ABI4* on PIN1 protein expression levels alter polar auxin transport, we treated the hypocotyl-root junction with radiolabeled naphthalene acetic acid (NAA), a synthetic auxin. NAA has been shown to be an excellent tool for the study of active efflux of auxin (Delbarre et al., 1996; Petrásek et al., 2006). [^{3}H]NAA transport was ~2-fold higher in *abi4* mutants compared with wild-type plants (Figure 10). In addition, very low auxin transport was observed in roots of *ABI4*-overexpressing plants. These results correlate with the

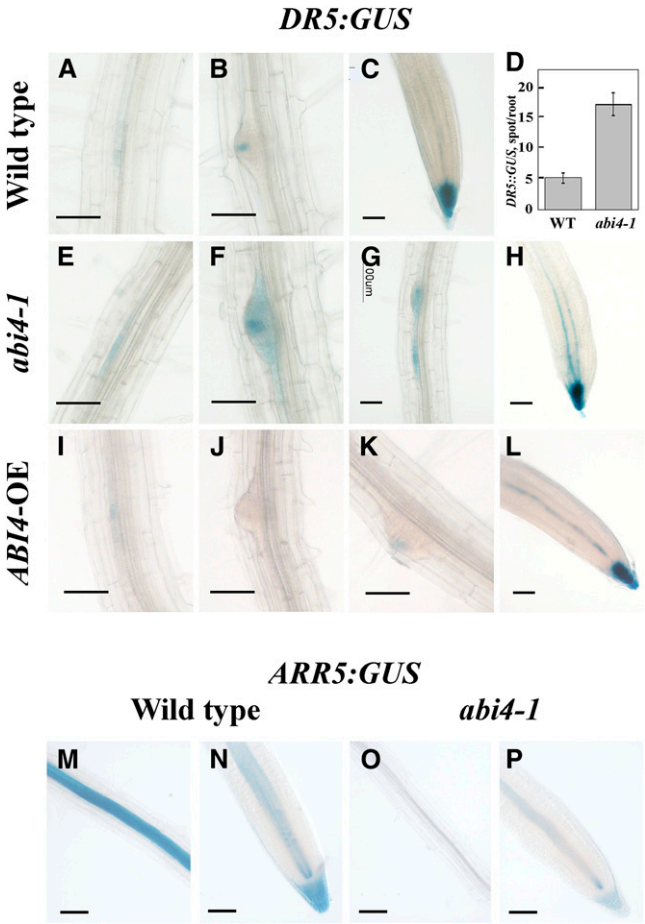

Figure 7. *ABI4* Affects Auxin and Cytokinin Distribution.

(A) to **(C)** and **(E)** to **(P)** GUS staining.
(A) to **(C)** Roots of seedlings of *DR5:GUS*/wild type.
(E) to **(H)** *DR5:GUS/abi4-1* mutant.
(I) to **(L)** *DR5:GUS/ABI4* overexpressor.
(M) and **(N)** *ARR5:GUS*/wild type.
(O) and **(P)** *ARR5:GUS/abi4-1* mutant. Bars = 50 μm.
(D) Number of stained foci in *DR5:GUS*/wild-type and *DR5:GUS/abi4-1* mutant seedlings. The data represent the mean ± SE of *n* = 3 independent experiments each containing 50 plants per treatment.

changes in PIN1 observed in the same genetic backgrounds. [^{3}H] NAA appears to have been transported via the polar auxin pathway because the levels observed in *abi4* and wild-type plants were almost fully diminished in the presence of the polar auxin transport inhibitor TIBA (Figure 10).

DISCUSSION

ABI4 Is Expressed in Roots and Affects LR Formation

We show here directly, both by qRT-PCR and histochemically using *Arabidopsis* plants expressing an *ABI4:GUS* reporter construct, that *ABI4* is expressed in mature regions of the root and that its pattern of expression correlates with root zones in which LRs develop (Figures 2 and 3). Expression decreased toward the younger regions of the root. An expression gradient was also observed in the root hair zone, with low promoter activity in the upper part of the zone and no detectable expression in the lower (younger) part or meristematic zones, including the root tips and LRP (Figures 2E and 2G). In mature root zones, *ABI4* is expressed in companion cells and to a lesser extent in the metaphloem and stele parenchyma (Figures 2H to 2J), with the extent of expression in the latter increasing with further development of the differentiated root (Figures 2H and 2I).

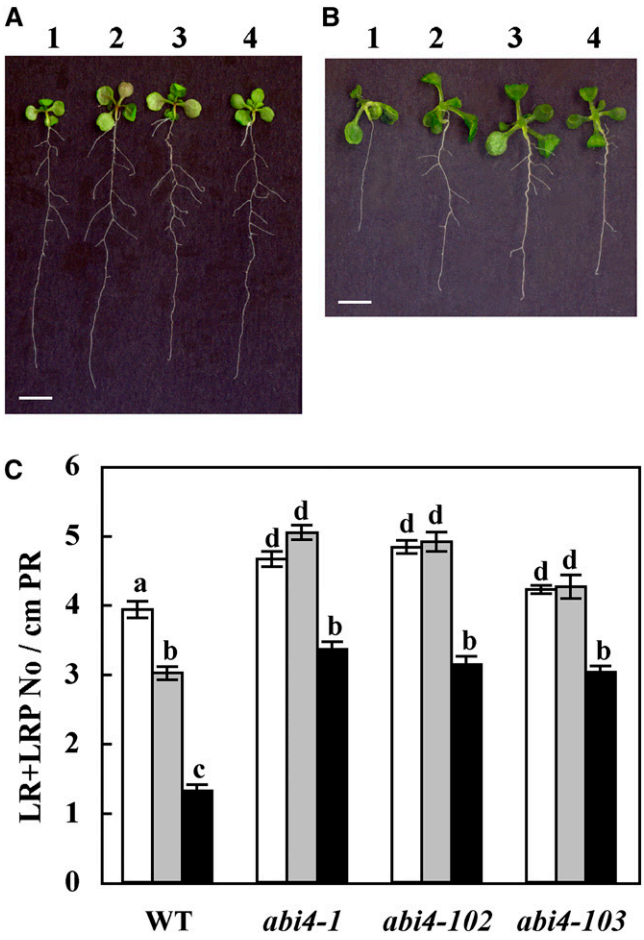

Figure 8. *abi4* Plants Are Insensitive to ABA and Cytokinin.

(A) and **(B)** One-week-old seedlings of the indicated lines were transferred to fresh 0.5× MS medium containing 10 μM ABA **(A)** or 10 μM zeatin **(B)**. Plants were photographed 1 week later. 1, wild type; 2, *abi4-1* mutant; 3, *abi4-102* mutant; 4, *abi4-103* mutant. Bars = 5 mm.
(C) LR density in *abi4* mutants. White bars, no treatment control; gray bars, ABA treatment; black bars, zeatin treatment. The data represent the mean ± SE of *n* = 3 independent experiments each containing 20 plants per treatment. Bars with different letters represent statistically different values by Tukey's HSD post-hoc test ($P \leq 0.05$). WT, wild type. [See online article for color version of this figure.]

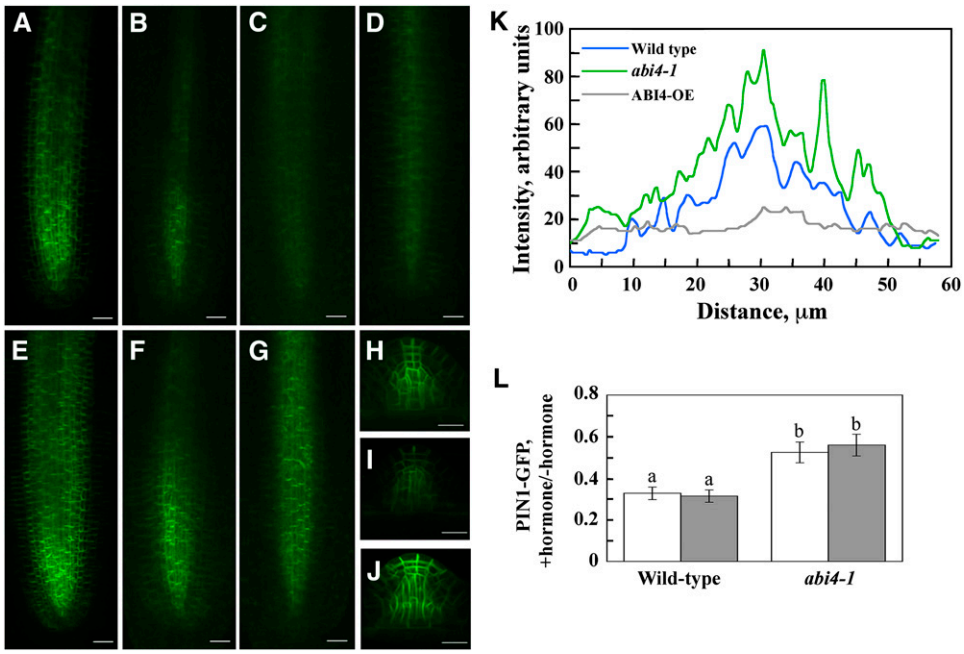

Figure 9. *ABI4* Affects PIN1 Distribution.

(A) to **(G)** PIN1-GFP was visualized in PR tips of 12-d-old *PIN1:PIN1-GFP*/wild-type (**[A]** to **[C]**), *PIN1:PIN1-GFP/ABI4* overexpressor **(D)**, and *PIN1:PIN1-GFP/abi4* mutant (**[E]** to **[G]**) seedlings with a fluorescent microscope.
(A), **(D)**, and **(E)** Untreated seedlings.
(B) and **(F)** Treated with 10 μM ABA for 24 h.
(C) and **(G)** Treated with 10 μM zeatin for 24 h. Bars = 20 μm.
(H) to **(J)** PIN1-GFP expression in untreated emerging LRs.
(H) *PIN1:PIN1-GFP*/wild type.
(I) *PIN1:PIN1-GFP/ABI4* overexpressor.
(J) *PIN1:PIN1-GFP/abi4* mutant. Bars = 20 μm.
(K) and **(L)** Quantification of the fluorescence intensity.
(K) Images shown in **(A)**, **(D)**, and **(E)** were scanned transversally using ImageJ 1.42q software. Presented pattern was taken 60 μm above root tips. Similar patterns were obtained when other sections were analyzed.
(L) Pixel intensities of four root images were determined using ImageJ 1.42q. Ratios of the values obtained for ABA-treated (white bars) or zeatin-treated (gray bars) plants to that of nontreated plants were calculated for both wild-type and *abi4-1* background. The data represent the mean ± SE of $n = 4$. Bars with different letters represent statistically different values by Tukey's HSD post-hoc test ($P \leq 0.05$).

ABI4 has been reported to be expressed in discrete developmental windows, mainly during seed maturation and in very young seedlings postgermination (Söderman et al., 2000; Arroyo et al., 2003). At later stages, *ABI4* is expressed at very low, albeit detectable, levels (Söderman et al., 2000; Arroyo et al., 2003). Other studies using *ABI4:GUS* expressing *Arabidopsis* (Arroyo et al., 2003; Bossi et al., 2009) did not see GUS staining in roots of seedlings at the postcotyledon stage. However, this may have been because the seedlings were being observed when the upper root zone had not yet reached the mature, *ABI4*-expressing stage or due to the use of low magnification/resolution to visualize the plants. On the other hand, a few reports have demonstrated the effects of mutations in *ABI4* on later developmental stages (Signora et al., 2001; Kaliff et al., 2007), indicating that *ABI4* is expressed in the roots and leaves of mature plants. Nevertheless, these studies did not directly demonstrate *ABI4* expression in these tissues.

Expression of *ABI4* in the root is modulated by plant hormones: it is induced by ABA and cytokinin and inhibited by auxin (Figure 3). Auxin is the key hormone affecting both initiation and development of LRs (Aloni et al., 2006; Fukaki et al., 2007; Osmont et al., 2007; Nibau et al., 2008). In agreement with the proposed inhibitory effect of *ABI4* on LR formation, treatment with the LR-promoting hormone IAA decreased the expression of *ABI4* (Figures 3A, 3C, and 3H). Similarly, inhibition of auxin transport by TIBA increased the expression of *ABI4* (Figures 3A, 3D, and 3I). Furtheremore, cytokinin and ABA are known to counteract auxin induction of LR formation (Bottger, 1974; Wightman et al., 1980). Treatment with either of these inhibitory hormones resulted in increased *ABI4* expression (Figures 3A, 3E, 3F, 3J, and 3K). It is interesting to note that, in germinating seedlings, *ABI4* expression is not induced by ABA (Söderman et al., 2000), suggesting that different signaling pathways might be involved in the regulation of *ABI4* expression in germinating seedlings and in mature roots.

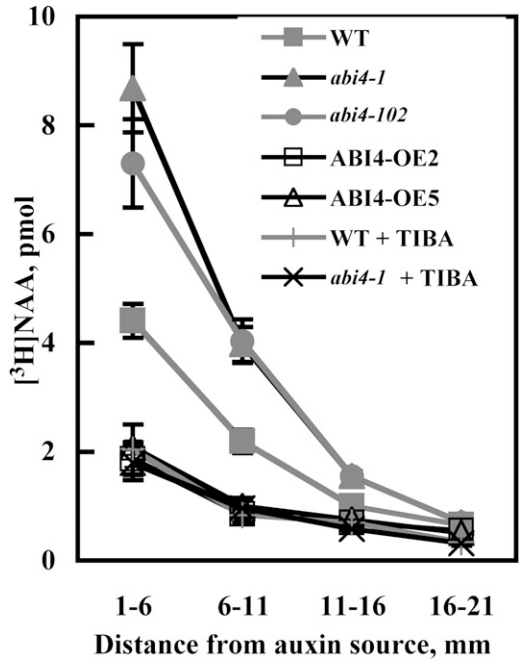

Figure 10. Polar Auxin Transport.

Agarose blocks containing 10 μM [^{3}H]NAA were placed on the shoot-root junction of the indicated 7-d-old seedlings. When indicated, blocks contained additional 10 μM TIBA. Roots were analyzed 10 h later as described in Methods. The data represent the mean ± SE of n = 3 independent experiments each containing 15 plants per treatment. WT, wild type.

Genetic manipulations affecting *ABI4* expression result in changes in root branching. Mutating *abi4* led to an increase of 25 to 35% in the number of LRs (Figure 1). In germinating seedlings, *ABI4* was highly expressed at the hypocotyl-root border (see Supplemental Figure 1 online; Arroyo et al., 2003) and, thus, probably inhibits LR emergence at early developmental stages. The decrease in LRPs and LRs in *abi4* mutants indicated that ABI4 inhibits the formation of LRPs. Similarly, overexpression of *ABI4* decreased LR formation (Figure 4). The inhibitory effect of *ABI4* overexpression on LR formation (Figures 4A and 4B) was in agreement with the increased number of LRs observed in the *abi4* mutants (Figures 1A and 1D). Analysis of LR developmental stage in *ABI4*-overexpressing plants (Figure 5A) and in *abi4* mutants (Figure 5B) confirmed that *ABI4* inhibits the establishment of new LRPs (stage A) and the growth of emerged LRs (stage D), whereas the intermediate stages B and C are less affected. Moreover, *ABI4* appears to decrease the initial growth rate of the newly emerged LRs (Figure 5C).

ABI4 Interactions with Auxin, Cytokinin, and ABA in Roots

Auxin is the key hormone promoting the development of LRPs and LRs (Aloni et al., 2006; Fukaki et al., 2007; Osmont et al., 2007; Nibau et al., 2008). ABA and cytokinin are known to counteract auxin activity by inhibiting LR formation (Aloni et al., 2006; De Smet et al., 2006; Wasilewska et al., 2008; Fukaki and Tasaka, 2009). Cytokinin has been suggested to inhibit LR formation by preventing establishment of the required auxin gradient via a decrease in the expression of PIN proteins (Laplaze et al., 2007; Ruzicka et al., 2009). On the other hand, the mechanism(s) governing ABA inhibition of LR formation is not understood. Our data suggest that *ABI4* mediates inhibition of LR formation by ABA and cytokinin via interference with both LRP formation and further growth of emerged (>0.5 mm) LRs (Figure 5). This latter stage has also been shown to be inhibited by the application of exogenous ABA (De Smet et al., 2003). LR development in *abi4* mutant plants is less sensitive to inhibition by ABA and cytokinin (Figure 8), suggesting that these hormones act upstream of *ABI4*. Mutants affected in the auxin influx carrier *AUX1* develop a decreased number of LRs (Marchant et al., 2002). Moreover, a double *pin1 pin3* mutant of the auxin-efflux transporters in *Arabidopsis* results in a 40 to 100% decrease in LRP number and to significant arrest in LR development (Benková et al., 2003). A triple *pin1 pin3 pin4* mutant failed to develop any organized LRPs (Benková et al., 2003).

ABI4 is known to play a role in the ABA-signaling pathway in germinating seedlings (Söderman et al., 2000). Here, we show that it is also expressed in roots at later stages of plant development (Figure 2). *ABI4* expression in the roots was induced by the root development inhibitory hormones ABA and cytokinin and was repressed by auxin, the LR-promoting hormone (Figure 3). Auxin treatment of *abi4* mutants resulted in an increased number of LRs (Figures 6F to 6I; see Supplemental Figure 5 online). The extent of this increase was similar to that observed in the respective mutants without addition of exogenous auxin (Figure 1), suggesting that mutating *abi4* does not alter the auxin response.

In addition, auxin treatment of the *ABI4*-overexpressing seedlings, which lack LRs, resulted in the development of some LRs (Figures 6Q and 6R). The number of LRs in Dex-induced *ABI4*-overexpressing plants was 30% of that in the auxin-treated wild-type and empty-vector plants (both with and without Dex) (Figures 6Q and 6R). These results suggested that *abi4* mutants are not affected in auxin perception or auxin response pathways. *ABI4* modulates LR development in a pathway that is distinct from that reported for *ABI3* (Brady et al., 2003): the *abi3* mutant displays wild-type-like root architecture, and its effects on LR formation can be seen only on the background of the *ERA1* mutation or following treatment with auxin. *ABI4* is expressed in upper (mature) regions of the root and not in meristematic tissues (Figure 2), whereas *ABI3* is expressed in LRs. In addition *ABI4* expression is reduced by auxin, while *ABI3* expression is enhanced (Brady et al., 2003), and *ABI3* mRNA levels in the roots were not affected in *abi4* mutants.

ABI4 may inhibit the formation of LRs by interfering with auxin distribution. Using the *GUS* reporter gene driven by the auxin-responsive synthetic *DR5* promoter, we showed a 3.5-fold increase in the number of GUS-stained foci in the genetic background of the *abi4-1* mutant compared with the expression of this reporter construct in the wild-type background (Figure 7D; see Supplemental Figure 6 online). In addition, root expression of the cytokinin level reporter *ARR5:GUS* construct (To et al., 2004) was markedly reduced in the *abi4-1* mutant background (Figures 7O and 7P) compared with the wild-type background (Figures 7M and 7N), suggesting that *ABI4* might affect cytokinin levels in the roots.

ABI4 expression zone in the mature (upper) root region correlates with the root zone in which LRs develop. *ABI4* is expressed mainly in phloem companion cells and to a lesser extent in the metaphloem and stele parenchyma cells (Figures 2H to 2J). *ABI4* inhibits LR formation in a non-cell-autonomous fashion; its expression in the phloem and in vascular parenchyma prevents LR initiation from pericycle cells adjacent to the xylem pole. This non-cell-autonomous function is most likely mediated by auxin. Moreover, auxin (Robert and Friml, 2009), ABA (Jiang and Hartung, 2008), and cytokinin (Hirose et al., 2008) are transported in the phloem. ABA is also synthesized in companion cells, where *ABI4* is highly expressed: the *Arabidopsis AAO3* gene encoding an ABA biosynthetic enzyme is also localized in phloem companion and stele parenchyma cells (Koiwai et al., 2004). Auxin is believed to act as a non-cell-autonomous signal, interacting with other signaling pathways to regulate developmental processes (Swarup et al., 2002). Our data suggest that *ABI4* mediates the interaction between the auxin, ABA, and cytokinin signaling pathways (Figure 11). Companion cells thus appear to be the preferred cell type for the regulatory proteins whose activities are affected or affect the balance between ABA, cytokinin, and auxin.

Levels of the auxin-efflux transporter PIN1 were reduced in *ABI4*-overexpressing plants (Figures 9D, 9I, and 9K) and were enhanced in the *abi4* mutants (Figures 9E to 9G, 9J, and 9K). These changes are expected to affect root auxin levels, thus affecting LR development. Indeed, direct measurements of auxin transport showed that polar auxin transport is enhanced in *abi4* mutants and inhibited in *ABI4*-overexpressing plants (Figure 10). Since *ABI4* encodes an AP2-domain transcription factor (Finkelstein, 1994), the *ABI4*-induced reduction of PIN1 protein levels is probably indirect. *PIN1* mRNA levels were not altered in *abi4* mutant or *ABI4*-overexpressing plants (see Supplemental Table 1 online), suggesting that *ABI4* does not directly regulate *PIN1* gene transcription. PIN proteins are known to be posttranscriptionally regulated (Abas et al., 2006; Malenica et al., 2007). Moreover, mutation of *MODULATOR OF PIN* results in a decrease in PIN1 protein levels without affecting *PIN1* gene expression (Malenica et al., 2007). Thus, ABI4 may transactivate genes affecting PIN1 levels.

Although ABA is known to inhibit LR formation (De Smet et al., 2006), our results describe a direct effect of the ABA-signaling pathway on auxin transport. Exogenous cytokinin and ABA inhibited PIN1 expression in roots (Figure 9). The extent of inhibition following application of these hormones was higher in wild-type plants than in the *abi4* mutants (Figure 9L), suggesting that ABA and cytokinins reduce PIN1 by both *ABI4*-dependent and *ABI4*-independent pathways. Cytokinin is known to counteract auxin activity and has been shown to negatively regulate PIN1, as reflected by a reduction in *PIN1:PIN1-GFP* signal in the *Arabidopsis* PR tip of cytokinin-treated plants (Laplaze et al., 2007; Ruzicka et al., 2009). The dampened reduction in PIN1 levels in *abi4* mutants by ABA and zeatin (Figure 9) correlated with the reduced of inhibitory effects of these hormones on LR formation in these plants (Figure 8), suggesting that *ABI4* activity interferes with polar auxin transport. This was supported by direct auxin transport assays (Figure 10). The increased PIN1 expression (Figure 9) and, thus, the predicated increase in auxin, is in agreement with the diffuse *DR5:GUS*/*abi4* expression pattern (Figures 7F and 7H) resembling that observed in auxin-treated wild-type plants (Benková et al., 2003).

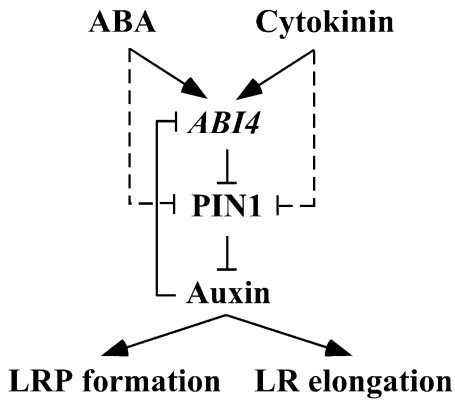

Figure 11. A Possible Mechanism for Modulation of LR Development by *ABI4.*

A simplified model is shown for the hormonal regulation of LR development in *Arabidopsis*. *ABI4* expression is enhanced by ABA and cytokinin and repressed by auxin. *ABI4*, in return, inhibits polar auxin transport in the root by decreasing the levels of the auxin efflux carrier PIN1, resulting in decreased auxin levels. Thus, *ABI4* and auxin form a feedback-inhibition loop. In addition to ABI4-dependent pathway, ABA and cytokinin can act in an ABI4-independent way (dashed lines). Auxin is essential for LR formation and for the elongation of emerged LRs; thus, reduction in auxin levels results in inhibition of LR formation. Arrows and T-bars represent enhancement and inhibition, respectively.

Our data suggest that *ABI4* mediates ABA and cytokinin auxin-antagonistic activity in the modulation of root development (Figure 11). *ABI4* expression is enhanced by ABA and cytokinin (Figure 3) and repressed by auxin, which is transported from the shoot to the root, or basipetally from the root tips toward the upper root sections. *ABI4*, in return, inhibits polar auxin transport in the root (Figure 10) by decreasing the levels of PIN1 (Figure 9), resulting in decreased auxin levels in the roots. Thus, *ABI4* and auxin form a feedback inhibition loop. Reduction of PIN1 by cytokinin and ABA was also observed in the *abi4-1* mutant (Figures 9F and 9G), suggesting an additional *abi4*-independent pathway. Moreover, the PIN1 levels in cytokinin- or ABA-treated *abi4* mutants were still high enough to support the development of LRs (Figure 8). Auxin is essential for both the establishment of LRPs (Aloni et al., 2006; Fukaki et al., 2007; Osmont et al., 2007; Nibau et al., 2008) and the elongation of emerged LR (Muday and Haworth, 1994). As a result, *abi4* mutant plants display an increased number of elongated LRs, whereas overexpression of *ABI4* results in a decreased number of LRPs and in inhibition of elongation of emerged LRs.

METHODS

Plant Material and Growth Conditions

The following *Arabidopsis thaliana* (Columbia ecotype) plants were obtained from the Arabidopsis Stock Center in Columbus, OH: *abi4* mutants, *DR5:GUS* (Ulmasov et al., 1997), *ARR5:GUS* (To et al., 2004),

and *PIN1:PIN1-GFP* (Benková et al., 2003). *Arabidopsis* 4C-S5 and pV-TOP (Craft et al., 2005) were obtained from I. Moore (Oxford University). *ABI4:GUS*, *pOp6:ABI4*, and *cauliflower mosaic virus 35S:ABI4* were constructed for this study. Seed sterilization and plant growth on solid 0.5× Murashige and Skoog (MS) + 0.5% (w/v) sucrose or in pots were as described previously (Shkolnik and Bar-Zvi, 2008). Hormone treatment was performed on Whatman No. 1 filter papers soaked with 0.5× MS medium supplemented with the indicated hormone. Each assay was performed with 50 seedlings from each line.

Constructs and *Arabidopsis* Plant Transformation

DNA corresponding to *ABI4* promoter or coding sequences was isolated using PCR and genomic DNA as the template and primers as listed in Supplemental Table 2 online. The *ABI4* promoter was subcloned upstream of the GUS coding sequence in pCAMBIA 1391Z (CAMBIA). The *ABI4* coding sequence was subcloned into pCAMBIA 1200 (CAMBIA) downstream of the cauliflower mosaic virus 35S promoter or into the pV-TOP vector (obtained from I. Moore) downstream of the Dex-inducible *pOp6* promoter (Craft et al., 2005). Constructs were introduced into *Agrobacterium tumefaciens* GV3101 and used in the transformation of wild-type Columbia or 4C-S5 *Arabidopsis* (Craft et al., 2005) plants, for pCAMBIA- or pV-TOP–based constructs, respectively (Clough and Bent, 1998). Transgenic plants were selected on plates containing hygromycin. Plants were selfed twice, and T3 homozygous plants were used in this study. At least five lines of independent transformants were assayed. Transgenic *Arabidopsis DR5:GUS*, *ARR5:GUS*, and *PIN1:PIN1-GFP* plants were used as the pollen source for crossing with emasculated wild-type or *abi4-1* mutant plants. Cross-bred offspring were selected on 1 μM ABA-containing media for the *abi4* trait and analyzed for GUS or GFP expression.

qRT-PCR Analysis

Total RNA was isolated using an Aurum Total RNA Mini kit (Bio-Rad Laboratories). The RNA concentration was estimated spectrally (Nano Drop ND-1000; Nano Drop Technologies). cDNA was synthesized using an ABgene Reverse-iT 1st Strand synthesis sit (ABgene). The reaction mixture contained 700 ng of total RNA and 400 ng of random decamers. Relative transcript levels were assayed by real-time PCR analysis using the 7300 real-time PCR system (Applied Biosystems). Primer sequences were designed by Primer-Express software Version 2.0 (Applied Biosystems). Wherever possible, one of the primers in each set was designed at an exon-exon border. All amplicon length ranges were between 75 and 90 bp. The reaction mixture contained 10 μL of Power SYBR Green PCR Master Mix (Applied Biosystems), 50 ng of cDNA (for endogenous control, as 18S RNA, a 1000-fold dilution was used), and 500 nM of each of the forward and reverse primers. The following default program was used: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min each, and a dissociation stage of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. RNA relative quantification analyses were performed using 7300 System SDS software (Applied Biosystems). The list of primers used is shown in Supplemental Table 2 online. The data represent the mean ± SE of $n = 3$ independent experiments. Each data point was determined in triplicates in each of the three biological replicates and presented as mean ± SE.

GUS Staining

Plant tissues were fixed in acetone for GUS staining as described previously (Weigel and Glazebrook, 2002). Pictures were taken with a Nikon camera (DXM1200F) using a stereoscope and microscope. Each treatment was performed using three biological replicates.

PIN1:PIN1-GFP Expression

Roots of plants expressing *PIN1:PIN1-GFP* (Benková et al., 2003) under different generic backgrounds were imaged using Zeiss LSM510 confocal laser scanning microscope. Image quantification was performed using ImageJ 1.42q software (W.S. Rasband, ImageJ, U. S. National Institutes of Health). Images are representative of 15 roots per each treatment with three biological replicates.

Root Sections

GUS-stained seedlings were fixed, dehydrated, and directionally embedded in Technovit 7100 resin (Heraeus Kulzer) (Beeckman and Viane, 2000). Sections (4 μm) were cut with a Reichert-Jung Ultracut microtome (Leica) using a glass knife, stained with 0.05% (w/v) ruthenium red, and mounted in Entellan mounting medium (Merck) before photography.

LR Developmental Stages

Seeds were germinated on plates containing 0.5× MS supplemented with 0.5% (w/v) sucrose, 0.5% (w/v) plant agar, and where indicated also 2 μM Dex. Ten-day-old seedlings were collected, and the four defined LR developmental stages (De Smet et al., 2003) were determined using a light microscope. Dex had no effect on the number or distribution of the determined developmental stages in wild-type plants or in plants transformed with empty vector. Three biological experiments containing each 20 plants per treatment were analyzed. Error bars represent SE.

LR Elongation Rate

Seedlings were grown vertically on 0.5× MS supplemented with 0.5% (w/v) sucrose and 1.5% (w/v) plant agar. The length of the first two visible LRs was marked every 24 h on the plate using color-coded markers. Root lengths at all time points were measured 9 d after emergence of the LRs. Growth rates were calculated from the difference in length after 24 and 48 h.

Root Auxin Transport Assay

[^{3}H]NAA (Nuclear Research Center) was a gift from J. Riov (The Hebrew University, Rehovot, Israel), and NAA was obtained from Duchefa Biochemie. Seedlings were grown on vertical plates containing 0.5× MS supplemented with 0.5% (w/v) sucrose and 1.5% (w/v) plant agar. Agar blocks (2 mm × 1 mm × 1 mm) containing 10 μM [^{3}H]NAA (4×10^4 cpm × nmol^{-1}) were placed on top of the plants at the shoot-root junction. Plates were incubated vertically for 10 h, roots were detached 1 mm below the auxin application site, 5-mm segments were cut, placed into vials containing Quicksafe A scintillation cocktail (Zinsser Analytic), and incubated at room temperature for 2 d in the dark. Radioactivity was determined in a Tri-Carb 2100TR scintillation counter (Packard Instruments). Three biological replicates, each containing 15 plants per treatment, were examined for each line. Data are presented as mean ± SE.

Statistical Analyses

Differences between groups were analyzed by Tukey's HSD post-hoc test (PASW Statistics Data Editor of the SPSS 17.0 software package; IBM Company).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM 129580 for *ABI4* mRNA, NM 106017 for *PIN1* mRNA, and NM 114679 for *ARR5* mRNA. The

Arabidopsis Genome Initiative locus identifier for *ABI4* is AT2G40220, AT1G73590 for *PIN1*, and AT3G48100 for *ARR5*.

Supplemental Data

The following materials are available in the online version of the article.

Supplemental Figure 1. GUS Staining of *ABI4:GUS* Plants.

Supplemental Figure 2. *ABI4* Expression in Root and Leaf Tissues.

Supplemental Figure 3. Quantification of Dexamethasone-Induced *ABI4* Overexpression in Transgenic *Arabidopsis* Plants Harboring the *pOp6:ABI4* Construct.

Supplemental Figure 4. Auxin Treatment of *abi4* Mutants.

Supplemental Figure 5. Lateral Root Formation in IAA-Treated *abi4* Mutants.

Supplemental Figure 6. *DR5:GUS* Expression Pattern Is Altered on the Genetic Background of the *abi4* Mutation.

Supplemental Figure 7. Lateral Root Formation in Cytokinin-Treated Wild-Type and *abi4* Mutant *Arabidopsis*.

Supplemental Table 1. Genes Which Steady State mRNA Levels Are Not Altered in Roots of *abi4* Mutants and *ABI4*-OE Plants.

Supplemental Table 2. Primer List.

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