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A plant microRNA regulates the adaptation of roots to drought stress

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ABSTRACT

Plants tend to restrict their horizontal root proliferation in response to drought stress, an adaptive response mediated by the phytohormone abscisic acid (ABA) in antagonism with auxin through unknown mechanisms. Here, we found that stress-regulated miR393-guided cleavage of the transcripts encoding two auxin receptors, TIR1 and AFB2, was required for inhibition of lateral root growth by ABA or osmotic stress. Unlike in the control plants, the lateral root growth of seedlings expressing miR393-resistant *TIR1* or *AFB2* was no longer inhibited by ABA or osmotic stress. Our results indicate that miR393-mediated attenuation of auxin signaling modulates root adaptation to drought stress.

1. Introduction

Drought stress (soil water deficit) is among the most common adverse environmental conditions limiting agricultural productivity worldwide. Plant roots are responsible for water uptake and are the first plant organ to encounter soil water deficiency. Accordingly, roots exhibit great architectural plasticity to help the plants to better adapt to water availability [1–3]. Under normal conditions lateral roots proliferate; under drought stress, both the initiation and the elongation of lateral roots are reduced [1–3], presumably facilitating primary root elongation for water uptake from deeper soil. Whereas the phytohormone auxin plays an essential role in promoting lateral root initiation and elongation [4,5], the stress hormone abscisic acid (ABA) inhibits lateral root generation [3,6,7]. Little is known, however, about the mechanisms underlying this antagonistic interaction between the ABA and auxin signaling pathways in regulating lateral root development.

In the auxin-signaling pathway, the binding of auxin to the F-box TIR1 family of auxin receptors promotes interactions between the receptors and Aux/IAA proteins [8]. One miRNA, miR393, targets the TIR1 family mRNAs for degradation [9–16]. This layer of regulation affects the auxin response in several developmental processes in plants when either the miRNA or its resistant targets are overexpressed [15,17]. Interestingly, the miR393 transcript level has been shown to be induced by ABA, cold, salt, or PEG treatment [11], implying a potential connection between stress and auxin signaling via this micro RNA. Nonetheless, this connection has not been well established, although miR393 was shown to be induced by a pathogen elicitor that initiates the cleavage of *TIR1* family mRNAs [18].

While studying root responses to drought stress and ABA, we found that *fry1*, a previously identified ABA hypersensitive mutant, is also hypersensitive to the inhibition of lateral root development by ABA. It also displays phenotypes reminiscent of auxin-resistant mutants. The *fry1* mutants exhibited enhanced cleavage of auxin receptor transcripts as well as increased expression of the ABA-induced miR393. We thus explored the possible link between miR393 and the response of roots to drought stress. Our results demonstrate that miR393-mediated attenuation of auxin signaling is essential for inhibition of lateral root growth by ABA or osmotic stress.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 was used in the experiments unless otherwise stated. The *fry1-1* mutant in the C24 background

Abbreviations: ABA, abscisic acid; TIR1, transport inhibitor response 1; AFB, Auxin-binding F-box; MS salt, Murashige and Skoog salt; GUS, beta-glucuronidase; PEG, polyethylene glycol

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and a T-DNA knockout line *fry1-6* (SALK_020882) in the Col-0 background were described previously [19,20]. Seeds were surface-sterilized with bleach and planted on half-strength MS media supplemented with 1% sucrose and 0.6% agar. After 3 days of cold treatment, the plates were incubated at 22 ± 1 °C under constant white light for seed germination and seedling growth. For the root growth assays with ABA or PEG treatment, four-day-old seedlings were transferred to ½ MS agar plates with or without 0.5 or 1.0 μM *cis*, *trans*-ABA (Cat. #A1049, Sigma, St. Louis) or −0.5 MPa PEG. The plates were incubated vertically for growth under the above-mentioned conditions. Upon completion of the treatment, the seedlings were photographed using a digital camera and lateral root lengths were measured. For the PEG treatment assays, PEG-infused agar plates were prepared by using 25% PEG (molecular weight 8000, Sigma, St. Louis) as described [21]. The soil-grown plants were kept in a growth room at 22 ± 1 °C with a 16-h light period.

2.2. Constructs and plant transformation

AFB2 genomic DNA was amplified using the primers CACCATTGATGTTCTCTAAAAACAA and GAATCCACACAAATGGCGGCGCATCC. *TIR1* genomic DNA was amplified using the primers CACC TAATTTATATGGTTTGAGTCAC and CGTTAGTAGTAATGATTTGCCTG. The fragments were ligated into the pENTR-D-TOPO vector (Invitrogen, Carlsbad, CA). After sequence confirmation, *AFB2* was cloned into the pMDC Gateway vector pMDC162 in frame with the *GUS* coding sequence, and *TIR1* was cloned into modified pMDC99 with *nos* terminator added through LR clonase recombination to generate *AFB2:GUS* and *TIR1*, respectively. Site-directed synonymous mutagenesis was carried out with primers CAATGAGGAGTTTGTGG ATGTCTTCATGTGAAGTC and AGACATCCACAAACTCCTCATTGTTTCA TACTTGCTCACA to generate *AFB2m:GUS* or with primers GCTGGAGACAATGCGTAGTTTGTGGATGTCTTCTTGTTCCG and CCACAAACTA CGCATTGTCTCCAGCTTTGAAGCATTGG to generate *TIR1m*. Strains of *Agrobacterium tumefaciens* GV3101 transformed by electroporation with these constructs were used for plant transformation.

2.3. Target gene cleavage product cloning

A modified 5′-RACE was performed according to the GeneRacer kit manual (Invitrogen, Carlsbad, CA). In brief, 5 μg of total RNA were ligated to an RNA adaptor using T4 RNA ligase before reverse transcription. First-round PCR products were diluted 100-fold and used for the second round amplification with a nested 5′ RACE primer and the *AFB2* gene-specific primer TCAACGGAGGAAAGTCAAAAAC. Final PCR products were gel-purified and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

2.4. RNA analysis

Total RNA was extracted from 12-day-old seedlings grown under constant light using the Trizol reagent (Invitrogen, Carlsbad, CA). For detection of either full-length or cleaved transcripts, 10 μg of total RNA were separated on 1.5% formaldehyde agarose gels. For miRNA detection, 30 μg of total RNA were fractionated by 15% polyacrylamide/8 M urea gels. A DNA oligonucleotide GGATCAATGCGATCCCTTTGGA, complementary to the *AtmiR393a* and *AtmiR393b* product miR393, was labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. RNA gel blots were autoradiographed to a phosphoimage screen, and the signals were quantified using ImageQuant 5.0 (Amersham, Piscataway, NJ). For RT-PCR analysis of miR393 precursors, the GGATCCAAAGGGATCGCATTG and GAATCCAAAGAGATAGCATG primers were, respectively, used to amplify *AtmiR393a* and *AtmiR393b* transcripts with Oligo-dT-primed first-strand cDNA. The PCR products were resolved with 4% agarose gel.

2.5. GUS staining and activity assay

Seedlings or tissues from transgenic plants were subjected to vacuum while being immersed in a staining solution (25 mM sodium phosphate buffer at pH 7.0, 10 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 0.1% Triton X-100, and 2 mM 5-bromo-4-choro-3-indolylb-D-glucuronide cyclohexylamine salt) for 10 min before staining at 37 °C. GUS staining was performed with 1-week-old seedlings for 0.5 h with the AFB2m:GUS line or for 5 h with the AFB2:GUS line. For the AFB2:GUS and AFB2m:GUS activity assays, the seeds were planted directly on ½ MS plates containing 1% sucrose with 0, 0.5 or 1.0 μM ABA.

3. Results

3.1. The ABA-hypersensitive mutant fry1 is also hypersensitive to ABA inhibition of lateral root development

The *fry1* (*fiery1*) mutants were isolated through their phenotype of super induction of the ABA- and stress-responsive gene *RD29A* [19]. *FRY1* encodes a bifunctional enzyme with both an inositol polyphosphate 1-phosphatase activity, which catalyzes the degradation of the second messenger inositol 1, 4, 5-trisphosphate (IP_3), and a nucleotidase activity, which degrades the adenosine 2′,(3′), 5′-bisphosphate [22]. Because ABA mediates the inhibition of lateral root growth by drought [3], we checked whether FRY1 could be involved in this response. Indeed, *fry1* mutants exhibited enhanced inhibition of lateral root growth in response to ABA (Fig. 1). In fact, *fry1* seedlings had dramatically reduced lateral root growth even without ABA (Fig. 1 and [20]). This observation may indicate a hypersensitive response to lower concentrations of endogenous ABA, similar to the ABA hypersensitive gene induction in the mutant [19].

3.2. Enhanced cleavage of AFB2 mRNA and elevated miR393 levels in fry1

We previously reported that *fry1* is less responsive to auxin in both lateral root induction and the activation of the auxin response reporter *DR5::GUS* as well as native auxin-responsive genes [20]. Since the F-box auxin receptors TIR1, AFB1, AFB2 and AFB3 are involved in root system morphology [15,23], we checked the transcript levels of these receptor genes in *fry1*. However, no significant differences were detected in the full-length mRNA levels of *TIR1*, *AFB1*, *AFB2*, and *AFB3* between the wild type and *fry1* (Fig. 2A).

It is known that *TIR1* family members are cleaved by miR393 [9–13]. Interestingly, whereas *AFB2* cleavage products were barely detectable in the corresponding wild types, these products accounted for 11.3 and 6.7 percent of the total *AFB2* transcript levels in *fry1-1* and *fry1-6*, respectively (Fig. 2A). The cleavage products of the three other family members were also detectable, but were not particularly accumulated in *fry1* mutants (Fig. 2A and data not shown). Using RNA isolated from 2-week-old seedlings, 5′-RACE further confirmed that the *fry1-6* mutant contained a higher level of *AFB2* cleavage products than the wild type (data not shown). These cleavage products were cloned, sequenced, and found to be cleaved predominantly at the miR393 target site (Fig. 3A and Supplementary Fig. 1).

Enhanced cleavage of auxin receptor transcripts in *fry1* suggests that *fry1* might have an elevated level of miR393. Indeed, the miR393 level was several-fold higher in *fry1* than in the wild type,

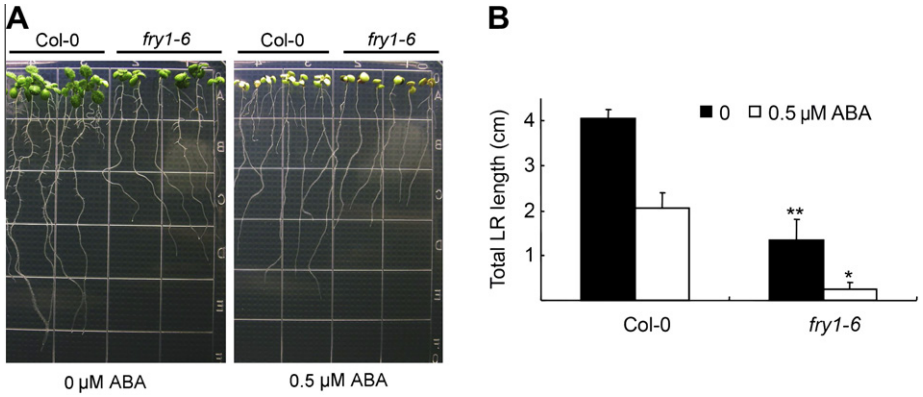

Fig. 1. *fry1* mutants are hypersensitive to ABA inhibition of lateral root growth. (A) Morphology of 2-week-old wild-type and *fry1-6* seedlings grown on ½ MS media with or without ABA. (B) Total lateral root (LR) lengths of the wild-type and *fry1-6* seedlings shown in (A). Data are means and standard errors ($n = 5$). * and ** denote significant differences between *fry1-6* and Col-0 at the 0.05 and 0.01 significance levels (Student's *t*-test), respectively.

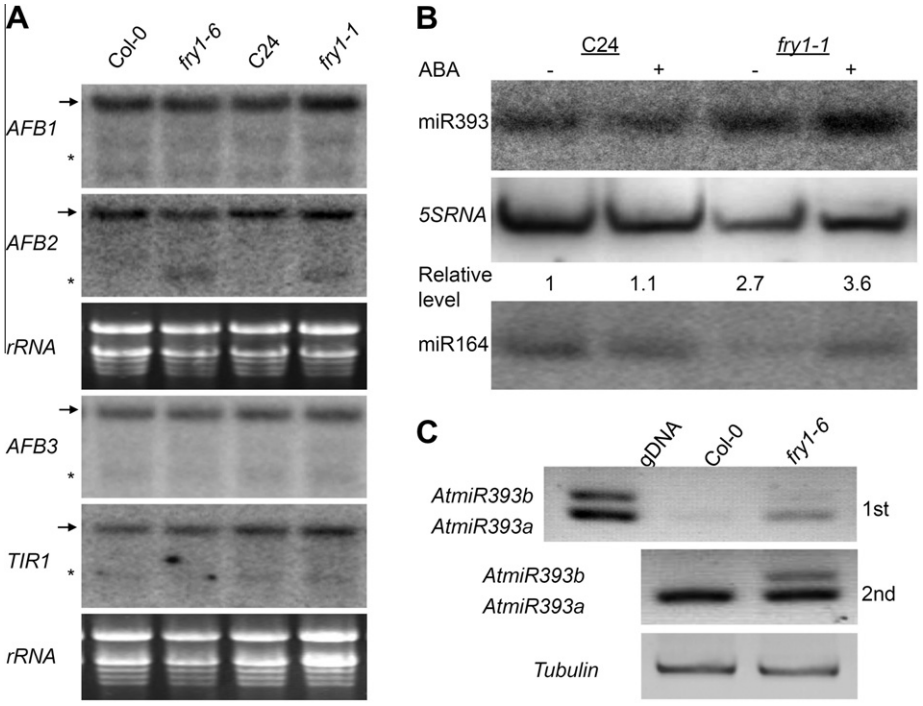

Fig. 2. Accumulation of AFB2 cleavage products and elevated miR393 and its precursor levels in *fry1*. (A) RNA blotting analysis of *TIR1* family members and their miR393 cleavage products in *fry1* mutants. Full-length transcripts are indicated with arrows and cleavage products are indicated with asterisks. rRNA was used as a loading control. (B) miR393 levels in *fry1-1* mutants. 5S RNA and miR164 were used as loading controls. The relative level data are the signal ratios of miR393 to the 5SRNA transcript. (C) RT-PCR of miR393 precursors *AtmiR393a* and *AtmiR393b* in *fry1-6*. PCRs were performed for 40 cycles in the first round (upper panel), after which the products were diluted 100-fold and then amplified for 35 cycles in the second round (middle panel). A Tubulin gene was used as the control.

particularly after ABA treatment (Fig. 2B). Nonetheless, compared with the strong induction of miR393 by ABA in a previous report [11], we could not detect a significant increase in the miR393 level in the wild-type seedlings upon ABA treatment (Fig. 2B). Since the Arabidopsis genome harbors two miR393 precursors, both giving identical mature miR393 [11,12,24], we checked for their potential contribution to the elevated miR393 level in *fry1*. While both precursors were barely detectable in the wild type, a significant level of *AtmiR393a* was detected in *fry1*. When the first round PCR product was diluted and subjected to a second round of amplification, the *AtmiR393b* transcript could be detected in *fry1* but not in the wild type, even after 35 cycles (Fig. 2C). Therefore, both miR393 precursors were induced to higher levels in the *fry1* mutant than in the wild type.

3.3. Expression of miR393-resistant AFB2 stabilizes AFB2 expression

We generated transgenic plants expressing the *AFB2* native promoter driven either the *AFB2:GUS* or *AFB2m:GUS* translational fusion constructs. The *AFB2m* gene contained synonymous mutations in the miR393 target site, which lowered the free energy such that the duplex formation was not favored (Fig. 3A). Since a certain level of miR393 was detected in seedlings grown under normal conditions (Fig. 3B), some difference in GUS activity between *AFB2:GUS* and *AFB2m:GUS* transgenic seedlings would be expected when the transcript is targeted by miR393. Indeed, the *AFB2m:GUS* activity was detected in multiple transgenic lines after 0.5 h staining at levels that were comparable to or even stronger than those in the 5-h stained *AFB2:GUS* seedlings (Fig. 3B and D). Therefore,

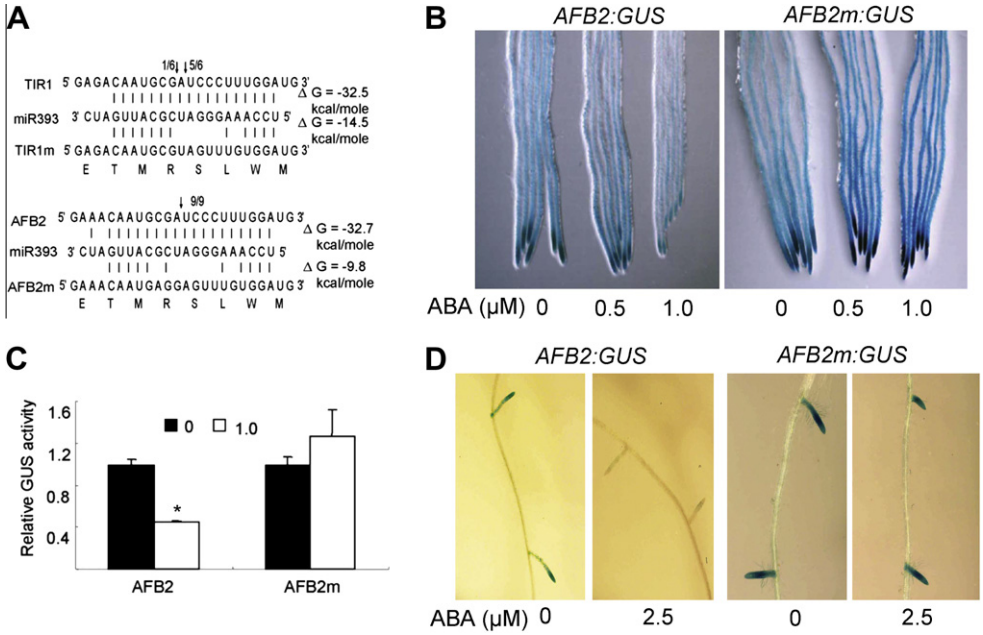

Fig. 3. ABA influences the stability of *AFB2* through miR393. (A) Cleavage sites and mutated sequences of *TIR1* and *AFB2*. The free energy of the native and mutated pairs is indicated. (B) GUS staining in 6-day-old *AFB2* or *AFB2m* transgenic seedlings treated with (right) or without (left) ABA at the indicated concentrations. (C) GUS staining in 6-day-old *AFB2* or *AFB2m* seedlings treated with 0 or 1.0 μM ABA. (D) GUS staining of lateral roots in 6-day-old *AFB2* or *AFB2m* transgenic seedlings treated with (right) or without (left) ABA at the indicated concentrations. * Denotes a significant difference between ABA treated and not treated at the 0.05 significance level (Student's *t*-test).

the mutated form of *AFB2m* is resistant to miR393-guided cleavage under normal conditions.

We then investigated the impact of ABA on *AFB2* expression in these reporter lines. After treatment for one week with 0.5 μM ABA, the *AFB2:GUS* staining was clearly reduced in the elongation zone and was slightly reduced at the root tips (Fig. 3B). With 1.0 μM ABA treatment, a dramatic decrease in *AFB2:GUS* staining was observed throughout the entire root (Fig. 3B). In contrast, the same ABA treatments did not reduce GUS staining in the *AFB2m:GUS* lines. In fact, the ABA treatments even slightly enhanced the *AFB2m:GUS* staining compared with the control (Fig. 3B). The GUS activities in these lines were quantified using proteins extracted from seedlings treated with or without 1.0 μM ABA. ABA treatment reduced the *AFB2:GUS* activity by more than 50%, but increased the *AFB2m:GUS* activity by around 30% (Fig. 3C). To further investigate AFB2 expression during lateral root growth, we stained seedlings with lateral roots and found AFB2:-GUS mainly at the lateral root tips. ABA treatment of these seedlings significantly reduced GUS activities in *AFB2:GUS* seedlings, yet it had little effect on GUS activities in *AFB2m:GUS* seedlings (Fig. 3D). These experiments were repeated several times with similar results obtained.

3.4. Expression of miR393-resistant AFB2 or TIR1 blocks lateral root inhibition by ABA or osmotic stress

With the above finding that ABA does not reduce the level of AFBm:GUS expression, we examined whether attenuation of auxin signaling through miR393 cleavage of auxin receptor transcripts is required for ABA-initiated inhibition of lateral root growth. As shown in Fig. 4A, ABA significantly inhibited lateral root growth in wild-type seedlings, but it was unable to inhibit the growth of lateral roots in both miR393-resistant *AFB2m* and *TIR1m* transgenic seedlings. After ABA treatment, the lateral root length decreased by about 60% in the wild-type but by less than 10% in the miR393-resistant seedlings (Fig 4B). Therefore, miR393-guided transcript cleavage of *TIR1* family members is essential for ABA inhibition of lateral root growth.

Since osmotic stress is an important component of drought stress and can be administered in a more quantitative way using polyethylene glycol (PEG) in agar media [21], we investigated if the inhibition of lateral growth by osmotic stress [3] also requires miR393-guided cleavage of TIR1 family transcripts. PEG treatment inhibited lateral root growth both in the wild-type and transgenic control plants but much less so in the miR393-resistant seedlings (Fig. 4C). Compared to plants in the non-stress control, the total lateral root length decreased by 38% and 25% in the PEG-treated *AFB2m* and *TIR1m* plants, respectively; whereas in *AFB2* and *TIR* plants, the total lateral root length decreased by 73% and 41%, respectively (Fig 4D).

4. Discussion

In plants, inhibition of lateral root growth combined with promotion of primary root growth are common adaptive responses to drought stress, yet the underlying mechanisms for these adaptations are unclear [3]. In this study, we first observed that the ABA-hypersensitive mutant *fry1* is also hypersensitive to ABA-regulated inhibition of lateral root growth (Fig. 1). Since *fry1* mutants are less sensitive to auxin [20], we hypothesized that the *FRY1* locus might mediate the antagonistic interaction of these two signaling pathways. It should be noted that *fry1* is distinct from other auxin-resistant mutants, such as *axr1*, *axr2*, and *ibs*, which have reduced sensitivity to both auxin and ABA [25,26]. Therefore, *FRY1* may underpin novel mechanisms responsible for the interaction between ABA and auxin signaling pathways.

Auxin is perceived by auxin-receptor TIR1 family members, which, upon binding to auxin, target Aux/IAA proteins, the repressors of auxin signaling, for degradation [8]. Given that *fry1* exhibits broad auxin-insensitive phenotypes, it is likely that *fry1* plants possess impaired auxin receptor functions. Auxin receptor transcripts are known targets of miR393-guided cleavage [9–13]. Intriguingly,

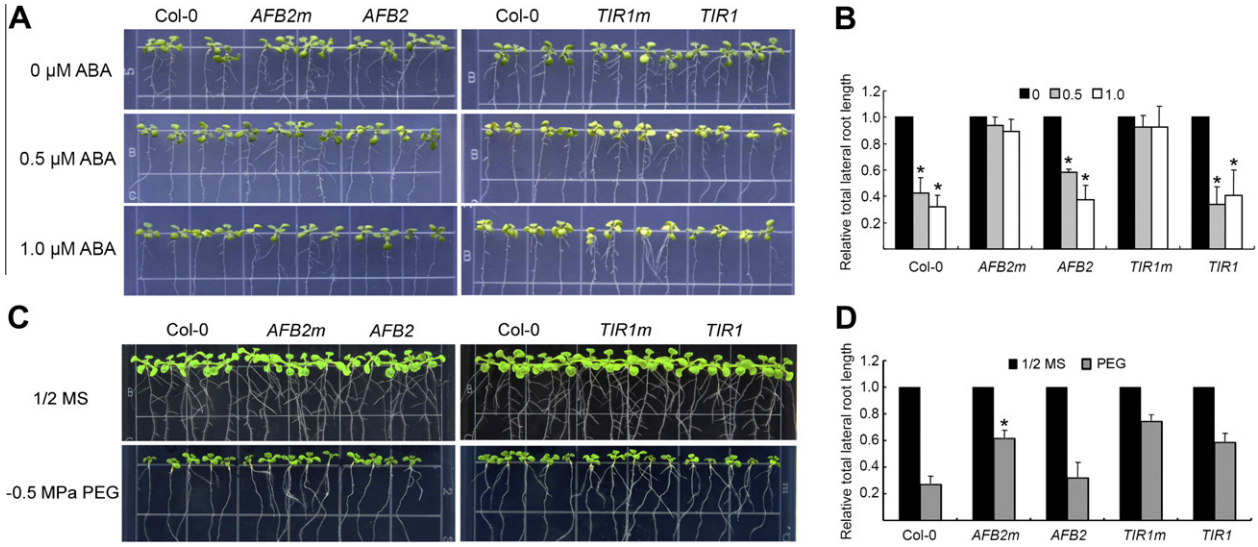

Fig. 4. Expression of *AFB2m* and *TIR1m* confers ABA and PEG insensitivity in lateral root growth. (A) Four-day-old wild type, *TIR1*, *TIR1m*, *AFB2*, and *AFB2m* transgenic seedlings were transferred from regular ½ MS media to MS media supplemented with the indicated concentrations of ABA. The pictures were taken 4 d after the transfer. (B) Relative lateral root lengths of the indicated seedling lines treated with or without ABA. Data are averages of two independent experiments with four seedlings for each line. * Denotes a significant difference between ABA treated and not treated at the 0.05 significance level (Student's *t*-test). (C) Four-day-old seedlings were transferred from the regular ½ MS medium to a medium with −0.5 MPa PEG. The pictures were taken ten days after the transfer. (D) Relative lateral root lengths of the indicated seedling lines treated with or without PEG. Data are averages of two independent experiments with four seedlings for each line. * denotes a significant difference between *AFB2m* and *AFB2* with PEG treatment at the 0.05 significance level (Student's *t*-test).

miR393 has been shown to be induced by ABA and various stresses [11,18]. Although we did not detect a strong ABA or stress induction of miR393 in wild-type seedlings under our experimental conditions, elevated miR393 levels were clearly seen in *fry1* mutants, particularly after ABA treatment (Fig. 2B and C). This suggests that the ABA hypersensitivity of *fry1* may lead to sensitized ABA induction of this microRNA. Similarly, we detected reduced expression of miR393 in the ABA-insensitive mutant *abi1* (H. Chen and L. Xiong, unpublished). These data indicate that miR393 and its precursors may be under complex regulation by ABA and other stimuli (Fig. 2C).

As expected in plants with elevated miR393 levels, significantly more cleavage products of *AFB2* were accumulated in *fry1* (Fig. 2A). However, we failed to amplify any *AFB3* cleavage products using RNA from young seedlings. In floral tissues, cleavage products of *AFB2*, *AFB3*, and *TIR1* but not *AFB1* were detected [10], suggesting that tissue-specific cleavage events might occur. This kind of spatially regulated cleavage of *TIR1/AFB* transcripts likely plays a predominant role in controlling localized auxin responses. It also implies that only a fraction of the total *TIR/AFB* transcript pool is targeted by miR393 for cleavage. This perhaps explains why no significant difference in total steady-state *TIR1/AFB* transcript levels was found between wild-type and *fry1* homogenized seedlings (Fig. 2A).

Direct evidence for *in vivo* cleavage of auxin receptor mRNA by ABA-induced miR393 was also obtained using *AFB2:GUS* transgenic plants. Upon ABA treatment, *AFB2:GUS* staining was reduced, whereas *AFB2m:GUS* staining was not (Fig. 3B–D). We therefore suggest that ABA acts through promoting the cleavage of *AFB2* transcripts rather than repressing *AFB2* transcription, although we could not rule out the possibility that miR393 may additionally inhibit translation of the TIR1/AFB proteins.

ABA destabilization of the *TIR1/AFB* transcripts via miR393-guided cleavage may represent a key mechanism responsible for ABA regulation of root adaptation to drought stress. Whereas the native *AFB2* and *TIR1* transgenic seedlings responded to ABA and PEG similarly to wild-type plants, miR393-resistant *TIR1m* and *AFB2m* seedlings were insensitive to the inhibition of lateral root development caused by ABA and PEG treatment (Fig. 4). Thus, it is the miR393 resistance of the auxin receptors that is responsible for the ABA insensitivity in these plants. Although TIR1 has been shown to be the primary auxin receptor modulating lateral root growth [23,27], it is not unexpected that miR393-resistant *AFB2m* also exhibited ABA- and PEG-insensitive lateral root growth, since *TIR1/AFB* genes express in a largely overlapping pattern and show certain functional redundancy [23]. Consistent with this notion, both *TIR1m* and *AFB2m* transgenic seedlings still displayed certain ABA responsiveness in lateral root growth (Fig. 4). Taken together, our results demonstrate that ABA modulates root adaptation to drought stress at least partially through up-regulation of miR393-targeted cleavage of auxin receptor transcripts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet. 2012.05.013.

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