



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.

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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].

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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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

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 $\frac{1}{2}$ 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 CAC-CATTGATGTTCTCTAAAAACAA and GAATCCACACAAATGGCGGC-CATCC. *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 ATGCTTCATGTGAAGTC and AGACATCCACAACTCTCATTGTTTCA TACTTGCTCACA to generate *AFB2m::GUS* or with primers GCTGGA-GACAATGCGTAGTTTGTGGATGCTTCTTGTTCG and CCACAACTA 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 TCAACGGAGGAAAGT-CAAAAAC. 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/8M 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 GGATCCAAAGGGATCGCAT TG 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-chloro-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 $\frac{1}{2}$ 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,

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