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Regulation of auxin response by miR393-targeted *transport inhibitor response protein 1* is involved in normal development in *Arabidopsis*

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Abstract miR393, which is encoded by *MIR393a* and *MIR393b* in *Arabidopsis*, post-transcriptionally regulates mRNAs for the F-box auxin receptors TIR1 (Transport Inhibitor Response Protein 1), AFB1 (Auxin Signaling F-box Protein 1), AFB2 and AFB3. However, biological functions of the miR393-*TIR1/AFBs* module in auxin response and plant development is not fully understood. In the study herein, we demonstrate that miR393 accumulated in response to exogenous IAA treatment, and its induction was due to enhanced *MIR393b* transcription but not *MIR393a*. Overexpression of a miR393-resistant form of *TIR1* (*mTIR1*) enhanced auxin sensitivity and led to pleiotropic effects on plant development including inhibition of primary root growth, overproduction of lateral roots, altered leave phenotype and delayed flowering. Furthermore, miR393 level was increased in *35S:mTIR1* plant, suggesting that *TIR1* promoted the expression of miR393 by a feedback loop. The interaction between miR393 and its target indicates a fine adjustment to the roles of the miR393-*TIR1* module, which is required for auxin responses in plant development.

Keywords microRNAs · miR393 · *Transport inhibitor response 1* (*TIR1*) · Auxin response · Development · *Arabidopsis thaliana*

Introduction

The microRNAs (miRNAs) are endogenous ~21-nucleotide noncoding RNAs that target complementary mRNA transcripts for cleavage or transcriptional repression (Bartel and Bartel 2003; Bartel 2004; Carrington and Ambros 2003; Mallory and Vaucheret 2004). In plants, miRNAs have been demonstrated to play a crucial role in various biological processes including embryogenesis, flowering, leaf and root development, and plant responses to biotic and abiotic stresses (Llave 2004; Sunkar and Zhu 2004).

The identity of miRNA targets suggests that several miRNAs play a role in auxin signaling. miR393 targets auxin receptors TIR1 and three closely related F-box proteins (Jones-Rhoades and Bartel 2004). The transcripts of several *Arabidopsis* auxin response factors (ARFs) are either directly or indirectly regulated by miRNAs (Allen et al. 2005; Kasschau et al. 2003; Rhoades et al. 2002). These miRNAs involved in auxin signaling have been shown to modulate plant auxin responses and the expression of auxin-induced genes (Mallory et al. 2005; Wang et al. 2005).

miR393 is a conserved family that has been identified in many plants. In *Arabidopsis,* this family is encoded by two loci, *MIR393a* and *MIR393b* (Jones-Rhoades and Bartel 2004; Jones-Rhoades et al. 2006). Four F-box genes, *TIR1* (At3g62980), *AFB1* (At4g03190), *AFB2* (At3g26810), and *AFB3* (At1g12820), have been identified and validated as targets of miR393 (Jones-Rhoades and Bartel 2004; Navarro et al. 2006; Parry et al. 2009). The TIR1/AFBs

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constitute a small subset of F-box-containing auxin receptors, and function as a component of the Skp1-Cullin1-F-box protein (SCF) ubiquitin ligase complexes. TIR1/AFBs regulate auxin signaling by proteolysis of auxin/indole-3-acetic acids (Aux/IAA) repressors, and by releasing the activities of auxin response factors (ARFs) (Dharmasiri et al. 2005a; Kepinski and Leyser 2005; Ruegger et al. 1998). Previous studies have shown that mutations in *TIR1/AFBs* genes result in decreased auxin sensitivity in plants. However, only quadruple *tir1/afb* mutants exhibit a severe embryonic phenotype and a variety of growth defects, which indicates that these genes have overlapping functions, and collectively mediate auxin response during plant development (Dharmasiri et al. 2005b).

It has been shown that *TIR1, AFB2,* and *AFB3* are negatively regulated by miR393 in response to pathogen attack, and overexpression of miR393 results in decreased levels of *TIR1* mRNA and enhanced antibacterial resistance (Navarro et al. 2006). More recently, it was reported that the miR393/*AFB3* regulatory module controls root system architecture in response to external and internal nitrate availability in *Arabidopsis* (Vidal et al. 2010).

However, it is not clear whether miR393 is regulated by auxin. To gain insights into miR393 regulation by auxin, we investigated the expression of two *MIR393* genes for their responses to auxin treatments in *Arabidopsis* seedlings. Our results showed that exogenous IAA treatment induced miR393 accumulation resulted from enhanced *MIR393b* transcription. Overexpression of a miR393-resistant form of *TIR1* (*mTIR1*) enhanced auxin sensitivity and led to pleiotropic effects. In addition, overexpression of *mTIR1* could promote miR393 expression by a feedback loop. Our data suggested that the miR393-mediated regulation of *TIR1* contributed to regulate auxin responses in plant normal development.

Materials and methods

Plant material, growth conditions and treatments

Arabidopsis thaliana mutant *tir1-1* and the *tir1-1 afb1-1 afb2-1 afb3-1* quadruple mutant were kindly provided by Mark Estelle (Section of Cell and Developmental Biology, The University of California, San Diego). *dcl1-9* [CS3828] was obtained from the ABRC. Both wild-type (*Columbia-0, Ws* ecotype), mutants and transgenic plants seeds were surface sterilized with 70% ethanol and 10% bleach. Sterilized seeds were sown on B5-agar plates. Plates were vernalized in darkness for 2 days at 4°C and then transferred to a growth chamber at 22°C and 70% humidity under a 16-h-light/8-h-dark photoperiod. After 7–10 days, seedlings were potted in soil and placed under the same conditions.

For root elongation and lateral root assays, 5-day-old seedlings germinated on B5-agar plates were transferred onto new agar medium with or without hormone and grown vertically under constant light for designated times. Lateral root numbers were counted under a dissection microscope. Root elongation was measured using the ImageJ software.

Constructs and generation of transgenic plants

To generate the *35S:MIR393* constructs, a 778- and a 755-bp fragment surrounding the miRNA sequence including the fold-back structure were amplified from genomic DNA with the primers for *35S:MIR393a* and *35S:MIR393b*, respectively. The amplified fragments were sequenced and cloned downstream of the CaMV (Cauliflower mosaic virus) 35S promoter in pCAMBIA13011.

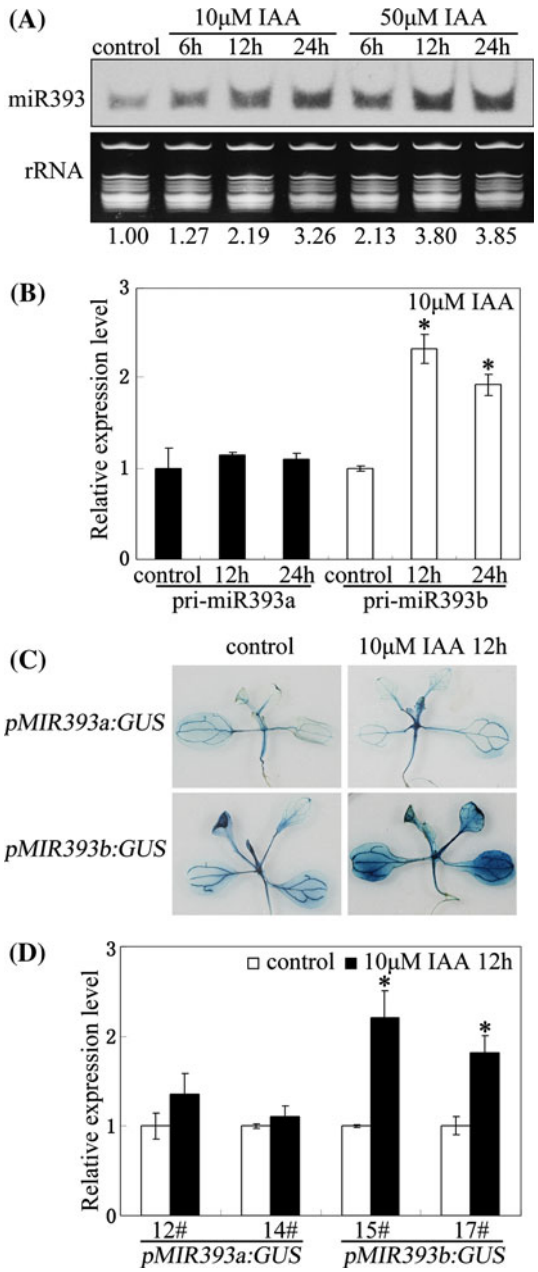
For the *promoter*:*GUS* constructs, 2.0- and 1.5-kb fragments upstream from the predicted fold-back of miR393 were amplified and sequenced. Fragments were double digested by *Pst*I/*Nco*I and substituted the 35S promoter before GUS exon in pCAMBIA1301 to obtain *pMIR393a*:*GUS* and *pMIR393b*:*GUS*, respectively. To generate the *35S*:*TIR1* constructs, the *TIR1* (At3g62980) coding sequence was amplified and cloned into the pCAMBIA13011 under a 35S promoter. For a miR393-resistant version of *TIR1* (*mTIR1*), mutations were introduced into the miR393 binding sequence by an overlapping PCR. The resulting product was digested and cloned into pCAMBIA13011.

All of the constructs described were electroporated into *Agrobacterium tumefaciens* GV3101, and used to transform *Arabidopsis* by the floral dip method (Clough and Bent 1998). Homozygous T3 seeds were used for further study.

All primers used in this work are listed in Supplementary Table 1. The clones used for vector construction were verified by sequencing.

Gene expression analysis

Total RNA was isolated from plant tissue using TRIzol reagent (Invitrogen, Shanghai, CHN), and treated with RNase-free DNaseI (TakaRa, Dalian, CHN). Treated RNA (1 μg) was used for the first-strand cDNA synthesis using PrimeScript RT Reagent Kit (TaKaRa). Real-time PCR was performed by the LightCycler 480 (Roche) with the SYBR Premix Ex Taq (Perfect Real Time) Kit (Takara). Primers of *TIR1* and *AFB1/2/3* were designed from each side of the miR393 cleavage site, and relative transcript levels were normalized using *UBQ5* as a standard. The primers used for qRT-PCR are also described in Supplemental Table 1.



◀**Fig. 1** Auxin treatment enhanced *MIR393b* expression. **a** RNA gel blot analysis of miR393 in response to exogenous IAA treatment. 10-day-old wild-type seedlings were transferred to liquid B5 medium containing IAA, and seedlings were harvested after 6, 12, and 24 h of treatment. 10 μg low molecular RNA was loaded and rRNA was stained by EtBr as loading control. *Numbers* indicate fold change relative to control sample. **b** qRT-PCR analysis of precursor transcripts of *MIR393* family members in response to exogenous IAA. Quantifications were normalized to the expression of *UBQ5*. The relative expression levels in control plants were set to 1.0. *Error bars* represent SD from three PCR results, and similar results were obtained in two independent experiments. *Asterisks* denote significantly different from the control plants ($P < 0.05$, Student's *t* test). **c** Response of *MIR393a* and *MIR393b* promoter:GUS to exogenous IAA treatment. 10-day-old homozygous transgenic seedlings grown on B5-agar plates were transferred to new agar medium with or without 10 μM IAA. Seedlings were stained for GUS activity after 12 h of treatment. **d** Quantification of *GUS* expression in response to IAA treatments in *pMIR393a:GUS* and *pMIR393b:GUS* transgenic plants. Two representative *transgenic lines* were analyzed. *Error bars* represent SD. *Asterisks* denote significantly different from the control *MIR393* promoter report lines ($P < 0.05$, Student's *t* test)

RNA blot analysis

RNA was extracted using TRIzol reagent (Invitrogen). Total RNA was separated on 1.1% formaldehyde-MOPS agarose gels. *TIR1* probe was randomly labeled with digoxigenin-dUTP (Roche) using a cDNA fragment spanning the miR393 cleavage site. For small RNAs, low molecular weight RNA was separated on 17% polyacrylamide gels under denaturing conditions (7 M urea). Blots were hybridized using either radioactively labeled or digoxigenin end-labeled locked nucleic acid (LNA) oligonucleotide probes with the sequence 5′-gAtcAatGcgAtcCctTtgGa-3′ (capital alphabets for LNA).

Histochemical detection of GUS activity

Histochemical localization of GUS staining was performed by incubating seedlings in a solution of 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 1 mM potassium ferricyanide, 0.1% Triton X-100, 0.1 M sodium phosphate buffer, pH 7.0 and 10 mM EDTA overnight at 37°C, followed by clearing with 70% ethanol.

Results

Auxin enhanced *MIR393b* expression

The miR393 levels in wild-type seedlings were determined to evaluate the effects of exogenous IAA treatments. Northern blot analyses showed that miR393 was induced at IAA concentrations of 10 and 50 μM. The increased level of miR393 was apparent after 12 h of exposure to 10 and 50 μM IAA, and the level continued to increase by approximately threefold after 24 h exposure (Fig. 1a).

MIR393a and *MIR393b* produce two identical mature miR393. To determine whether the responsiveness of *MIR393* to IAA is locus-specific, we measured precursor abundance of *MIR393a* and *MIR393b*. The precursor transcripts of *MIR393b*, but not those of *MIR393a*, were elevated under the IAA treatment (Fig. 1b). We also generated promoter:GUS transgenic lines with ~2-kb putative promoter sequences upstream of the predicted fold-back structure of *MIR393a* and *MIR393b*. In transgenic seedlings with no IAA treatment, GUS activity was observed in the shoot apical meristem, and vascular bundles of roots and leaves. After a 12 h treatment with 10 μM IAA, only *pMIR393b:GUS* transgenic seedlings showed an increase

of GUS intensity, especially in leaves and hypocotyls (Fig. 1c). qRT-PCR analysis of *GUS* expression revealed a twofold increase in two *pMIR393b:GUS* transgenic lines after 12 h of 10 μM IAA treatment (Fig. 1d). These data indicated that the increased level of miR393 primary transcripts after the IAA treatment most probably due to the enhanced transcription of *MIR393b*.

To elucidate the effect of miR393 accumulation, we also analyzed mRNA levels of *TIR1*, *AFB1*, *AFB2* and *AFB3* in *Arabidopsis* seedlings after exogenous IAA treatment. The results of real time RT-PCR did not show any remarkable changes in *TIR1/AFBs* transcripts after 12 h treatment with 10 μM IAA (Fig. 2a). Next, we examined the transcription of *TIR1/AFBs* using our previously described reporter lines *TIR1/AFBs* promoter:GUS (She et al. 2010). Quantitative GUS expression is shown in Fig. 2b. We found that the activities of the *TIR1* and *AFB1* promoters were elevated about 1.6-fold after 10 μM IAA treatment, indicating exogenous IAA increased transcription of *TIR1* and *AFB1*. GUS staining also showed that exogenous IAA was able to transcriptionally induce expression of *TIR1* in roots of *pTIR1:GUS* seedlings after 0.5 and 6 h treatments (Fig. 2c). According to these data, we proposed that the posttranscriptional repression of *TIR1* by miR393 might contribute to the homeostasis of *TIR1* mRNA in response to a high concentration of IAA treatment.

Interaction of miR393 and *TIR1* modulates plant auxin sensitivity

To investigate the role of miR393, we generated transgenic *Arabidopsis* plants harboring constructs of *35S:MIR393*, 35S:*TIR1* and *35S:mTIR1* (a miR393-resistant form of *TIR1*). Northern blot data showed more than threefold increases of miR393 accumulation in these transgenic *35S:MIR393a* and *35S:MIR393b* lines (Fig. 3a). Using qRT-PCR analysis, we detected moderate decrease of the *TIR1* F-box family mRNA levels in all of these lines, which indicated the same effects of *At-MIR393a* and *At-MIR393b* overproduction (Fig. 3b). The magnitude of the decrease in their mRNA levels varied among different F-box genes, and *AFB1* exhibited a partial resistance to miR393. For the *35S:mTIR1* construct, we introduced six mutations into the miR393 target site in the *TIR1* coding sequence without alteration of the corresponding amino acid sequence ET-MRSLW (Fig. 3c). Northern blot analysis revealed a significant increase of *TIR1* mRNA abundance in *35S:TIR1* and a further doubling of the mRNA levels in *35S:mTIR1* (Fig. 3d). An introduced point mutation in *mTIR1* also created an *Nsb*I restriction site, which allowed us to distinguish RT-PCR products of *mTIR1* transcripts from those of *TIR1* (Supplemental Fig. 1). Our results suggested that release of *mTIR1* transcripts from miR393 cleavage

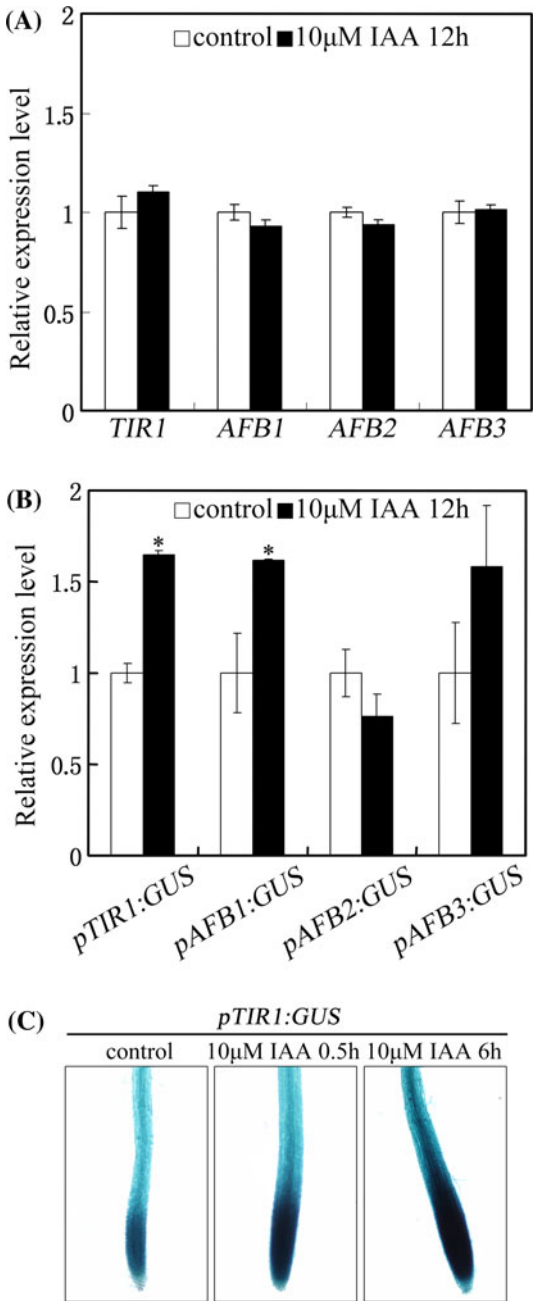


Fig. 2 miR393 posttranscriptionally regulates *TIR1/AFBs* transcripts in response to exogenous IAA. **a** qRT-PCR analysis of *TIR1/AFBs* transcripts in response to auxin treatments. 10-day-old transgenic seedlings grown on B5-agar plates were treated with 10 μM IAA for 12 h. Quantifications were normalized to the expression of *UBQ5*. The relative expression levels in control plants were set to 1.0. *Error bars* represent SD from three PCR results, and similar results were obtained in two independent experiments. **b** Quantification of *GUS* expression in response to IAA treatments in *pTIR1:GUS*, *pAFB1:-GUS*, *pAFB2:GUS*, and *pAFB3:GUS* transgenic plants. 10-day-old homozygous transgenic seedlings grown on B5-agar plates were transferred to new agar medium with or without 10 μM IAA. *Error bars* represent SD from three PCR results. **c** Response of *TIR1* promoter:GUS to exogenous IAA treatment. Seedlings were stained for GUS activity after 0.5 or 6 h of 10 μM IAA treatment

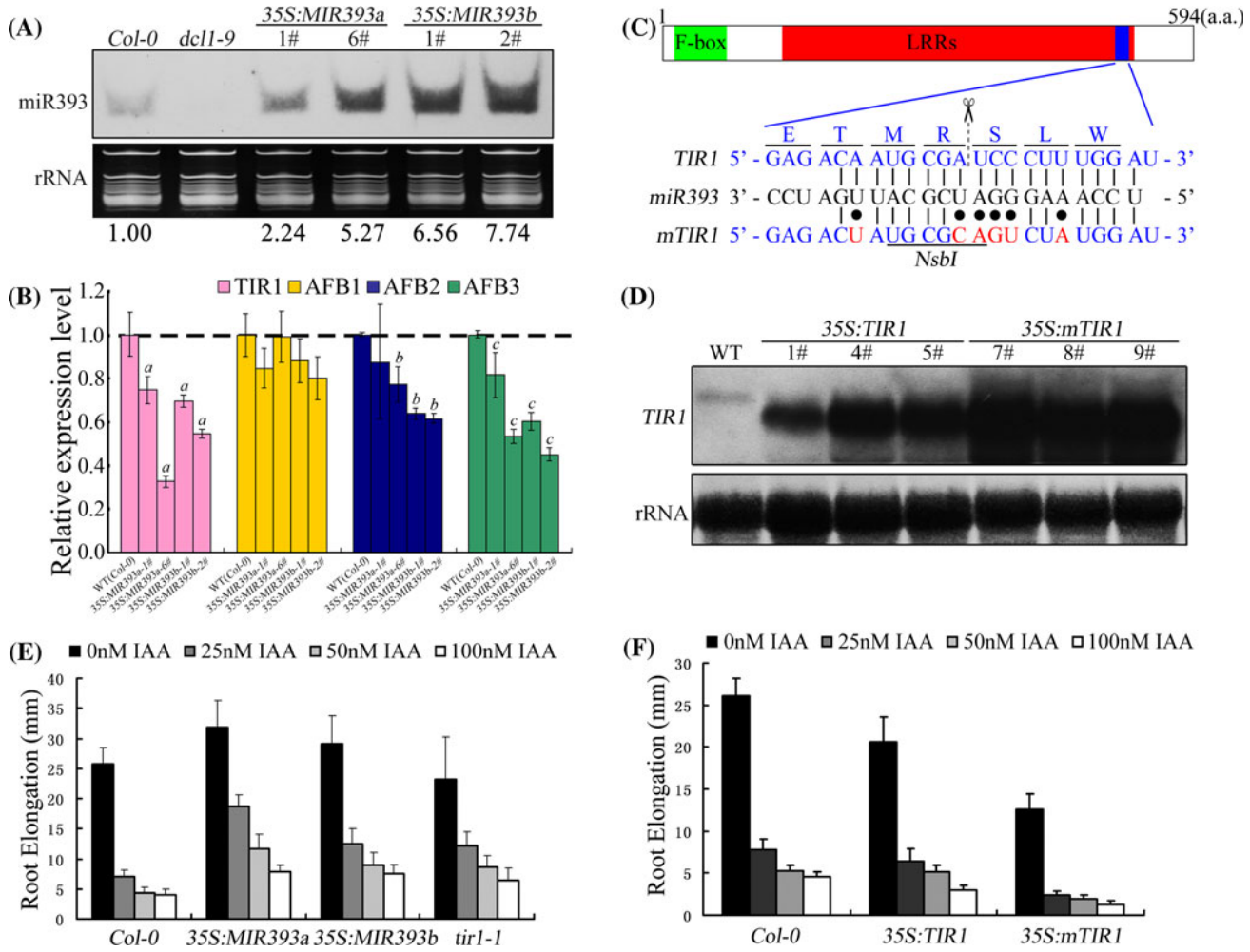


Fig. 3 Altered *TIR1* levels mediated by miR393 regulate plant auxin sensitivity. **a** Overexpression of miR393 in transgenic *Arabidopsis* plants. RNA gel blot analysis of miR393 levels in the wild-type and two representative *transgenic lines*. The blots also included RNA from the *dcl1-9* mutant as a negative control. **b** Detection of *TIR1/AFBs* transcripts in *35S:MIR393* transgenic plants by real-time RT-PCR. Quantifications were normalized to the expression of *UBQ5*. The relative expression levels in wild-type plants were set to 1.0. *Error bars* represent SD from three PCR results, and similar results were obtained in two independent experiments. *a*, *b*, and *c* denote significantly different from wild-type *Col-0* ($P < 0.05$, Student's *t* test) for *TIR1*, *AFB2* and *AFB3*, respectively. **c** Diagram of *mTIR1* expression constructs. The introduced point mutations in the miR393 target site of *TIR1* are shown. Introduced point mutation in the miR393 complementary sequence creates an *Nsb*I site and is *underlined*. **d** RNA gel blot analysis of *TIR1* mRNA. 40 μg total RNA extracted from *35S:TIR1*, *35S:mTIR1* and wild-type plants were loaded and rRNAs on blot membrane were visualized by methylene blue trihydrate staining as a loading control. **e** Effects of decreased *TIR1* levels on plant auxin sensitivity. 5-day-old seedlings on B5-agar plates were transferred to new agar medium containing IAA. Root length was measured after 5 days growth. *Error bars* represent SD. There are significant differences between wild-type and *transgenic lines* as well as *tir1-1* under 0, 25, 50 and 100 nM IAA treatments, as determined by ANOVA ($P < 0.05$). **f** Effects of increased *TIR1* levels on plant auxin sensitivity. Root length was measured after 5 days growth. *Error bars* represent SD. For *35S:mTIR1*, there are significant differences from wild-type at all IAA concentration points ($P < 0.01$). For *35S:TIR1*, only at 25 and 100 nM IAA ($P < 0.05$), as determined by ANOVA

resulted in over-accumulation of the full-length transcripts, and confirmed that *TIR1* mRNA is under negative regulation of miR393.

To assess auxin responses, we determined the effect of exogenous IAA on root elongation. 5-day-old seedlings were transferred onto new B5-agar plates containing different concentrations of IAA, and primary root length was measured after 5 days. Both *35S:MIR393* and *tir1-1* displayed reduced inhibition of primary root growth compared to the control (Fig. 3e). Opposite results have been obtained with *35S:TIR1* and *35S:mTIR1* seedlings. Root elongation of *35S:TIR1* plants were slightly shorter than wild-type under 25 and 50 nM IAA, but became significantly shorter compared with *Col-0* under 100 nM IAA condition ($P < 0.05$, ANOVA) (Fig. 3f). Further, *35S:mTIR1* plants displayed an additive increase in auxin sensitivity compared to *35S:TIR1*, so that root elongation was inhibited strongly even under low concentration of 25 nM IAA. These results

demonstrated that overexpression of miR393 led to decreased *TIR1* levels and reduced plant auxin sensitivity, while overexpression of *mTIR1* enhanced auxin sensitivity. These data indicated that the balance between miR393 and *TIR1* expression regulated auxin responses.

Altered *TIR1* levels mediated by miR393 affect early auxin-responsive gene expression

It has been well known that primary auxin-response gene transcription can be activated by exogenous IAA (Hagen and Guilfoyle 2002). The changes of auxin sensitivity regulated by the miR393-*TIR1* module prompted us to determine whether these transgenic lines altered the expression of auxin-responsive genes. We used qRT-PCR to monitor the transcripts of three primary auxin-response genes *DFL1/GH3.6* (At5g54510), *AXR5/IAA1* (At4g14560) and *MSG2/IAA19* (At3g15440). The basal expression of all of these genes were slightly repressed in miR393 overexpression lines, and further repressed in *tir1-1* and *tir1/afb* quadruple seedlings. In contrast, we observed slightly increased transcripts of these genes in *35S:TIR1* lines and a further increase in *35S:mTIR1* (Fig. 4a–c, black columns).

To reveal the effect of the miR393-*TIR1* regulatory module on auxin response, we tested the expression levels of these genes under exogenous IAA treatment. After 12 h treatment with 10 μM IAA, these genes exhibited substantial changes in transcript abundance in all of these seedlings compared with their own control samples respectively. It showed that *35S:MIR393* inhibited the upregulation of the three auxin responsive genes compared with wild-type, while *35S:TIR1* and *35S:mTIR1* enhanced the expression of these genes when exogenous IAA was applied (white columns). These data suggested that miR393-mediated *TIR1* regulation influenced the expression of some primary auxin-responsive genes, which contributed to the alteration of plant auxin sensitivity.

Ectopic expression of miR393-resistant *TIR1* causes pleiotropic effects

To determine the in vivo consequences of disrupting miR393 regulation, phenotypic analysis was performed in *35S:mTIR1* transgenic plants. Ectopic overexpression of *mTIR1* led to pleiotropic effects including shorter primary roots, more lateral roots, altered leaf phenotype and delayed flowering time, while overexpression of wild-type *TIR1* had mild effects on morphology.

We measured the primary root length of 5-day-old transgenic *35S:MIR393*, *35S:TIR1* and *35S:mTIR1* seedlings and the lateral root numbers of 10-day-old transgenic seedlings under long day conditions on vertically oriented

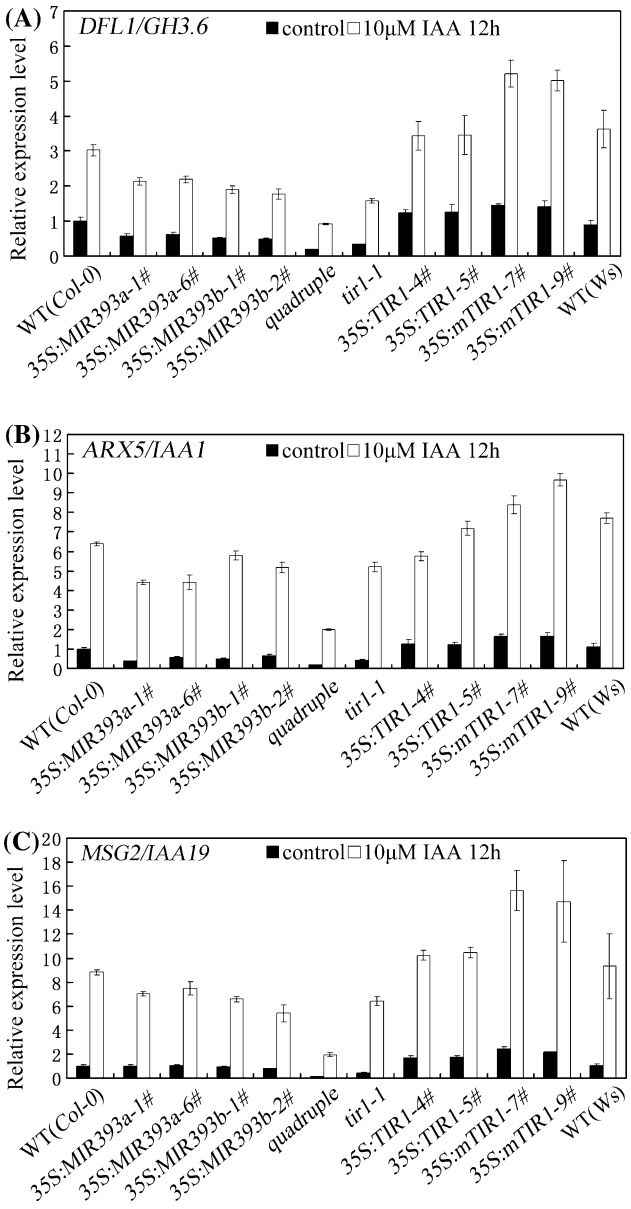


Fig. 4 Transcript levels of three primary auxin-responsive genes in response to exogenous IAA. Relative mRNA levels of *GH3.6* **a**, *IAA1* **b** and *IAA19* **c** were measured by qRT-PCR. 10-day-old seedlings grown on B5-agar plates were transferred to B5 liquid medium containing 10 μM IAA for 12 h. Quantifications were normalized to the expression of *UBQ5* and the relative mRNA levels in control *Col-0* plants were set to 1.0. *Error bars* represent SD from three PCR results, and similar results were obtained in two independent experiments

plates. It showed that transgenic *35S:TIR1* and *35S:mTIR1* seedlings exhibited phenotypes opposite to those of *35S:MIR393* lines, as well as *tir1-1* lines. Compared with wild-type plants, *35S:MIR393* seedlings displayed slightly longer primary root and fewer lateral roots, while *35S:TIR1* seedlings had shorter primary roots and more lateral roots (Table 1, Fig. 5). Furthermore, *35S:mTIR1* exhibited more

Table 1 Quantitative effects of altered *TIR1* levels mediated by miR393 on root growth

Genotype	Primary root length (mm)[a]	Lateral root numbers[b]
Wild-type (*Col-0*)	8.1 ± 0.7	6.2 ± 1.4
tir1-1	7.8 ± 1.6	1.9 ± 0.9*
35S:miR393a-1	9.7 ± 1.2*	5.0 ± 1.7
35S:miR393a-6	9.8 ± 1.7*	3.8 ± 1.3*
35S:miR393b-1	7.8 ± 2.0	5.1 ± 1.6
35S:miR393b-2	8.6 ± 1.3	3.1 ± 1.3*
35S:TIR1-4	6.9 ± 1.4*	14.7 ± 2.7*
35S:TIR1-5	6.6 ± 0.8*	16.8 ± 5.2*
35S:mTIR1-7	5.1 ± 1.4*	25.4 ± 4.9*
35S:mTIR1-9	3.9 ± 0.4*	31.3 ± 5.9*

* Significantly different from wild-type (ANOVA, $P < 0.05$). Data are mean ± SD, $n = 15$

[a] Primary root length (mm) per plant of 5-day-old seedlings grown under continuous light

[b] Lateral root numbers per plant of 10-day-old seedlings grown under continuous light

dramatic changes in both average root length and lateral root number than *35S:TIR1*. Along with the observation that *35S:mTIR1* accumulated more *TIR1* transcripts than *35S:TIR1*, our results provided evidence that regulation of *TIR1* mRNA levels by miR393 is an important control point for primary root elongation and lateral root initiation.

We also showed that 5-day-old *35S:TIR1* seedlings displayed downward cotyledons and upward petioles, while cotyledons in *35S:mTIR1* were more severely curled (Fig. 6a–c). 10-day-old *35S:mTIR1* but not *35S:TIR1* plants produced downward curly true leaves with long and twisted petioles (Fig. 6d–f). 4-week-old *35S:mTIR1* plants displayed extremely curled leaves with margins bending to the abaxial surface. Furthermore, the rosette leaves from *35S:mTIR1* were much smaller in size and fewer in number than that of wild-type plants, while *35S:TIR1* plants had normal rosette leaves (Fig. 6g–j). After 8-weeks growth, *35S:mTIR1* plants displayed an obvious delay in bolting and flowering time, whereas the *35S:TIR1* seedlings displayed no developmental anomalies except for reduced silique numbers (Fig. 6k). Moreover, we found that *35S:mTIR1* plants exhibited strong apical dominance, with only one shorter shoot observed in all transgenic lines. Meanwhile, the plant vegetative phase was prolonged in *35S:mTIR1* lines (Fig. 6k).

In *35S:MIR393* plants, no other changes of development and growth were observed, except that the time producing the first leaf with the serrate margins was slightly delayed (Supplemental Fig. 2).

Possible feedback regulation of miR393 expression

Feedback loops in which miRNA-regulated genes regulate the transcription of their miRNA have been described in a number of animals and plants (Kim et al. 2007; Varghese and Cohen 2007; Wu et al. 2009). In order to determine whether miR393 is regulated by its target *TIR1*, we analyzed the effect of *TIR1* level on accumulation of mature miR393 using *35S:TIR1*, *35S:mTIR1*, *tir1-1* and *tir/afb* quadruple mutants. The level of miR393 was elevated about 1.5-fold in 10-day-old *35S:mTIR1* but not in *35S:TIR1* plants. Conversely, it was reduced about 30% in *tir1-1* and *tir/afb* quadruple mutants (Fig. 7). These data demonstrated that miR393 was positively regulated by the level of *TIR1*, and the expression of *TIR1* was modulated by a negative feedback loop.

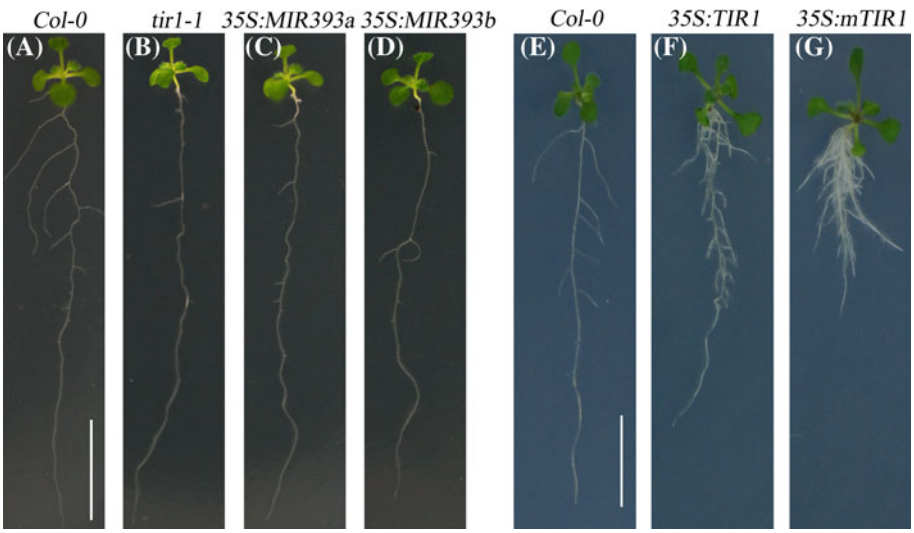


Fig. 5 Ectopic expression of miR393-resistant *TIR1* promotes lateral root production. 10-day-old wild-type *Col-0* **a**, **e**, *tir1-1* mutant **b**, transgenic *35S:MIR393a* **c**, *35S:MIR393b* **d**, *35S:TIR1* **f** and *35S:mTIR1* **g** plants grown on B5-agar plates were photographed. *Scale bar* = 10 mm

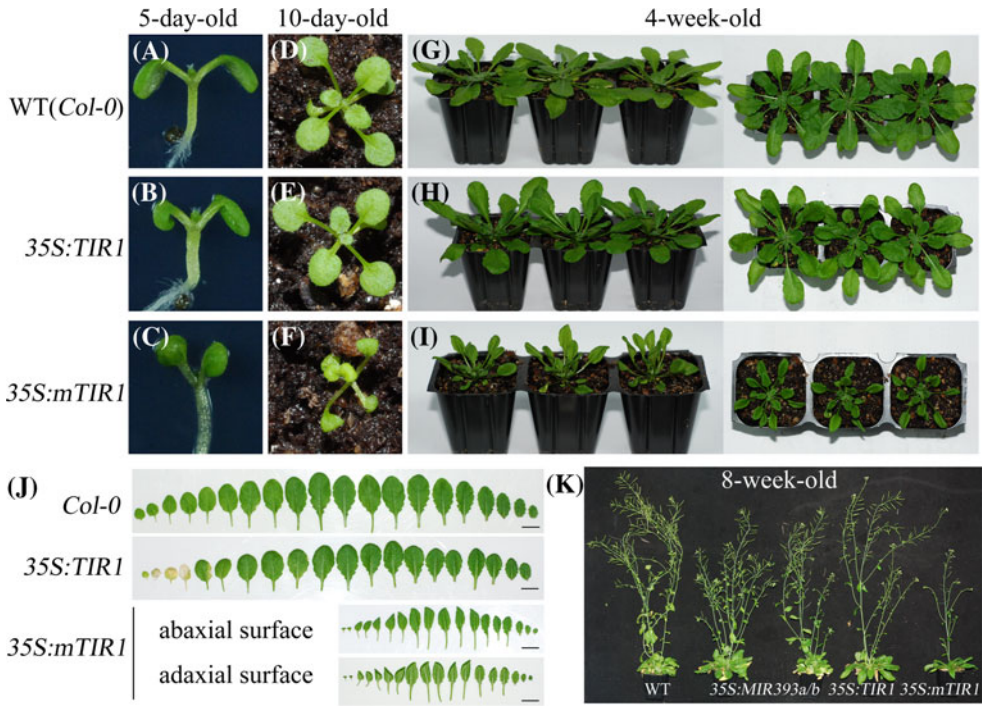


Fig. 6 Ectopic expression of miR393-resistant *TIR1* causes pleiotropic effects. **a–c** 5-day-old *35S:mTIR1* transgenic plants displayed downward cotyledons and upward petioles. Seedlings of wild-type, *35S:TIR1* and *35S:mTIR1* plants grown on B5-agar plates were photographed. **d–f** 10-day-old *35S:mTIR1* plants displayed downward curly true leaves with long and twisted petioles. Wild-type, *35S:TIR1* and *35S:mTIR1* plants grown in pots were photographed. **g–i** The rosette leaves from 4-week-old *35S:mTIR1* were much smaller in size and fewer in number. Wild-type, *35S:TIR1* and *35S:mTIR1* transgenic plants grown under short-day conditions were photographed with side view (*left*) and top view (*right*). **j** Margins of *35S:mTIR1* rosette leaves were bending toward the abaxial surface. Leaves from 4-week-old wild-type, *35S:TIR1* and *35S:mTIR1* transgenic plants were cut and arranged in the order of appearance. Both sides of *35S:mTIR1* leaves are shown. *Scale bar* = 1 cm. **k** 8-week-old *35S:mTIR1* plants exhibited strong apical dominance

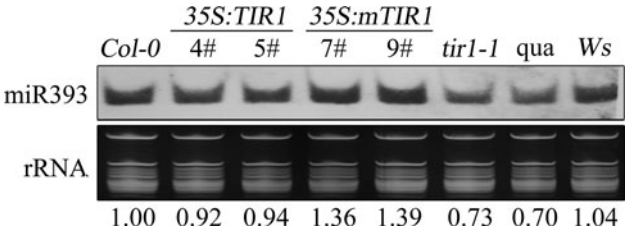


Fig. 7 Regulation of miR393 expression by *TIR1*. Small RNA gel blot analysis of miR393 in the wild-type *Col-0, Ws*, *35S:TIR1*, *35S:mTIR1 transgenic lines* and *tir1-1*, *tir/afb* quadruple mutants. *Numbers* indicate the fold changes relative to *Col-0*

Discussion

Auxin regulation of miR393 expression

Several plant miRNAs have been predicted to regulate mRNAs involved in auxin signaling pathway. To date, however, only miR164 and miR390 have been reported to response to IAA (Guo et al. 2005; Marin et al. 2010; Yoon et al. 2010). In this work, we show that miR393 in *Arabidopsis* seedlings can be induced by exogenous IAA application. Northern blot analysis revealed a 2- to threefold increase of miR393 accumulation after 12 h IAA exposure. Analysis of precursor transcripts and promoter:GUS reporter lines confirmed that miR393 accumulation could be controlled at the transcriptional level. Unlike most auxin-responsive genes, which can rapidly regulate their transcription after a short period of treatment, the level of miR393 increased only at high IAA concentration and a long time course. The slower kinetics of miR393 induction may create a homeostatic mechanism to maintain the transcripts of *TIR1/AFBs* in response to high concentration of IAA treatment.

Another feature of miR393 accumulation in response to auxin is the locus-specific control of its transcription. There are two *MIR393* loci: *MIR393a* (At2g39885) and *MIR393b* (At3g55734), which produce two identical mature miR393. Recent studies provided evidence that closely related miRNAs, which are predicted to target the same genes, have in fact different functions during development or stress responses (Li et al. 2008; Sieber et al. 2007). Previous studies showed that a flagellin-derived peptide induces miR393 accumulation, which results from increased transcription of *MIR393a* (Navarro et al. 2006). Our results indicated that *MIR393b* specifically induced

their transcription in response to exogenous auxin treatment. Since the expression of the *MIR393* family was under the control of their corresponding promoters, we proposed that the locus-specific control of *MIR393* transcription can provide an additional layer of regulation for the auxin signaling network through repressing target gene expression.

The interaction between miR393 and *TIR1*

According to the results of 5′-RACE assay, four F-box genes, *TIR1*, *AFB1*, *AFB2*, as well as *AFB3* have been validated to be the targets of miR393 (Jones-Rhoades and Bartel 2004; Navarro et al. 2006). In the present study herein, we showed that overexpression of miR393 decreased *TIR1* mRNA level, while miR393 resistant form of *TIR1* (*mTIR1*) resulted in over-accumulation of the *mTIR1* transcripts. These data further confirmed that *TIR1* mRNA is under negative regulation by miR393.

The regulation of *TIR1* mediated by miR393 can also be observed during plant development. Using promoter:GUS analysis, it has been found that *TIR1, AFB2*, and *AFB3* are broadly transcribed throughout the plant, while their corresponding GUS fusion proteins are restricted to the primary and lateral root tips, young leaves and young flower buds, indicating significant posttranscriptional regulation of these genes (Dharmasiri et al. 2005b; Parry et al. 2009). As a prime candidate for this regulation, it is reasonable to propose that a developmentally based regulation of miR393 production, which in turn controls the level of its target gene transcripts, is required for the auxin-related developmental processes.

Negative and positive feedback loops of miRNA/target regulons have been described for the miRNA homeostasis regulation in plants (Marin et al. 2010; Rajagopalan et al. 2006; Wu et al. 2009). In *Arabidopsis*, miR172b and miR156a are also found to be positively regulated by the their targets (Wu et al. 2009). Interestingly, we also observed that the accumulation of miR393 was increased in *35S:mTIR1* plants, and was reduced in *tir1-1* and *tir/afb* quadruple mutants (Fig. 7), though the related mechanism was unclear. We tested the TIR1 transcript levels in *35S:TIR1*, *35S:mTIR1* and *tir1-1* plants and found that the *TIR1* level in *tir1-1* plants was not obviously different from the level in wild-type (Supplementary Fig. 3). According to the previous reports, the *tir1-1* mutant, which is caused by a glycine to aspartate substitution at position 147, is affected in auxin response (Ruegger et al. 1998). We proposed that the change of miR393 level might be due to deficient function of TIR1 protein.

Function of the miR393-*TIR1* regulatory module

Auxin is a vital hormone that regulates many aspects of plant development. Individual cells interpret auxin largely by a signaling pathway that involves the F-box protein TIR1 as an auxin receptor. Auxin-dependent TIR1 activity leads to ubiquitination-based degradation of Aux/IAAs, and triggers predefined changes in developmental programs (Vanneste and Friml 2009).

It is well known that auxin can inhibit root growth and promote lateral root production. We analyzed the effect of overexpressing miR393 and miR393-resistant *mTIR1* on root development. *35S:MIR393* seedlings displayed slightly longer primary root and fewer lateral roots, while *35S:mTIR1* and *35S:TIR1* seedlings had shorter primary roots and more lateral roots. These data suggested miR393-mediated *TIR1* regulation was critical for plant root development.

In addition to the root phenotypes, we did not observe apparent developmental defects in our *35S:MIR393* plants, which were exhibited by *tir1/afb* mutants. Similar results have been reported by other researchers (Jones-Rhoades and Bartel 2004; Navarro et al. 2006). Our further analysis revealed that overexpression of miR393 only decreased *TIR1*, *AFB2* and *AFB3* transcripts, but not *AFB1*. A plausible explanation is that *AFB1*, one member of the auxin receptor F-box family, is partially resistant to miR393-guided negative regulation (Navarro et al. 2006). Since the *TIR1/AFBs* function in a redundant fashion to mediate auxin response, it is not surprising to find that *35S:MIR393* plants displayed no dramatic defects in development.

On the other hand, plants expressing a miR393-resistant version of *TIR1* had dramatically increased *TIR1* mRNA levels and altered accumulation of many auxin responsive genes when compared with *35S:TIR1*. These expression changes were correlated with developmental abnormalities such as downward curly leaves, dwarfed stature, as well as growth retardation. Our results differ from a previous report describing the expression of a *mTIR1* with four mutations, which has no effect on the pattern of GUS staining and plant phenotypes. However, no convincing data had been shown to explain the same effect of *pTIR1:mTIR1-GUS* as *pTIR1:TIR1-GUS* (Parry et al. 2009). In order to avoid the effect of constitutively expressed promoter, we have also generated the *pTIR1:mTIR1* (in *tir1-1* background) transgenic lines and have got 10 independent T1 transgenic plants. We found that 6 of them displayed the pleiotropic developmental defects, including downward curled leaves and upward petioles (supplemental Fig 4).

Furthermore, our *35S:mTIR1* plants phenocopy the artificial target mimics plants (MIM 393) reported by Weigel Lab (Todesco et al. 2010). In plant, miRNA target

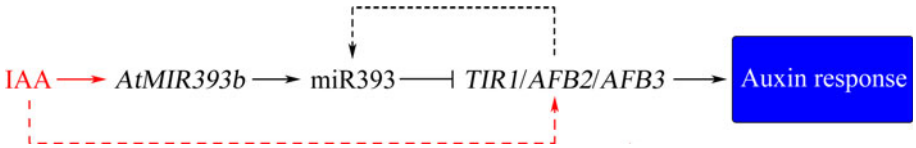


Fig. 8 The model for the regulation of auxin response by miR393/*TIR1*. The schematic diagram illustrates that auxin triggers a transcriptional induction of *AtMIR393b*, leading to increased miR393 levels. The posttranscriptional repression of *TIR1/AFBs* genes by miR393 alters plant auxin responses. The possible feedback regulation between TIR1 and miR393 expression provides a homeostatic model in which miR393 and *TIR1* abundance are tightly regulated

mimicry is an endogenous mechanism used to negatively regulate the activity of a specific miRNA family, through the production of a false target transcript that can not be cleaved (Todesco et al. 2010). Up to now, this technique has been successfully exploited to the study of miRNA functions (Gou et al. 2011; Wang et al. 2008, 2009; Wu et al. 2009). These data further indicates the important role of miR393 in plant development.

In contrast, no obvious phenotypic changes were observed in the *35S:TIR1* transgenic lines, though the mRNA level of *TIR1* was 7–30-fold higher than that of wild-type (Supplementary Table 2). This is probably because transgene-derived *TIR1* mRNAs are still repressed by relative abundant endogenous miR393. Our results implied that disrupting miR393-directed *TIR1* regulation rather than an extra copy of *TIR1* gave rise to severe developmental consequences in *35S:mTIR1* lines. In order to explain the pleiotropic effects in *35S:mTIR1*, we examined the genome-wide response in 10-day-old seedlings by Affymetrix ATH1 microarrays. Using a per-gene variance of $\log_2$ (fold change) >1 and FDR (Q value) <0.01, we found 291 genes were upregulated and 72 genes were downregulated (Supplemental Table 3). These data demonstrated that ectopic expression of a miR393-resistant *TIR1* resulted in the collective misregulation of many downstream genes, suggesting the function of the miR393-*TIR1* module is critical for interpreting local auxin signals which are required for proper plant development.

In summary, we propose a model for the regulation of auxin response by miR393/*TIR1* (Fig. 8). Auxin triggers a transcriptional induction of *AtMIR393b*, leading to increased miR393 level. The posttranscriptional repression of *TIR1/AFBs* genes by miR393 alters plant auxin responses. Meanwhile, high level of *TIR1* promotes miR393 expression by a feedback loop. The specific regulation of *MIR393* expression and the interaction between miR393 and its target indicates a fine adjustment to the roles of the miR393-*TIR1* module, which is required for auxin responses in plant development. Elucidation of the mechanism of the miR393-*TIR1* regulatory module and their eventual functions is still an important task for future research.

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