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# Auxin regulation of the *microRNA390*-dependent transacting small interfering RNA pathway in *Arabidopsis* lateral root development

**Eun Kyung Yoon[1], Ji Hyun Yang[1], Jun Lim[2], Soo Hwan Kim[3], Seong-Ki Kim[4] and Woo Sung Lee[1,*]**

[1]Department of Biological Science and the Basic Science Research Institute, Chun-chun Dong 300, Jang-An Gu, Sungkyunkwan University, Suwon, 440-746, [2]Department of Bioscience and Biotechnology, Konkuk University, Seoul, 143-701, [3]Department of Biological Science, Yonsei University, Wonju, 220-710 and [4]Department of Life Science, Chung-Ang University, Seoul, Korea, 156-756

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## ABSTRACT

***MicroRNA* (*miR*)*390* cleaves the non-coding *TAS3* precursor RNA for the production of *tasiRNA-ARF*, a group of an endogenous *trans*-acting small-interfering RNAs which cleave the transcripts of *auxin response factor (ARF) 3/4. miR390*-cleaved *TAS3* RNA is polymerized and diced into *tasiRNA-ARF* by RNA-dependent RNA polymerase6 (RDR6) and Dicer-like4 (DCL4), respectively. *tasiRNA-ARF*-dependent post-transcriptional gene silencing (PTGS) of *ARF3/4* is involved in auxin-mediated polarity establishment in the development of aerial lateral organs, such as leaf and flower. To understand how auxin regulates *ARF4* expression, we examined auxin responsiveness of *miR390* expression, which comprises a regulatory step for the biogenesis pathway of *tasiRNA-ARF* (the *tasiRNA-ARF* pathway), in *Arabidopsis thaliana* lateral root (LR) development. The results of this study provide evidence that *miR390* expression is sensitive to TIR1-dependent transcriptional regulation and auxin concentration, and also that mutual negative-regulation between the *tasiRNA-ARF* pathway and *ARF4* modulates the spatiotemporal expression of *ARF4*. We propose that, together with auxin concentration sensing through *miR390* transcription, the *tasiRNA-ARF* pathway mediates the auxin response and ARF4-mediated LR developmental processes.**

## INTRODUCTION

During the development of multicellular organisms, cells detect local concentrations of pattern-forming substances within a concentration gradient and respond by exhibiting the corresponding developmental fates. In plants, the phytohormone auxin is considered to be a pattern-forming substance which participates in many aspects of plant development, including morphogenic triggering of lateral organs. Various lateral organs appear to adopt a similar pattern of auxin distribution with high concentrations at the apexes of the respective primordia (1–3). However, our knowledge of how auxin concentrations are translated into pattern formation is rudimentary.

Once delivered into target cells, auxin binds to the F-box protein TIR to initiate its downstream signaling pathway (4,5), although, formally, alternative auxin signaling pathways may also exist. Auxin signaling employs two classes of antagonistic regulatory transcription factors: auxin/indole-3-acetic acid (AUX/IAA) and auxin response factor (ARF) (6,7). There are 23 known *ARFs* in *Arabidopsis*, and this diversity suggests that they have roles in different tissues and/or developmental stages (8). Similarly, AUX/IAA forms a large family with 29 members (9). When auxin levels are low, AUX/IAA represses ARF activity by directly binding to ARF. Conversely, when auxin levels are high, degradation of AUX/IAA is induced and the released ARF becomes active in transcriptional control (10,11). Direct auxin binding causes the AUX/IAA protein to interact with $SCF^{TIR}$, a member of an E3 ubiquitine ligase complex, inducing degradation (12,13).

*To whom correspondence should be addressed. Tel: +82 31 290 7004; Fax: +82 31 290 7015; Email: wslee@yurim.skku.ac.kr

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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In the cambium cells of the poplar tree, the distribution of AUX/IAA depends on auxin concentration, suggesting that the *AUX/IAA* genes have roles in interpreting auxin concentration (14). Among the 23 family members of ARFs in *Arabidopsis*, *ARF3* (*ETT*) and its close homologue *ARF4* (*ARF3/4*) are known to be transcriptional repressors (11), and are implicated in pattern formation in gynoecium (15) and leaf primordium development (1,16,17). As abaxial determinants, ARF3/4 have been suggested to be mediators for auxin signaling to partition adaxial and abaxial domains during leaf primordium formation (17). It has thus been hypothesized that ARF3/4 interprets auxin concentrations in the leaf.

A unique feature of *ARF3/4* regulation is that its transcripts are post-transcriptionally cleaved by *tasiRNA-ARF*, an endogenous transacting small-interfering RNA (18–21). Biogenesis of *tasiRNA-ARF* (the *tasiRNA-ARF* pathway) is initiated by cleavage of the protein-noncoding *TAS3* RNA by *miR390*. The *miR390* gene in *Arabidopsis* comprises two family members: *miR390a* and *miR390b* (22). The *miR390*-cleaved transcript bound to Argonaute7 (AGO7) (22) is used as a template for polymerization by RNA-dependent RNA polymerase6 (RDR6). The resulting double-stranded RNA is cleaved in phase by Dicer-like4 (DCL4) to generate 21-nucleotide-long *tasiRNA-ARF*. Defects in this pathway cause phenotypic abnormalities in flower gynoecium formation (15) and leaf heteroblasty (23), suggesting that regulation of *tasiRNA-ARF*-dependent *ARF3/4* expression plays a role in the development of these lateral organs. Furthermore, sided adaxial localization of *tasiRNA-ARF* correlates with the restriction of *ARF3/4* localization in the abaxial domain during leaf development in *Arabidopsis* (17,23–25). The degree of conservation observed in land plants suggests that the *tasiRNA-ARF* pathway plays a fundamental role in plant development (26). It was recently demonstrated that *tasiRNA-ARF* forms a concentration gradient across the adaxial (higher level) and abaxial domains (lower level) during leaf development in *Arabidopsis* (27). *tasiRNA-ARF* was suggested to be mobile by demonstrating that the localization of *tasiRNA-ARF* is different from the place of its biogenesis. In contrast to the gradient distribution of *tasiRNA-ARF* and broad distribution of *miR390*, *ARF3* expression occurs in the abaxial domain with a sharp boundary (28). Although the molecular mechanism underlying the boundary formation of *ARF3* is not understood, a connection to *tasiRNA-ARF* has been suggested (27,28). It was recently shown that *miR390* is the restrictive factor for the *tasiRNA-ARF* accumulation in maize shoot (29,30). Based on the roles of *tasiRNA-ARF* in auxin-triggered morphogenesis in lateral organs, we hypothesized that the *tasiRNA-ARF* pathway interacts with auxin signaling. To determine how auxin signaling and the *tasiRNA-ARF* pathway might interact, we used lateral root (LR) development in *Arabidopsis* as a model system. Auxin is known to be the primary trigger of LR development, but the *tasiRNA-ARF* pathway has not been reported to act in LR development. Auxin accumulates in the founder cells adjacent to the xylem pole prior to initiation of anticlinal and periclinal cell divisions of these cells to form primordia. Following the formation of early primordia, autonomous meristems of three to five cell layers are established (31–34). Further cell divisions and changes of cellular architecture lead to about eight cell layers of primordium at which point the LR emerges out of the primary root epidermis. Spatio-temporal correlation of auxin maxima with the initiation of LR suggests that auxin concentration is an instructive signal for positioning the site of initiation (35,36). In order to gain insight into how auxin regulates the *tasiRNA-ARF* pathway and *ARF3/4* expression, we examined how *miR390* expression is regulated by auxin. We suggest that *miR390* expression plays a role in sensing auxin concentration, potentially leading to *tasiRNA-ARF*-dependent post-transcriptional gene silencing (PTGS) of *ARF4* and LR development.

## MATERIALS AND METHODS

### Plant materials and growth conditions

Following germination on Murashige and Skoog (MS) media, plants (*Arabidopsis thaliana* ecotype *Columbia-0*) were germinated on MS media and then the plants were grown under long-day conditions (16 h-light/8 h-dark) at 22°C for two weeks. The plants were transferred to media containing the designated hormones for the indicated length of time. To analyze the effect of the 26S proteasome inhibitor MG132, 10-day-old seedlings were dipped into MS media containing 10 μM IAA and/or 10 μM MG132 for 6 h. The numbers of emerged LR were counted by eye. The p*MIR390a:GUS* and p*MIR390b:GUS* constructs are described in Montgomery *et al.* (22) and the seeds carrying these constructs were kindly provided by Dr James Carrington.

### RT-PCR, qRT-PCR and northern analysis

RNA was extracted with Trizol reagent (MRC) and poly d(T) cDNA was prepared from 2 μg of total RNA with MMLV reverse transcriptase (Fermantas) and quantified on the Chromo-4-apparatus (Bio-Rad) using the Power SYBR green PCR Master Mix (Applied Biosystems). Cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers used in this study are listed in Supplementary Table 1.

For northern blot analyses, total RNA (15 μg) extracted from the roots was transferred to Hybond-$N^+$ membranes (Amersham Biosciences). Hybridization was performed at 65°C using Rapid-Hyb buffer (Amersham Biosciences) with probes labeled with $^{32}$P-dCTP using the Random Primers DNA Labeling System (Invitrogen). Blots were washed once for 20 min in 2× SSC and 0.1% SDS at room temperature; once for 15 min in 0.5× SSC and 0.1% SDS at 65°C; and once for 15 min in 0.1× SSC and 0.1% SDS at 65°C. Low molecular weight (LMW) RNAs were precipitated with 0.5% PEG8000 and 0.5 M NaCl, separated in a 15% denaturing polyacrylamide gel, and subjected to blot hybridization analysis. DNA oligonucleotides complementary to *miR390* and *tasiR-ARF* were end-labeled with [γ-$^{32}$P] ATP with T4

polynucleotide kinase (TaKaRa) for hybridization. After hybridization at 45°C, the membrane was washed twice in a non-stringent solution [3× SSC, 25 mM $NaHPO_4$ (pH 7.5), 5% SDS and 10× Denhardt's solution] and once in a stringent solution (1 × SSC and 1% SDS) at 45°C. The membrane was dried and exposed to X-ray film at −70°C.

### Transient expression assay using protoplasts

*Arabidopsis* protoplasts were prepared from three-week-old seedlings. Leaves were collected and soaked in an enzyme solution (1% cellulose R-10, 0.25% macerozyme R-10, 400 mM mannitol, 8 mM $CeCl_2$, 5 mM MES at pH 5.7) at 22–25°C for 3–4 h. Transient expression assays using *Arabidopsis* mesophyll protoplasts were performed according to the method of Yoo *et al.* (37). The transfected protoplasts were lysed in lysis reagent (Promega), and luciferase activity was assayed as described by the manufacturer (Promega).

### Whole mount *in situ* hybridization

A DNA fragment of 0.45 kb containing the *tasiR-ARF* cleavage site of the *ARF4* gene was used to make digoxigenin-labeled antisense/sense RNA probes. The sense and antisense RNA probes were labeled with digoxigenin-11-UTP using SP6 and T7 polymerase (Ambion) and the hybridized probe was detected by using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche). Whole mount *in situ* hybridization was performed according to the method of Jan Hejátko *et al.* (38). *tasiR-ARF* and *miR390* were identified using the locked nucleic acids (LNA) oligonucleotide probe (IDT): 5′- T(+G)GGG(+T)CT T(+A)CAA(+G)GTCA(+A)GAA-3′ and 5′-GG(+C) GC(+T)AT(+C)CC(+T)CC(+T)GA(+G)CT(+T)-3′, respectively (23,26).

## RESULTS

### *miR390* expression in response to auxin and auxin concentration

To examine how *miR390* expression is regulated by auxin concentration, *miR390* levels were monitored in the wild-type *Col-0* seedling roots exposed to the different IAA concentrations (Figure 1A). The results showed that the levels increased only at high concentrations such as 10 and 50 μMs (Figure 1A). Consistent with this, the levels increased as the duration of exposure was extended at the fixed concentration of 10 μM, whereas at 10 nM, the levels remained relatively unchanged, suggesting that *miR390* transcription is induced at high auxin concentrations. In contrast to *miR390*, IAA up-regulated *tasiRNA-ARF* levels remained relatively constant throughout the different IAA concentrations, when compared to the untreated control (Figure 1B, upper). The IAA (10 μM)-induced up-regulated level of *tasiRNA-ARF* remained constant during the exposure period of 24 h (Figure 1B, bottom). *tasiRNA-ARF* was not detected over the basal level in *rdr6-11* mutants, where the *tasiRNA-ARF* pathway is blocked (Figure 1B, bottom). *In situ* hybridization of *tasiRNA-ARF* using LNA antisense probe shows that when compared to *Col-0*, overall *tasiRNA-ARF* accumulation is significantly weak in *tir1-1*, further substantiating auxin control of *tasiRNA-ARF* level (Figure 1C). The response to high IAA concentrations seems to be restricted to *miR390*, and does not appear for *ARF4*, *RDR6*, *DCL4* or *AGO7* (Supplementary Figure 1). These results collectively suggest that *miR390* expression is a potential regulatory step for auxin regulation of the *tasiRNA-ARF* pathway.

We monitored promoter activity of the *miR390* genes to investigate how the expression and localization of *miR390* are regulated by auxin. Promoter activity (GUS signal) in both p*MIR390a : GUS* and p*MIR390b : GUS* seedlings was observed in the primary root and LR primordia (Figure 2A and B). The auxin-responsive synthetic promoter DR5 was used an indirect measure of IAA concentration, and showed a weak concentration maximum at the LR apex (Figure 2C). Whereas the GUS signal in the LR primordia of p*MIR390a : GUS* appears to be inversely correlated with DR5:GUS expression, the GUS signal in p*MIR390b : GUS* primordia positively correlates with DR5 : GUS expression (Figure 2A and B). These results imply that promoter activity of the *MIR390b* gene may correlate with a putative auxin gradient present in the LR primordium. Whole-mount *in situ* hybridization analyses with an LNA antisense probe revealed that mature *miR390* is broadly distributed within the LR primordia of wild-type *Col-0* seedlings, also in an auxin-dependent manner, in both earlier (upper) and later (bottom) stages of LR development prior to emergence (Figure 2D). Unlike the promoter activities, the distribution of mature *miR390* appears to be restricted to the LR (Figure 2D). In addition, the distribution of mature *miR390* in response to auxin was significantly broader than that of GUS expression, implying that the site of *miR390* transcription is different from the site of *miR390* processing and/or final localization. Similar to the distribution of mature *miR390*, *tasiRNA-ARF* was also broadly distributed in LR primordia (Figure 2E), suggesting that *miR390* is spatially associated with *tasiRNA-ARF* accumulation. These results collectively show that *miR390* expression is induced by auxin in a concentration-dependent manner, leading to a speculation that auxin may influence the activity of the *tasiRNA-ARF* pathway through its concentration-dependent regulation of *miR390* expression. *miR390* expression was shown to be sensitive only to auxin, and not to the other hormones such as abscisic acid, gibberellins and cytokinin (Figure 2F), suggesting that *miR390* expression and possibly the *tasiRNA-ARF* pathway are specifically involved in the auxin response. In contrast, RT-PCR analyses showed that expression of *HYL1*, which is involved in biogenesis of microRNA, and *TAS3* was induced by the various hormones applied including auxin (Supplementary Figure 2).

To further analyze auxin regulation of *miR390b* expression, we performed a transient transcription assay in *Arabidopsis* protoplasts transformed with a p*MIR390b : LUC* construct. The results show that

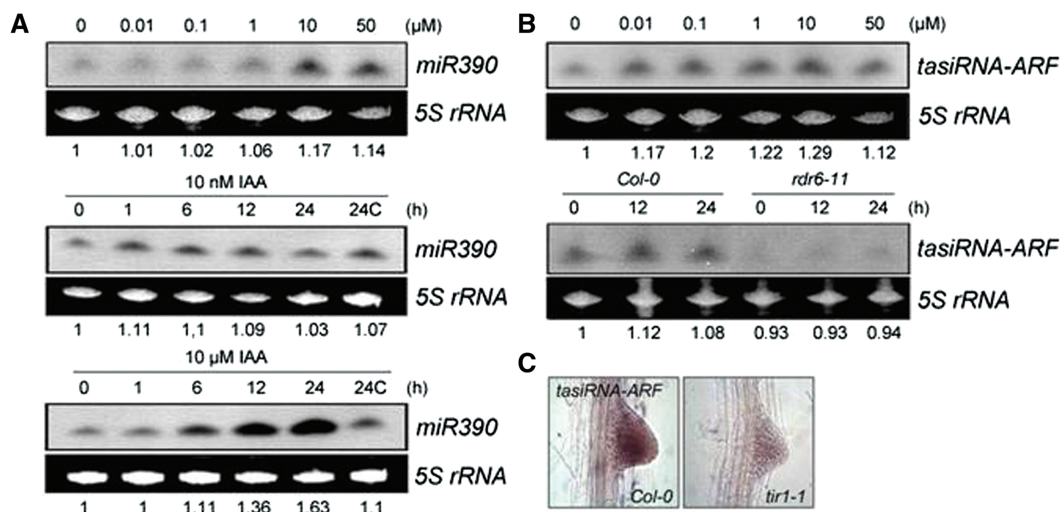


**Figure 1.** Auxin responsiveness of *miR390* and *tasiRNA-ARF*. (**A**) Northern blot analyses of *miR390* levels in *Col-0* seedling roots exposed to different IAA concentrations for 12 h (upper), and seedling roots exposed to 10 nM (middle) or 10 μM (bottom) IAA for different durations. (**B**) Northern blot analyses of *tasiRNA-ARF* in *Col-0* seedling roots exposed to different IAA concentrations for 12 h (upper), and in *Col-0* and *rdr6-11* seedling roots exposed to 10 μM IAA for 24 h (bottom). (**C**) *In situ* hybridization of *tasiRNA-ARF* using a LNA antisense probe in LRs of *Col-0* and *tir1-1*.

luciferase activity increased with exogeneous IAA (Figure 3A). Consistent with this, qRT-PCR analyses indicate that the levels of the precursor-*MIR390b* are up-regulated by IAA in *Col-0* seedling roots exposed to IAA (10 μM) for 24 h (Figure 3B). In addition, up-regulation by IAA was inhibited in the mutant *tir1-1* in which TIR1-mediated protein degradation is compromised (Figure 3B). Consistent with these results, MG132, a protein degradation inhibitor, was shown to decrease *miR390* levels in both the absence and presence of exogenous IAA (Figure 3C). *In situ* hybridization also showed that *miR390* expression appeared to be restricted to the LR base in *tir1-1*, while it was more broadly dispersed in *Col-0* (Figure 3D). It was also shown that miR390 was accumulated mainly in the matured LR region toward the LR apex in *Col-0* seedling roots treated with the auxin transport inhibitor N-1-naphthyphthalamic acid (NPA), while was broadly dispersed in LR in the untreated control (Figure 3E). These results collectively indicate that *miR390* expression is regulated by both TIR1-dependent auxin signaling and auxin transport.

### Mutual negative-regulation between the tasiRNA-ARF pathway and ARF4

To test the possibility of *tasiRNA-ARF* involvement in *ARF4* regulation, we compared the levels of *ARF4* transcripts in *Col-0* and *rdr6-11* seedling roots exposed to exogenous IAA (10 μM) for 12 or 24 h (Figure 4A). In both *Col-0* and *rdr6-11*, *ARF4* transcript levels increased at 12 h. However, after 24 h exposure to IAA, they decreased in *Col-0*, whereas an increase was observed in *rdr6-11*, indicating that *tasiRNA-ARF*-mediated PTGS is affecting *ARF4* expression in *Col-0*. A transient transcription assay, which was performed by transfecting the p*ARF4:LUC* construct into *Arabidopsis* protoplasts, showed that *ARF4* promoter activity was up-regulated by IAA (Figure 4B), suggesting that transcriptional control is involved in the auxin-mediated up-regulation of *ARF4* expression. The higher auxin sensitivity observed for *ARF4* compared to *RDR6* may explain the initial *ARF4* up-regulation followed by PTGS in *Col-0* (Figure 4C). It required 10 μM IAA to attain a 6-fold increase of *RDR6* transcripts compared to the untreated control, whereas only 10 nM was required to attain a 7-fold increase of *ARF4* transcripts. These results show that auxin-dependent *ARF4* expression is modulated through transcriptional up-regulation and *tasiRNA-ARF*-dependent down-regulation.

To understand the auxin-dependent regulatory network of *ARF4* expression, temporal changes in *ARF4* transcript levels were evaluated in *Col-0* and *rdr6-11* seedlings over 72 h-periods, following exposure to 2,4-D (10 μM), a chemically stable form of auxin (Figure 5A). In *Col-0* seedlings, an initial increase in *ARF4* transcript level was observed at 6 h, followed by a slight decrease and maintenance of levels during 24–72 h. In contrast, in *rdr6-11*, *ARF4* transcript levels increased more dramatically at 6 h than *Col-0* and peaked at 24 h, followed by a continuous decrease without maintenance of a constant level. This implies that the *tasiRNA-ARF* pathway provides the regulation to modulate *ARF4* expression at a constant level. This finding is consistent with the *in situ* hybridization signal of the enlarged *ARF4* domain in *rdr6-11*, when compared to *Col-0* (Figure 5B). To examine whether the *tasiRNA-ARF* pathway is regulated by ARF4, the mRNA levels of *RDR6*, *AGO7* and *DCL4* were compared between *Col-0*, *arf4-2* and *ARF4-OX*, in which *ARF4* is overexpressed under the control of 35S promoter (Figures 5C and 6A). qRT-PCR analyses showed that while the levels of *AGO7* and *DCL4* mRNAs were not significantly different between *Col-0*

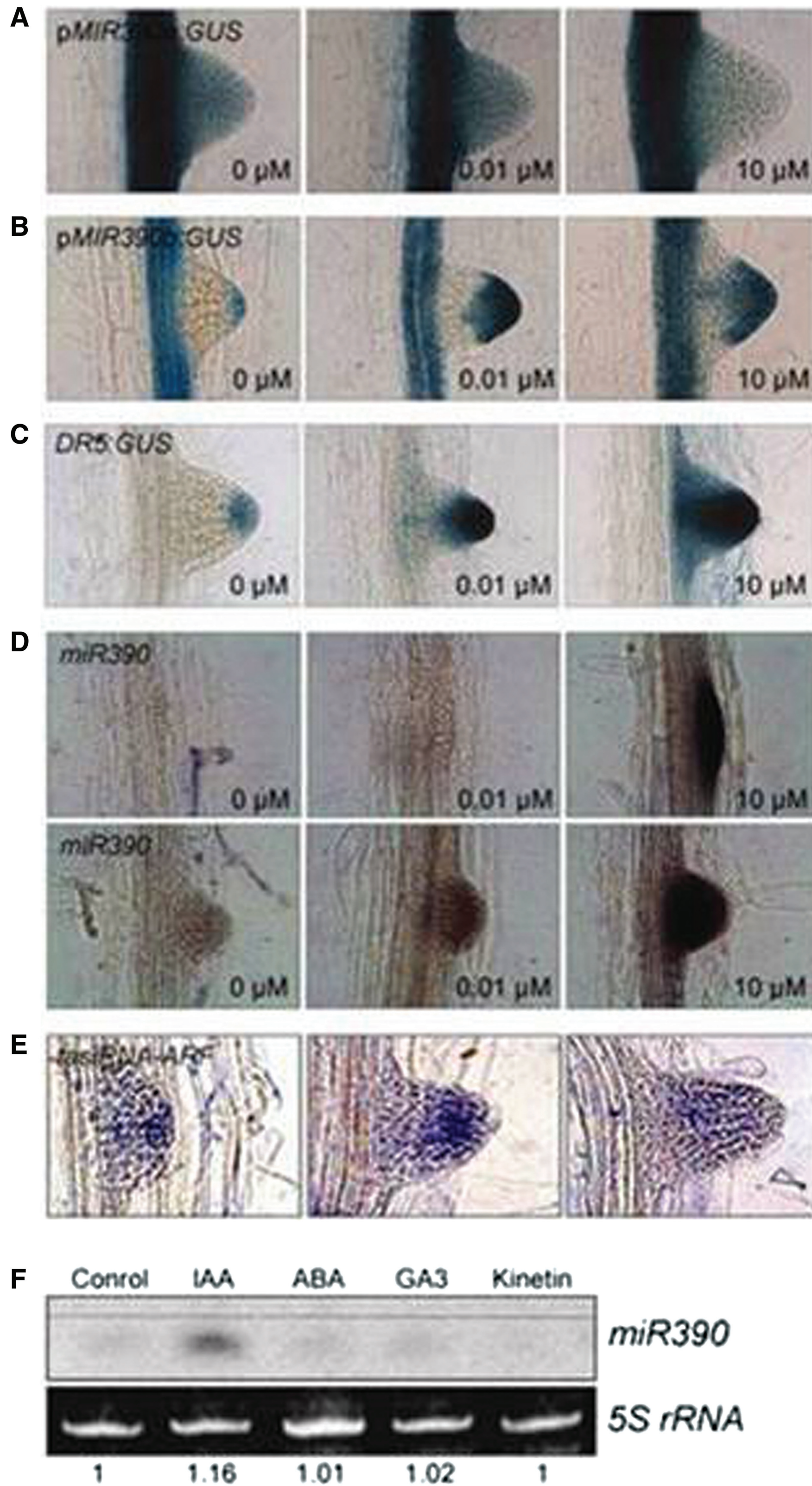


**Figure 2.** *miR390* expression in response to auxin concentration. The promoter activity of p*MIR390a:GUS* (**A**) and p*MIR390b:GUS* (**B**) seedlings. GUS activity was observed in the LR primordia of seedlings treated with the designated concentrations of IAA for 12 h, at comparable developmental stages. (**C**) GUS activity driven by the DR5 promoter in LR primordia of seedlings at comparable developmental stages, treated with the designated concentrations of IAA for 12 h. (**D**) *In situ* hybridization analyses of *miR390* distribution in LR primordia at early (upper) and later pre-emergence stages (bottom) in *Col-0* seedlings treated with the designated concentrations of IAA for 12 h. (**E**) *In situ* hybridization of *tasiRNA-ARF* using a LNA antisense probe at different developmental stages of *Col-0* LR. (**F**) Northern analyses of the levels of *miR390* in *Col-0* seedling roots exposed to various hormones (each 10 μM) for 12 h.

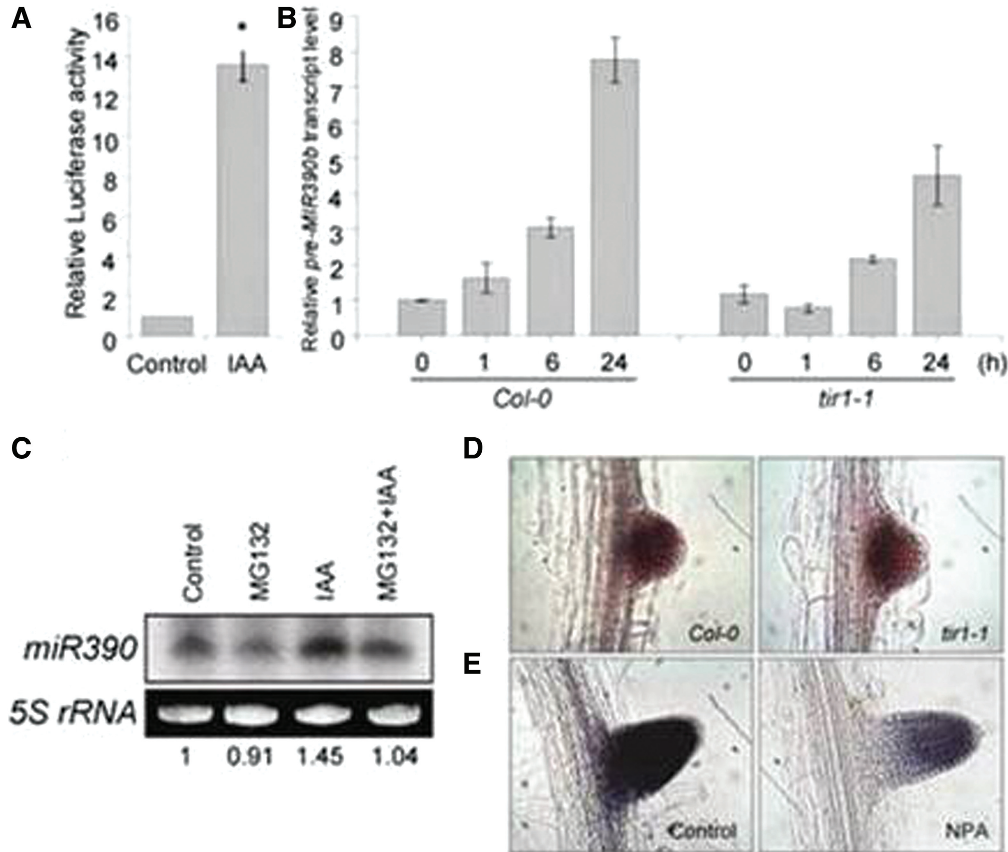


**Figure 3.** Transcriptional control of *miR390*. (**A**) Transient transcription assay in *Arabidopsis* protoplasts transformed with the DNA construct p*MIR390b:LUC*. Luciferase activity was measured in protoplasts incubated with 10 µM IAA for 1 h. The asterisks indicate a significant difference from the control ($P < 0.01$, Student's *t*-test). Bars represent the standard deviations of three independent experiments. (**B**) qRT-PCR analyses of precursor-*MIR390b* in *Col-0* and *tir1-1* seedling roots exposed to IAA (10 µM) for 24 h. Bars represent the standard deviations of three independent experiments. (**C**) Assay of *miR390* transcription in seedling roots incubated with 10 µM of the proteasome inhibitor MG132 and/or IAA for 6 h. The numbers indicate the relative abundance of gene transcripts compared to the control lane, defining the band intensity of the first lane (Control) as 1. (**D**) *In situ* hybridization of *miR390* using a LNA antisense probe in *Col-0* and *tir1-1* (D), and in *Col-0* seedlings exposed to NPA for 12 h (**E**).

and *arf4-2*, the level of *RDR6* mRNA was high in *arf4-2* and was at a basal level in *ARF4-OX* (Figure 5C). In addition, *miR390* levels, measured by northern hybridization, were repressed in *ARF4-OX*, and were higher in *arf4-2*, albeit less significantly (Figure 5D). These results indicate that ARF4 inhibits the activity of the *tasiRNA-ARF* pathway by repression of *RDR6* and/or *miR390*, forming a feedback loop. To examine ARF4 repression of *miR390* expression during LR development, the distribution of *miR390* was compared between *arf4-2* and *Col-0* by *in situ* hybridization (Figure 5E). In *arf4-2*, the *miR390* signal was observed to appear at the LR initiation site, and a tail of expression stemming from the LR apex of the fully mature LR was also observed (Figure 5E, bottom). In contrast, in *Col-0*, *miR390* was not detected in the initiation site, and there was no tailing expression of *miR390* in the apex region in the fully matured LR (Figure 5E, upper). The intensity of *miR390* level in the fully matured LR was weaker in *ARF4-OX* than in *Col-0*, further supporting ARF4 repression of *miR390* (Figure 5F). These results suggest that the distribution of *miR390* is associated with ARF4 repression of *miR390*. Together with the *tasiRNA-ARF*-dependent regulation of *ARF4* expression described above (Figure 5B), these results suggest that mutual negative regulation between the *tasiRNA-ARF* pathway and ARF4 determines the distributions of *ARF4* and *miR390*. We speculate that the ability to maintain the levels of *ARF4* transcripts in *Col-0* may represent a dynamic and balanced up- and down-regulation of *ARF4* expression throughout the developmental stages.

## ARF4 mediates LR development

To investigate ARF4 participation in LR development, expression of genes which are involved in the pattern-formation of lateral organs, such as *HD-ZIPIII* and *YABBY*, was compared between *arf4-2* and *ARF4-OX* (Figure 6A). RT-PCR analyses showed that the *YABBY* genes *FILAMENTOUS FLOWER* (*FIL*) and *YAB3* were up-regulated in ARF4-OX, while they were repressed in *arf4-2* (Figure 6A). In contrast, the *HD-ZIPIII* genes *PHABULOSA (PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*) were shown to be insensitive to ARF4 regulation (Figure 6A). Consistent with the

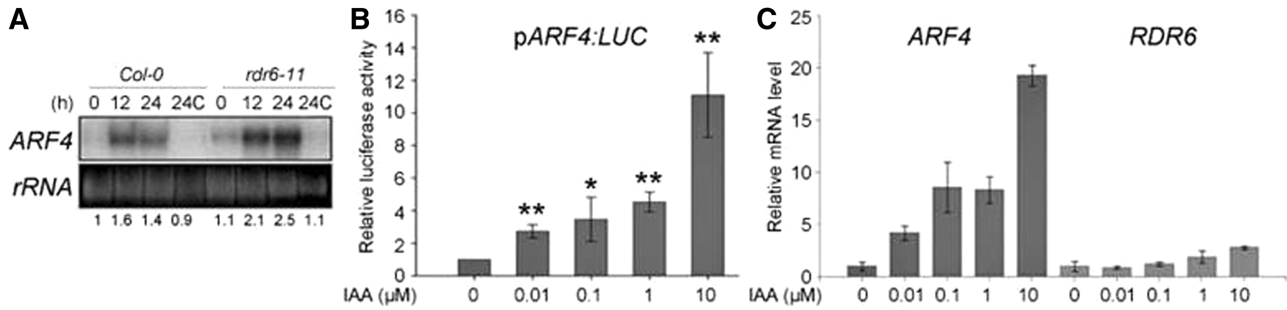


**Figure 4.** Auxin regulation of *ARF4* expression. (**A**) Northern blot analyses of the levels of *ARF4* transcripts in *Col-0* and *rdr6-11* seedlings grown on 10 μM IAA for the indicated time periods. 24C, untreated control. (**B**) Transient transcription assays of *ARF4*. The plasmid carrying the p*ARF4:LUC* DNA construct was transfected into *Arabidopsis* protoplasts which were subsequently incubated with the indicated concentrations of IAA for 1 h. Bars represent the standard deviations of three independent experiments. The asterisks indicate a significant difference from the 0 μM control (*$P < 0.05$, **$P < 0.01$, Student's *t*-test) (**C**) qRT-PCR analyses of the changes of *ARF4* and *RDR6* transcript levels in seedling roots exposed to different concentrations of IAA for 12 h. Bars represent the standard deviations of three independent experiments.

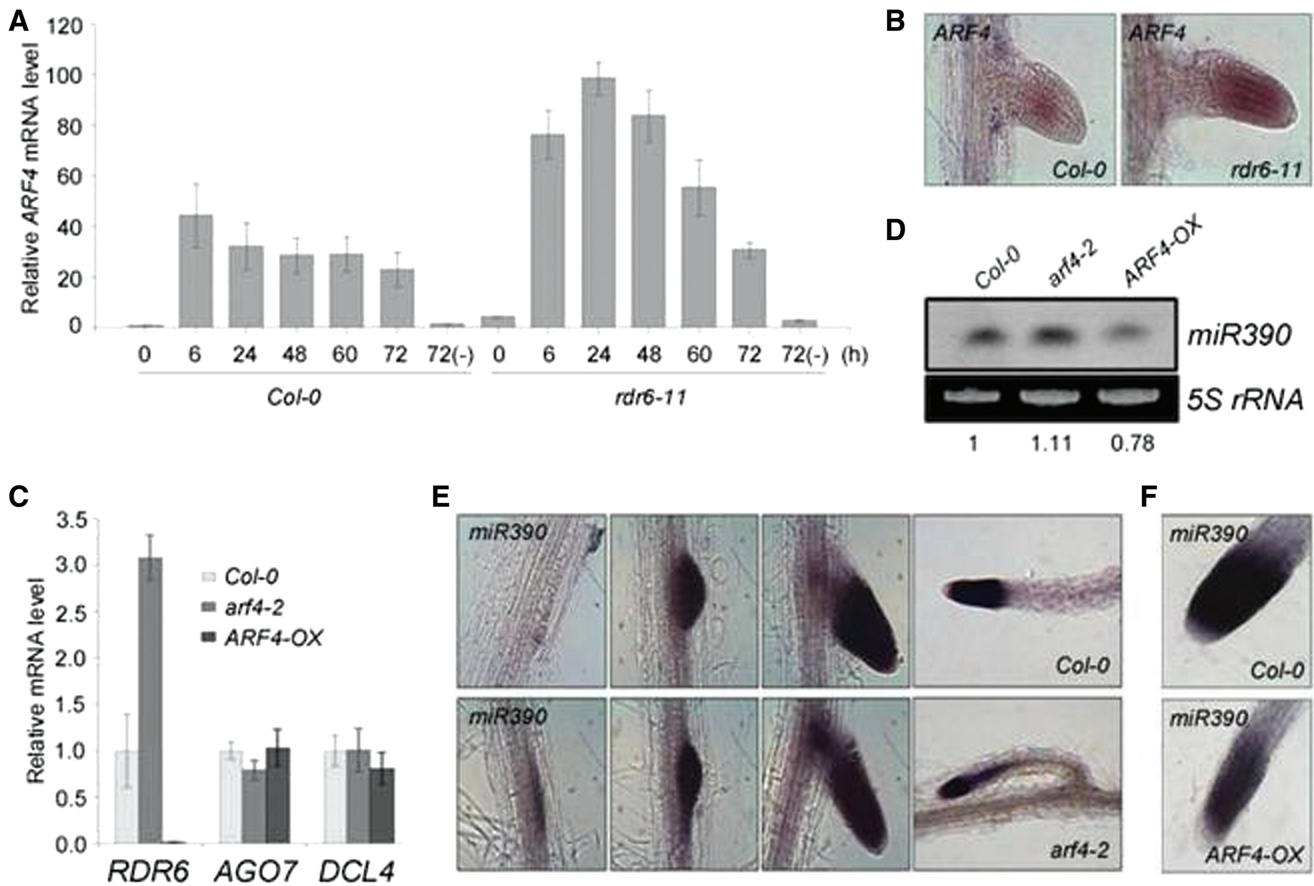


**Figure 5.** Regulatory interaction between *miR390/tasiRNA-ARF* and *ARF4*. (**A**) qRT-PCR analyses of temporal changes in *ARF4* expression over a 72 h period, in *Col-0* and *rdr6-11* seedling roots exposed to 2,4-D (10 μM) for the designated times prior to qRT-PCR analysis. 72C, untreated control. Bars represent the standard deviations of three independent experiments. (**B**) *In situ* hybridization analyses of *ARF4* transcripts in *Col-0* and *rdr6-11*. (**C**) qRT-PCR analyses of *RDR6*, *AGO7* and *DCL4* expression in *arf4-2* and *ARF4-OX* seedling roots grown in auxin-free media. Bars represent the standard deviations of three independent experiments. (**D**) Northern blot analyses of *miR390* expression in *arf4-2* and *ARF4-OX* seedling roots grown in auxin-free media. The numbers indicate the relative abundance of gene transcripts compared to the control lane, defining the band intensity of the first lane (*Col-0*) as 1. (**E**) *In situ* hybridization of *miR390* at comparable developmental stages in *Col-0* (upper) and *arf4-2* (bottom). (**F**) *In situ* hybridization analyses of *miR390* expression at the LR apex regions in *Col-0* and *ARF4-OX*.

RT-PCR analyses, *in situ* hybridization of a *YABBY FIL* gene indicates that its expression is up-regulated in *ARF4-OX*, but repressed in *arf4-2* (Figure 6B). These results suggest that ARF4-up-regulation of *FIL* is involved in auxin regulation of LR development. The results of an LR assay indicate that the *tasiRNA-ARF* pathway and ARF4 are involved in LR development. The numbers of emerged LR in *rdr6-11* and *arf4-2* were

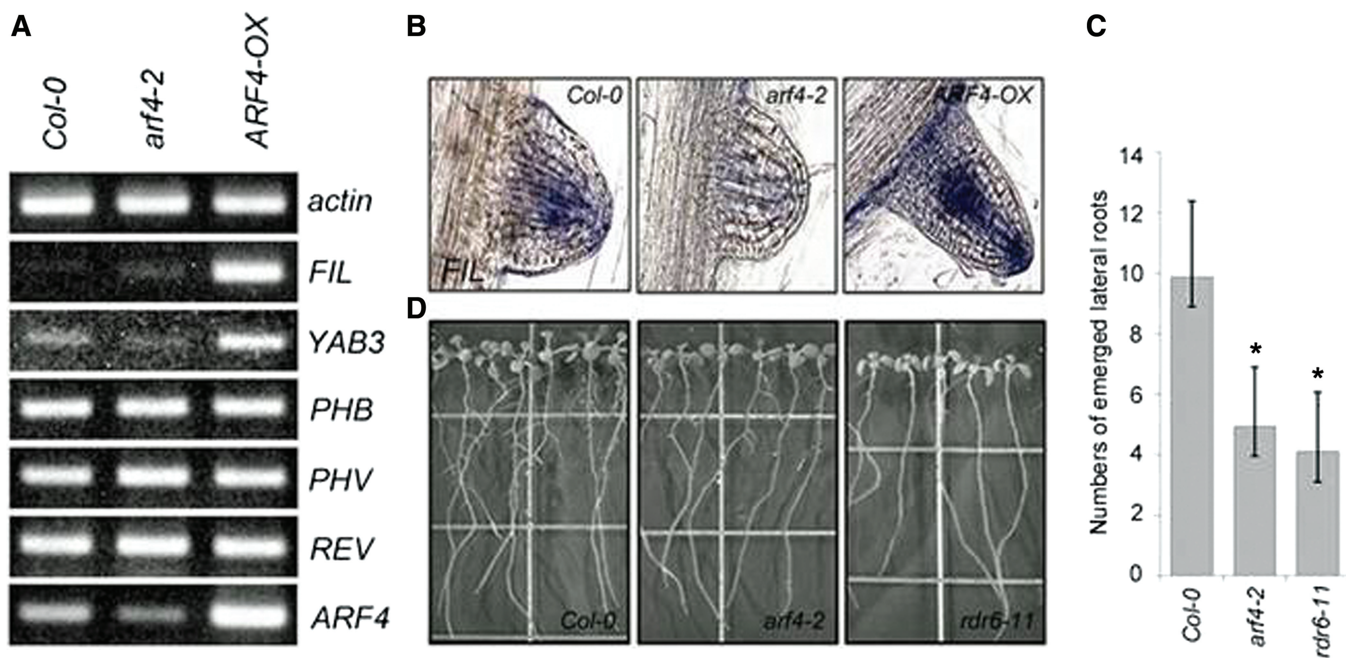


**Figure 6.** ARF4 control of the *YABBY* genes. (A) RT-PCR analyses of the *YABBY* and *HD-ZIPIII* genes in *arf4-2, ARF4-OX* and *Col-0*. (B) *In situ* hybridization of *FIL* transcripts using an antisense probe in *arf4-2, ARF4-OX* and *Col-0*. (**C**, **D**) Numbers and picture of emerged LRs following 10-d growth of germinated seedlings in auxin-free media. The numbers are an average of 10 seedlings for each line (*Col-0*, *arf4-2*, *rdr6-11*). The asterisks indicate a significant difference from the wild-type control ($P < 0.01$, Student's *t*-test). Standard errors are indicated.

less than in *Col-0* by ~50%, while the lengths of primary roots were relatively constant in these plants (Figure 6C and D). It was also noted that growth of the emerged LRs in *rdr6-11* was significantly retarded, compared to *Col-0* and *arf4-2*. The number of LRs in the ARF4-OX line was higher than in *Col-0* (Supplementary Figure 3). These results collectively suggest that *tasiRNA-ARF* regulation of *ARF4* expression plays a role in LR development. In conclusion, this study shows that the *tasiRNA-ARF* pathway and ARF4 mediate auxin regulation of LR development, and proposes that the auxin signal interacts with *miR390* expression, the *tasiRNA-ARF* pathway and *ARF4* expression during LR development.

## DISCUSSION

In this study, we have analyzed how the auxin signal interacts with the *tasiRNA-ARF*-dependent PTGS of *ARF4* during LR development. We suggest that *miR390* expression is a potential regulatory component for the sensing of auxin concentration and for the modulation of *ARF4* expression and *tasiRNA-ARF* level. Additionally, auxin signal is also known to regulate *miR164* expression, which down-regulates the NAC1 transcription factor during LR initiation (39). Accumulation of data identifying other hormone-responsive *microRNAs* such as *miR393* (ABA), *miR160* (auxin) or *miR159* (GA) suggest that *microRNAs* may be a key component in hormonal regulation of development in various organs (40–42). We previously reported that auxin-sensitive *miR167* down-regulates *ARF8* and *OsGH3-2*, a free auxin conjugating enzyme, to regulate the free auxin level in cultured rice cells (43). Taking into consideration that *microRNA* target genes are also subject to transcriptional control, what could be the developmental role of *microRNA*-mediated down-regulation of target genes? In triple mutants of *miR164*, where *miR164* expression is completely abolished, the expression domain of *CUC*, a target of *miR164*, becomes less precise, leading to the varying degrees of abnormal phenotypes in inflorescence meristem and flower primordia (44). Thus, it has been suggested that *miR164* reduces fluctuations of its target transcripts to increase developmental precision and stability. Stabililization of developmental processes by *microRNA* has also been proposed in animal development (45). Therefore, we speculate that *miR390*-mediated modulation of the levels of *tasiRNA-ARF* and *ARF4* transcripts may play a role in stabilizing developmental processes during LR development.

Our findings in this study suggest that the expression of *ARF4* and *tasiRNA-ARF* are mutually dependent (Figure 5B and E), leading to a speculation that distribution of *ARF4* is defined by *tasiRNA-ARF*. This study also suggests that the capacity to maintain constant levels of *ARF4* transcripts is derived from a complex regulatory network involving *ARF4* transcription, PTGS of *ARF4* and ARF4 repression of *RDR6*/*miR390* (Figure 5A). The *tasiRNA-ARF* pathway may provide the ability to fine-tune the spatiotemporal expression of *ARF4* in response to auxin, and thus we propose that the *tasiRNA-ARF* pathway may play a role in buffering and increasing precision of ARF4-mediated developmental processes. We propose that transcriptional control alone is not sufficient for the accurate positioning and expression

of *ARF4*. To make the regulatory network even more complicated, *miR390* maturation and *tasiRNA-ARF* biogenesis may also participate in the spatiotemporal regulation of *ARF4* expression (27). It is interesting to note that ARF4 repression of *RDR6*, but not *AGO7* and *DCL4*, may represent an additional regulatory step in regulating the *tasiRNA-ARF* pathway (Figure 5C).

Our results suggest that the level of *miR390* expression appears to reflect auxin concentration, and thus may act as a sensor of auxin concentration. *miR390* transcription is regulated by TIR1-dependent auxin signaling, implying that degradation of an AUX/IAA may play a role in sensing auxin concentration. Identification of this putative AUX/IAA and its auxin-dependent distribution should help us understand the mechanism underlying *miR390* sensing of auxin concentration. It is also possible that different *AUX/IAA* genes are involved in *miR390* transcription at different developmental stages. We speculate that the *tasiR-ARF* and *ARF4* transcripts are auxin-directed positional signals that specify subregions within LR. Conservation of the *tasiR-ARF* pathway in land plants suggests that PTGS-mediated modulation of *ARF4* expression may be a common and fundamental event in auxin-mediated LR development in plants. Although deviations are likely to occur depending on plant lineages and developmental contexts, conveyance of the auxin signal to development through *tasiRNA-ARF*-dependent PTGS of *ARF4* may represent a conserved pathway underlying the development of lateral organs in plants.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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