J. Plant Biol. (2010) 53:150–154
DOI 10.1007/s12374-010-9100-4

ORIGINAL RESEARCH

Auxin and Abscisic Acid Responses of *Auxin Response Factor 3* in *Arabidopsis* Lateral Root Development

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Received: 25 January 2010 / Revised: 3 February 2010 / Accepted: 5 February 2010 / Published online: 19 March 2010

Abstract As in the cases of the embryo and many lateral organs, lateral root (LR) development is triggered by the phytohormone auxin. LR development is also known to be inhibited by abscisic acid (ABA). Here we show that an auxin response factor ARF3, which participates in pattern formation of aerial lateral organs, such as leaf or flower, is up-regulated by auxin during LR development in *Arabidopsis*. This study demonstrates that *ARF3* expression is regulated at the transcriptional level and by posttranscriptional gene silencing (PTGS), which is mediated by a group of trans-acting small-interfering RNA (*tasiRNA-ARF*). The *tasiRNA-ARF* pathway and *ARF3* expression are sensitive to auxin and ABA treatment, suggesting that *tasiRNA-ARF*-dependent PTGS of *ARF3* is linked to auxin promotion of LR development and to ABA inhibition.

Keywords Lateral root · Auxin · Abscisic acid · tasiRNA-ARF · ARF3

Introduction

Among its numerous roles in plant development, auxin plays an important role in patterning and establishing axes throughout the life cycle of a plant. Establishments of a concentration gradient and maxima play a critical role in auxin-mediated developmental processes (Friml et al. 2003). A potential role of auxin as a morphogen has been suggested in the developments of lateral organs (Benková et al. 2003; Reinhardt et al. 2003; Blilou et al. 2005; De Smet et al. 2007). During lateral root (LR) development, auxin moves from the base within the primary root, through the primordium interior to the primordium apex where it forms an 'auxin maxima' (Benková et al. 2003). Auxin induces cell divisions in the pericycle founder cells to establish a meristem (Laskowski et al. 1995; Himanen et al. 2002; Casimiro et al. 2003). Auxin-mediated organ patterning is also demonstrated in gynoecium (Nemhauser et al. 2000) and leaf primordium formation (Benková et al. 2003; Friml et al. 2003; Pekker et al. 2005). In both cases, ARF3 (ETTIN) and the functionally redundant ARF4 appear to be crucial in conveying auxin signaling into the pattern formation. As a mediator of auxin signaling, ARF3/4 has been suggested to be an abaxial determinant important for the partitioning of adaxial and abaxial domains during leaf primordium formation (Pekker et al. 2005). Sided abaxial localization of the *ARF3/4* transcripts and auxin influx carrier AUX1 further supports the role of ARF3/4 in auxin-mediated pattern formation (Reinhardt et al. 2003).

A group of transacting-siRNA (*tasiRNA-ARF*) which cleaves *ARF3/4* transcripts constitutes one method by which proper polarization of domain-specification transcription factors is ensured during leaf primordium formation, in addition to antagonization of ARF3/4 function in the abaxial domain by the activity of certain adaxial determinants (Garcia et al. 2006; Nogueira et al. 2007). Localization of *tasiRNA-ARF* in the adaxial domain correlates with the sided localization of ARF3/4 in the abaxial domain (Garcia et al. 2006; Nogueira et al. 2007). Participation of *tasiRNA-ARF* in ARF3/4-mediated asymmetry formation suggests that the *tasiRNA-ARF*-mediated posttranscriptional gene silencing (PTGS) pathway (designated as 'the *tasiRNA-ARF* pathway') is linked to auxin signaling. Systemic mobility of *tasiRNA-ARF* around the meristem and primordia is suggested to be important in

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Table 1 The primer and the probe sequences used in this study

Gene name	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
qActin	GAAAAGATCTGGCATCACACTTTATA	AACGATTCCTGGACCTGCCTCATC
qARF3	CCCACACCAAATGTTCCTCT	CAACACTTGTTCGGATGGTG
promoter ARF3	TCTAGATGACTTTTACCCTCTCAGCCAA	AAGCTTCGTTTACTTTGATGCCGTTGGA
SGS3	CTGGTCCAATGTCTAAGGAA	CGGAATCATTCTCTTCCTCA
actin	TGGCATCATACTTTCTACAA	CCACCACTGAGCACAATGTT
TAS3	GGAAAACATAACCTCCGTGA	GTCAACCATACATCAATAAC
RDR6	CATGCATTATGATGCAGCTG	AATGAGTTGCCCGTCGTACA
AGO7	GTTATCTTCATGGGAGCTGA	CCTTCACTCCTAAATGGCTA
DCL4	TCATAGAGATTCCTCCCGAA	CCAAGATCCCTATTCAAGGA
miR390	GGCGCTATCCCTCCTGSGCTT	
tasiR-ARF	TGGGGTCTTACAAGGTCAAGA	

establishing polarity during the leaf primordium formation (Chitwood et al. 2009). *tasiRNA-ARF* is derived from the protein-noncoding RNA precursor *TAS3* (Allen et al. 2005; Axtell and Bartel 2005; Yoshikawa et al. 2005; Garcia et al. 2006). Following *miR390*-Argonaute7 (AGO7)-mediated cleavage of *TAS3* (Montgomery et al. 2008), RNA-dependent RNA polymerase 6 (RDR6) generates dsRNA by polymerization, using the *miR390*-cleaved *TAS3* as template (Yoshikawa et al. 2005; Montgomery et al. 2008). The *dsRNA* intermediate is cleaved into 21-nucleotides in phase by RNaseIII-like Dicer-like4 (DCL4) to generate *tasiRNA-ARF* (Allen et al. 2005; Yoshikawa et al. 2005). The *tasiRNA-ARF* pathway is also known to be involved in young-to-adult leaf phase changes in *Arabidopsis*, implying that ARF3/4 is functional throughout the various developmental stages (Fahlgren et al. 2006; Hunter et al. 2006). The *tasiRNA-ARF* pathway is known to be conserved among land plants (Allen et al. 2005; Axtell et al. 2007), suggesting that it plays a fundamental and common role in plant development.

In contrast to auxin, abscisic acid (ABA) is known to play a role in inhibiting LR development (Sunkar and Zhu 2004). However, despite expectations that ABA may interact with auxin in response to various stresses, whether and how auxin and ABA interact during the course of LR development is not understood. This study was directed to examine whether *tasiRNA-ARF*-dependent PTGS of *ARF3* is regulated jointly by these hormones. We suggest that PTGS of *ARF3* is tightly regulated by auxin and ABA.

Materials and Methods

Plant Materials and Growth Conditions

Plants (*Arabidopsis thaliana* ecotype *Columbia-0*) were germinated on Murashige and Skoog (MS) media, and grown under long-day conditions (16 h light/8 h dark) at 22°D for 2 weeks. The plants were transferred to media

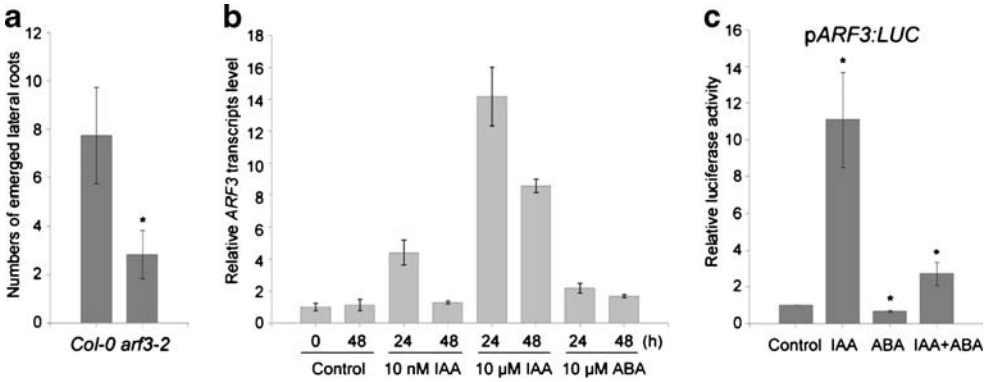

Fig. 1 *ARF3* expression is regulated by auxin and ABA. **a** Numbers of emerged LRs following 10 days growth of germinated seedlings. The *asterisks* indicate a significant difference from the wild-type control ($P<0.01$, Student's t test; $n=10$). Standard errors are indicated. **b** qRT-PCR analyses of *ARF3* transcript level in seedling roots exposed to IAA or ABA for 24 or 48 h. *Bars* represent the standard deviations of the three independent experiments. **c** Transient transcription assays of *ARF3*. *Arabidopsis* protoplasts were transfected with the p*ARF3:LUC* reporter and was subsequently incubated with IAA and/or ABA for 1 h. The *asterisks* indicate a significant difference from the control (*$P<0.05$, Student's t test). *Bars* represent the standard deviations of three independent experiments

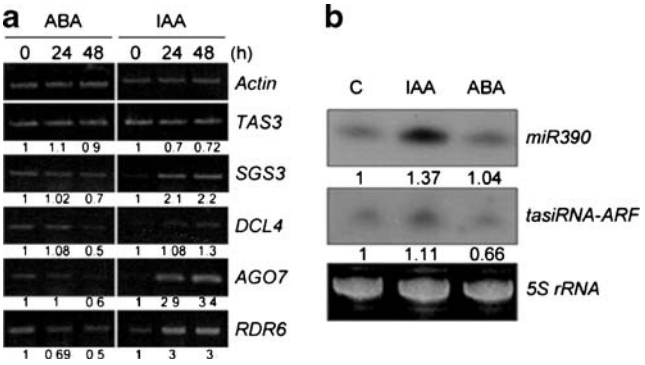

Fig. 2 ABA down-regulates specific regulatory factors in *tasiRNA-ARF* biogenesis. **a** RT-PCR analysis of the PTGS-regulatory component genes. mRNA was prepared from the roots exposed to 10 μM IAA or 10 μM ABA for 24–48 h. Intensity of the bands was normalized, taking the intensity of the 0 h bands as 1. **b** Detection of *miR390* and *tasiRNA-ARF* by Northern hybridization in seedling roots exposed to 10 μM ABA or 10 μM IAA for 12 h

containing 10 μM ABA or 10 μM IAA for the indicated length of time. *Col-0* (CS6000), *arf3*-2 (CS24604), and *rdr6-11*(CS24285) seeds were obtained from *Arabidopsis* Biological Resource Center (ABRC). *pARF3:ARF3-GUS* and *pARF3:ARF3m-GUS* seeds were kindly provided by Dr. James Carrington. Transient gene expression assays using *Arabidopsis* protoplasts were performed as previously described by Yoo et al. (2007).

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 (Fermentas) and quantified on the Chromo-4-apparatus (Bio-Rad) using the Power SYBR green polymerase chain reaction (PCR) Master Mix (Applied Biosystems). Cycling conditions were performed as described by the manufacturer (Applied Biosystems).

Low molecular weight RNAs were separated in a 15% denaturing polyacrylamide gel, and subjected to blot hybridization analysis. The oligonucleotide sequences used in this study are listed in the Table 1.

GUS Histochemical Localization Assay

Histochemical GUS localization of transgenic *Arabidopsis* plants was performed as described by Jefferson et al. (1987). Seedlings grown in MS medium for 12 days were directly placed in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) and incubated at 37°C overnight.

Results and Discussion

To evaluate whether ARF3 participates in LR development, the numbers of emerged LRs were counted in wild-type *Col-0* and mutant *arf3-2* seedlings at 10 days following germination (Fig. 1a). The numbers were significantly less in *arf3-2* than *Col-0*, suggesting that ARF3 plays a role in LR development. To examine how *ARF3* expression responds to auxin and ABA, quantitative reverse transcription (qRT)-PCR analyses was performed to measure *ARF3* mRNA levels in *Col-0* seedling roots exposed to these hormones (Fig. 1b). The results show that the *ARF3* transcript level increases upon auxin treatment in a concentration-dependent manner, but the basal level observed in hormone-untreated plants remained unchanged upon 10 μM ABA treatment. To further examine the *ARF3* transcriptional response to these hormones, *ARF3* transcription was analyzed by transient transcription assay in *Arabidopsis* protoplast cells which are transformed with p*ARF3:LUC* and incubated with IAA and/or ABA (Fig. 1c). The results show that transcription was up-regulated by auxin, and down-regulated by ABA. To test

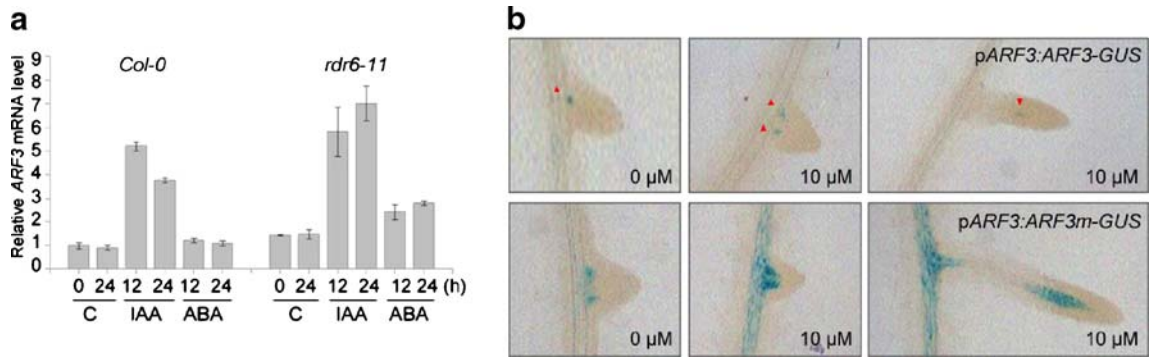

Fig. 3 *TasiRNA-ARF*-dependent PTGS of *ARF3*. **a** qRT-PCR analyses of *ARF3* expression in *Col-0* and *rdr6-11* which were exposed to 10 μM IAA or 10 μM ABA for the designated periods. **b** GUS activity driven by the p*ARF3:ARF3-GUS* reporter (*upper*) or the p*ARF3:ARF3m-GUS* reporter (*bottom*) in LR primordia at the comparable developmental stages in the absence or presence of IAA for 12 h. *Red arrowheads* mark *GUS* signals, respectively

whether ABA and auxin interact to transcriptionally regulate *ARF3*, *ARF3* transcription was analyzed in cells incubated with both auxin and ABA. The activity was higher than the level of ABA-incubated cells, but it was lower than that of auxin-incubated cells. These results suggest that ABA suppresses the auxin-dependent transcriptional up-regulation of *ARF3*.

To analyze whether *tasiRNA-ARF* biogenesis is regulated by auxin and ABA, genes encoding *tasiRNA-ARF* pathway components were examined for their transcript levels present in *Col-0* seedling roots which were exposed to 10 μMs of IAA or ABA (Fig. 2a). RT-PCR analyses showed that *RDR6*, *AGO7*, and *DCL4* were up-regulated by auxin and, down-regulated by ABA (Fig. 2a). In contrast, the level of TAS3 RNA appears to be insensitive to these hormones, indicating that components downstream of TAS3 biogenesis are subject to the hormonal regulations. *miR390* levels were observed to increase upon auxin treatment, but not in ABA-treated seedlings (Fig. 2b). *tasiRNA-ARF* levels were observed to be up-regulated by auxin and down-regulated by ABA (Fig. 2b). These observations collectively suggest that, similar to *ARF3* expression, *tasiRNA-ARF* biogenesis is a target for auxin and ABA in LR development.

The data above indicate that transcriptional and *tasiRNA-ARF*-dependent PTGS regulation of *ARF3* expression is sensitive to auxin and ABA. To investigate how these regulations contribute to *ARF3* expression, the level of *ARF3* transcript was compared between *Col-0* and the *rdr6-11* mutant, where posttranscriptional regulation is compromised (Fig. 3a). While the levels increase by auxin in both *Col-0* and *rdr6-11*, the level in *rdr6-11* is significantly higher, suggesting that *tasiRNA*-dependent cleavage of *ARF3* transcripts is blocked in *rdr6-11*. Compared to auxin, the increases in *ARF3* transcript levels were less significant in ABA-treated seedlings (Fig. 3a), suggesting that ABA repression of *ARF3* transcription is taking place. To verify whether *tasiRNA-ARF*-dependent PTGS of *ARF3* expression plays a role in LR development, we compared the distribution of *ARF3* transcripts between the lines carrying p*ARF3:ARF3-GUS* constructs which are either sensitive (p*ARF3:ARF3-GUS*) or resistant (p*ARF3:ARF3m-GUS*) to *tasiRNA-ARF* cleavage (Fig. 3b). In the p*ARF3:ARF3m-GUS* line, the *GUS* signal is observed to have strong expression in the emerging and fully emerged regions toward the apex in the LR, in addition to the vasculature region of the primary root in the line carrying. Weaker GUS signals were observed in these respective localizations in the p*ARF3:ARF3-GUS* line. It was also noted that the intensity of the GUS signal was stronger in auxin-treated seedlings than untreated seedlings. These results confirm that *tasiRNA-ARF*-dependent cleavage of *ARF3* transcripts occurs during the course of LR development. Although the mechanism of how ARF3 plays a role in LR development is not understood, we speculate that based on the localizations of the GUS signals, ARF3 is involved in the emerging and elongation stages of LR development. The results in this study demonstrate that both transcription and posttranscriptional regulation are involved in auxin-dependent localization and levels of *ARF3* transcripts. We recently reported that ARF4 represses expression of *miR390* to establish a feedback loop (Yoon et al. 2009). These lines of evidences suggest that the level and localization of ARF3/4 expression are determined by multiple layers of regulation. We speculate that the accurate level and localization of ARF3/4 are important in auxin-mediated LR development.

This study indicates that *tasiRNA-ARF* biogenesis and *ARF3* expression is subject to auxin and ABA regulation during LR development, implying that as a stress hormone, ABA modulates LR development by suppressing auxin regulation of LR development. We speculate that *tasiRNA-ARF*-dependent PTGS of *ARF3* may provide a convergent point for crosstalk between auxin and ABA. There is accumulating evidence of hormonal regulation of small RNA biogenesis (Navarro et al. 2006; Reyes and Chua 2007; Guo et al. 2005; Patrick et al. 2004; Yang et al. 2006). ABA is known to up-regulate the expression of several microRNAs such as *miR393* and *miR159* (Navarro et al. 2006; Reyes and Chua 2007). *miR393* cleaves transcripts of the auxin receptor *TIR1*, suggesting that TIR-mediated auxin signaling may also be a place for crosstalk between auxin and ABA. These observations, together with the hormonal regulation of *tasiRNA-ARF*-dependent PTGS of ARF3 described in this study, show that small RNAs are closely linked to plant development and/or stress physiology through plant hormones. Considering that the *tasiRNA-ARF* pathway is conserved in land plants, its regulation by auxin and ABA may represent a conserved regulatory network of *tasiRNA-ARF*-dependent lateral organ development in plants.

Acknowledgments We thank Dr. James Carrington and the *Arabidopsis* Stock Center for providing the *Arabidopsis* seeds. We thank Dr. Choong-Ill Cheon for assistance with the transient gene expression experiments. This study was supported by Faculty Research Fund, Sungkyunkwan University, 2006.

References

Allen E, Xie Z, Gustafson A, Carrington J (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell 121:207–221

Axtell MJ, Bartel DP (2005) Antiquity of microRNAs and their targets in land plants. Plant Cell 17:1658–1673

Axtell MJ, Snyder JA, Bartel DP (2007) Common functions for diverse small RNAs of land plants. Plant Cell 19:1750–1769

Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115:591–602

Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme K, Scheres B (2005) The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature 433:39–44

Casimiro I, Beeckman T, Graham N, Bhalerao R, Zhang H, Casero P, Sandberg G, Bennett MJ (2003) Dissecting *Arabidopsis* lateral root development. Trends Plant Sci 8:165–171

Chitwood DH, Nogueira FT, Howell MD, Montgomery TA, Carrington JC, Timmermans MC (2009) Pattern formation via small RNA mobility. Genes Dev 23:549–554

De Smet I, Tetsumura T, De Rybel B, Frey NF, Laplaze L, Casimiro I, Swarup R, Naudts M, Vanneste S, Audenaert D, Inzé D, Bennett MJ, Beeckman T (2007) Auxin-dependent regulation of lateral root positioning in the basal meristem of *Arabidopsis*. Development 134:681–690

Fahlgren N, Montgomery TA, Howell MD, Allen E, Dvorak SK, Alexander AL, Carrington JC (2006) Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in *Arabidopsis*. Curr Biol 16:939–944

Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jürgens G (2003) Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. Nature 426:147–153

Garcia D, Collier SA, Byrne ME, Martienssen RA (2006) Specification of leaf polarity in *Arabidopsis* via the trans-acting siRNA pathway. Curr Biol 16:933–938

Guo HS, Xie Q, Fei JF, Chua NH (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for *Arabidopsis* lateral root development. Plant Cell 17:1376–1386

Himanen K, Boucheron E, Vanneste S, de Almeida, Engler J, Inzé D, Beeckman T (2002) Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell 14:2339–2351

Hunter C, Willmann MR, Wu G, Yoshikawa M, de la Luz Gutiérrez-Nava M, Poethig SR (2006) Trans-acting siRNA-mediated repression of ETTIN and ARF4 regulates heteroblasty in *Arabidopsis*. Development 133:2973–2981

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Laskowski MJ, Williams ME, Nusbaum HC, Sussex IM (1995) Formation of lateral root meristems is a two-stage process. Development 121:3303–3310

Montgomery TA, Howell MD, Cuperus JT, Li D, Hansen JE, Alexander AL, Chapman EJ, Fahlgren N, Allen E, Carrington JC (2008) Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. Cell 133:128–141

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JD (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312:436–439

Nemhauser JL, Feldman LJ, Zambryski PC (2000) Auxin and ETTIN in *Arabidopsis* gynoecium morphogenesis. Development 127: 3877–3888

Nogueira FT, Madi S, Chitwood DH, Juarez MT, Timmermans MC (2007) Two small regulatory RNAs establish opposing fates of a developmental axis. Genes Dev 21:750–755

Patrick A, Alan H, David CB, Nicholas PH (2004) Modulation of floral development by a gibberellin-regulated microRNA. Development 131:3357–3365

Pekker I, Alvarez JP, Eshed Y (2005) Auxin response factors mediate *Arabidopsis* organ asymmetry via modulation of KANADI activity. Plant Cell 17:2899–2910

Reinhardt D, Pesce ER, Stieger P, Mandel T, Baltensperger K, Bennett M, Traas J, Friml J, Kuhlemeier C (2003) Regulation of phyllotaxis by polar auxin transport. Nature 426:255–260

Reyes JL, Chua NH (2007) ABA induction of miR159 controls transcript levels of two MYB factors during *Arabidopsis* seed germination. Plant J 49:592–606

Sunkar R, Zhu JK (2004) Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. Plant Cell 16:2001–2019

Yang JH, Han SJ, Yoon EK, Lee WS (2006) Evidence of an auxin signal pathway, microRNA167-ARF8-GH3, and its response to exogenous auxin in cultured rice cells. Nucleic Acids Res 34:1892–1899

Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2:1565–1572

Yoon EK, Yang JH, Lim J, Kim SH, Kim SK, Lee WS (2009) Auxin regulation of the microRNA390-dependent transacting small interfering RNA pathway in *Arabidopsis* lateral root development. Nucleic Acids Res. doi:10.1093/nar/gkp1123

Yoshikawa M, Peragine A, Park MY, Poethig RS (2005) A pathway for the biogenesis of *trans*-acting siRNAs in *Arabidopsis*. Genes Dev 19:2164–2175