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# MicroRNAs Regulate Auxin Homeostasis and Plant Development

The phytohormone auxin (principally indole-3-acetic acid [IAA]) is a key regulator of cell expansion and division that plays numerous roles in plant growth and development, including stem elongation, phototropic and gravitropic responses, apical dominance, and lateral and adventitious root formation. Auxin can stimulate or inhibit cell growth depending on its concentration and location within the plant, and its interactions with other hormone signaling pathways. In stems and coleoptiles, applied auxin induces cell elongation at low ( $\mu\text{M}$ ) concentrations but inhibits elongation at higher (mM) concentrations. Auxin produced in the shoot apex inhibits the formation of lateral buds (apical dominance), but transported to the hypocotyls and root it promotes the production of adventitious and lateral roots, respectively. It has also been found to play a role in morphogenesis and patterning in leaves and floral organs. Normal plant development thus involves carefully controlled fluctuations in auxin biosynthesis, transport, accumulation, and degradation, which fine-tune auxin concentrations in specific tissues at specific stages of development to achieve the appropriate response.

In the past decade, there has been a growing understanding of the importance of protein degradation via the ubiquitin-proteasome pathway in regulating developmental processes and maintaining cellular homeostasis in eukaryotic organisms, and this pathway has been found to play a key role in auxin signaling in higher plants (Moon et al., 2004). Within the past several years, microRNA-mediated regulation of gene expression has emerged as another key regulator involved in numerous developmental processes (Bartel, 2004). In this issue of *The Plant Cell*, three reports from independent groups present data showing that auxin homeostasis and related developmental processes in *Arabidopsis* depend on microRNA-mediated regulation of key components of auxin signaling.

The effects of auxin on plant development are mediated by several transcription factor families, including the auxin response factors (ARFs) and NAC-domain transcription factors. Sorin et al. (pages ■■■) show that ARGONAUTE1 (AGO1), a key player in microRNA pathways, regulates auxin-induced adventitious root formation associated with its effect on the expression of *AUXIN RESPONSE FACTOR17* (*ARF17*) and auxin-inducible *GH3* genes that are presumed targets of *ARF17*. Meanwhile, Mallory et al. (pages ■■■) show that plants expressing a form of *ARF17* that is resistant to transcript cleavage mediated by the microRNA miR160 produce high levels of *ARF17* mRNA and have altered accumulation of *GH3*-like mRNAs associated with numerous dramatic growth defects. Finally, Guo et al. (pages ■■■) show that miR164-directed cleavage of *NAC1* mRNA affects auxin regulation of lateral root development and suggest a model for how microRNA-mediated regulation may function in maintaining auxin homeostasis.

### AGO1, ARF17, AND ADVENTITIOUS ROOTS

MicroRNAs are endogenously encoded ~22 nucleotide RNAs that target complementary mRNA transcripts for cleavage or translational repression via the RNA-induced silencing complex (RISC) (reviewed in Bartel, 2004). In plants, microRNAs are processed from hairpin precursor RNAs by an RNase III endonuclease known as DICER-LIKE1 (Park et al., 2002; Reinhart et al., 2002). The current model postulates that mature microRNAs are incorporated into the RISC, bind target mRNAs based on complementarity, and guide cleavage of mRNA targets with perfect or near perfect complementarity and translational repression of targets with lower complementarity (Bartel, 2004). The translational repression mechanism is common in animals but does

not appear to play a major role in plants. The identity and activities of RISC components are incompletely understood, but in all eukaryotes examined, a member of the AGO protein family is a principal RISC component (Hammond et al., 2001).

*Arabidopsis* AGO1 is the founding member of this family of proteins. Homozygous *ago1-1* mutants show a severely abnormal phenotype characterized by unexpanded, pointed cotyledons and narrow, almost succulent, rosette leaves (Bohmer et al., 1998). The plants remain very small and produce a single shoot with a terminal inflorescence. The sepals and petals are also narrow and pointed, the pistils and stamens are abnormal, and the plants are completely sterile. Because AGO1 is a core component of the RISC, the pleiotropic *ago1* phenotypes are thought to result from the overaccumulation of many microRNA-targeted messages; indeed, a variety of microRNA targets are overexpressed in *ago1* mutants (Vaucheret et al., 2004). To begin to dissect the pleiotropic defects of *ago1*, Sorin et al. focused on *ago1* hypocotyls. They found that *ago1* mutants also exhibit resistance to picloram (a synthetic auxin)-induced hypocotyl elongation and fail to form adventitious roots on the hypocotyl in response to endogenous auxin or applied synthetic auxin 1-naphthalene acetic acid (NAA). Interestingly, this defect was specific to shoots; both auxin-induced inhibition of primary root growth and stimulation of lateral root development were similar in mutant and wild-type plants.

Analysis of auxin content and the rate of auxin biosynthesis in *ago1* mutants relative to *superroot2* (*sur2*) mutants (which overproduce auxin and develop numerous adventitious roots on the hypocotyls) and in *ago1 sur2* double mutants suggested that AGO1 influences the overall regulation of auxin homeostasis in the hypocotyl. The investigation of several auxin-inducible genes revealed that the expression of several *GH3* genes in particular was

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downregulated in *ago1* mutant hypocotyls. Finally, the authors found that *ARF17* was significantly overexpressed in mutant hypocotyls. The authors suggest a model in which *ARF17* negatively regulates *GH3* gene expression and adventitious root formation in Arabidopsis, through its regulation by *AGO1*-dependent microRNA-mediated repression.

The importance of microRNA regulation of *ARF17* is also revealed by the work of Mallory et al. Rhoades et al. (2002) previously identified *ARF17* as a potential target of microRNA miR160, and Vaucheret et al. (2004) showed that *ARF17* mRNA levels were increased *ago1* mutants. Mallory et al. set out to investigate the nature of this regulation. The authors created transgenic plants expressing a modified version of *ARF17* that was resistant to cleavage mediated by miR160 but that did not change the amino acid sequence. Plants expressing this construct produced increased levels of cleavage-resistant *ARF17* mRNA and altered accumulation of several *GH3*-like mRNAs and exhibited dramatic defects in cotyledon, leaf, root, and flower development. Although the work of Sorin et al. focused on adventitious root development, the dramatic phenotypes of both the *ago1* and microRNA-resistant *ARF17* expressing plants suggest that *ARF17* and its regulation by a microRNA are crucial for the proper development of many organs.

### OTHER ROOTS AND OTHER PLANTS

The work of Sorin et al. and Mallory et al. suggest that *AGO1* functions with miR160 to repress *ARF17*, to allow proper auxin homeostasis and development. In a related article, Guo et al. found that miR164-directed cleavage of *NAC1* mRNA downregulates auxin signals for lateral root development. Xie et al. (2000) previously showed that *NAC1* functions in auxin induction of lateral root development, and Rhoades et al. (2002) identified *NAC1* as a potential target of microRNA regulation by miR164. Guo et al. confirmed that *NAC1* mRNA cleavage occurs in vivo and is directed by miR164. Similar to the approach of Mallory et al., they created

transgenic plants expressing a cleavage-resistant form of *NAC1* mRNA. These plants accumulate *NAC1* mRNA and significantly increase initiation of lateral roots, suggesting that microRNA-mediated cleavage is an important negative regulator of *NAC1* in wild-type plants. Xie et al. (2000) showed that *NAC1* is a transcriptional activator of the auxin-responsive genes *DBP*, which encodes another DNA binding protein of unknown function, and *AIR3*, which encodes a protein with homology to subtilisin-like proteases, and *NAC1* activity is associated with lateral root production.

Also in this issue, Inukai et al. (pages ■■■) show that *CROWN ROOTLESS1* (*CRL1*) is essential for crown root formation in rice and that it is a target of an ARF in the auxin signaling pathway. This work raises the intriguing question of whether the rice ARF (and, thus, its target *CRL1*) is subject to microRNA-mediated regulation. Although Arabidopsis is capable of forming adventitious roots, they do not play an important role in normal plant development in the species, or indeed in many other dicots. By contrast, rice and other monocots produce numerous crown roots, which are a type of adventitious root that is one of the dominant root types of cereals. The regulation of adventitious root formation in rice therefore is of considerable interest. Jones-Rhoades and Bartel (2004) found that numerous microRNAs, including miR160 and miR164, are conserved in rice and Arabidopsis. Another report in this issue, by Sunkar et al. (pages ■■■), presents the isolation and characterization of 35 microRNAs in rice, which include 13 new families that are not conserved in Arabidopsis. This presents an area that is ripe for further investigation.

### COMPLEXITIES...

MicroRNAs regulate many genes, and the readout is complex. For example, Sorin et al. found that disruption of *AGO1* resulted in overproduction of *ARF17* and reduced *GH3* expression in the hypocotyl. Mallory et al. similarly found that expression of microRNA-resistant *ARF17* was associated with overproduction of *ARF17* mRNA and reductions in *GH3* expression, and although

altered phenotypes were seen in root as well as shoot development, auxin responsiveness was maintained in roots and adventitious rooting was not reported. The phenotypic differences in the two studies are likely due to a general impairment of all microRNAs in the *ago1* mutant (Vaucheret et al., 2004) versus the misregulation of a single target in the Mallory et al. study. It is reasonable to expect that *ago1* mutant phenotypes result from the collective misregulation of many targets, including not only *ARF17*, but also *ARF6*, *ARF8*, *ARF10*, *ARF16*, *NAC1*, and *TIR1*, to name a few. However, there are intriguing similarities between *ago1* mutants and miR160-resistant *ARF17* plants, suggesting that *ARF17* overexpression contributes to a subset of the *ago1* phenotypes. Together, these studies suggest that microRNA-mediated regulation of *ARF17* is a major facet of auxin homeostasis and plant development, which functions at least in part via an effect on *GH3* gene expression.

It is notable that the results of Sorin et al. suggest that *AGO1* influences auxin homeostasis primarily in the apical part of the plant and not in the root (i.e., *ago1* mutants are affected in adventitious root but not lateral root formation) and that a target of *AGO1* activity in the hypocotyl is *ARF17*. On the other hand, Guo et al. show that microRNA-mediated regulation of *NAC1* is part of the auxin response pathway in the root, affecting lateral root development. Because *AGO1* is involved in the microRNA regulation of many targets (Vaucheret et al., 2004), this difference could suggest that *ago1* defects in root auxin homeostasis are compensated for by the effects of misregulation of targets with opposing functions, such as might be imagined if activating ARFs that are microRNA targets (*ARF6* and *8*) are opposed in some tissues by repressing ARFs that are microRNA targets (*ARF10*, *16*, and *17*). Also, although only *AGO1* has been implicated in microRNA functioning to date, Arabidopsis has a family of 10 *AGO* proteins, which may have evolved to serve specialized functions (Carmell et al., 2002). It is possible that *ARF17* and other microRNA targets are regulated by different *AGO* proteins in different tissues or stages of development.

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This could explain some of the differences in the results of Sorin et al. and Mallory et al.; in the *ago1* mutant, one or more other AGO proteins may be acting on *ARF17* in the root, whereas the overexpressed microRNA-resistant *ARF17* used by Mallory et al. may be resistant to the activity of all AGO proteins.

## AUXIN HOMEOSTASIS

All three reports (Guo et al., Mallory et al., and Sorin et al.) provide evidence that microRNAs regulate auxin homeostasis in *Arabidopsis*. The evidence for this is severalfold. First, and most obviously, disruption of *AGO1* or of the microRNA-mediated cleavage of *NAC1* target mRNA caused dramatic effects on auxin induction of adventitious and lateral root formation, respectively. Second, the *GH3* genes that are the putative targets of *ARF17* encode auxin-conjugating proteins (Staswick et al., 2005), and regulation of these genes may provide an important mechanism for controlling levels of free IAA (active auxin). *ARF17* may act as a repressor of some *GH3* genes because overaccumulation of *ARF17* mRNA is correlated with reduced levels of some *GH3* mRNAs. Reducing microRNA-mediated cleavage of *ARF17* mRNA therefore might be expected to increase levels of free IAA, as repression of *GH3* transcription would be increased, reducing the level of the corresponding auxin-conjugating proteins and resulting in less conjugation of IAA. However, Sorin et al. measured a significant reduction in endogenous levels of not only conjugated but also free IAA in *ago1* mutant plants. They noted that auxin biosynthesis was slightly lower in the mutant relative to wild-type plants, which could be due to a direct effect of *AGO1* on regulation of auxin biosynthesis or an indirect effect of disrupting overall auxin homeostasis.

Both Mallory et al. and Guo et al. also investigated the question of whether auxin affects microRNA levels. Mallory et al. isolated total RNA from wild-type seedlings at 0, 0.3, 2, and 24 h after treatment with 10  $\mu$ M IAA and could see no appreciable effect of the IAA treatment on levels of the miR160, miR164, or miR167 microRNAs (all

of which have been implicated in auxin signaling). In addition, levels of *ARF17* mRNA and the *ARF17* mRNA cleavage product did not appear to be affected by auxin treatment. Thus, it is clear that levels of these microRNAs are not dramatically responding to IAA as do many of the mRNAs encoding other repressors acting in auxin responses, such as the *Aux/IAA* transcriptional repressors and the *GH3*-like auxin conjugating enzymes. Mallory et al. concluded that "regulation by these three miRNAs may instead be needed to set components of the auxin-response machinery to proper levels so that tissues can respond appropriately to auxin."

Guo et al. also measured auxin induction of expression of miR164 in wild-type plants and were able to detect a subtle difference in levels after synthetic auxin treatment. *NAC1* is an early auxin response gene, and an increase in *NAC1* mRNA was seen within 30 min after treatment with the synthetic auxin NAA at a concentration of 2  $\mu$ M. Similar to the results of Mallory et al. with IAA, the authors did not observe any change in miR164 levels under these conditions. However, Guo et al. report a consistent  $\sim$ 1.5-fold increase of miR164 (but not of miR163, which is not implicated in auxin responses) at 6 to 8 h after treatment with 10  $\mu$ M NAA. Importantly, the increase in miR164 at 6 to 8 h was coincident with an increase in the levels of the *NAC1* mRNA cleavage product and a reduction in *NAC1* full-length mRNA, which increased by approximately twofold at 2 to 4 h after 10  $\mu$ M NAA treatment, and then decreased to approximately one-tenth the control level at 6 to 8 h after treatment. The specificity and reproducibility of this response was further confirmed by similar analysis using transgenic plants expressing cleavage-resistant *NAC1* mRNA and three auxin-insensitive mutants (see Figure 6 in Guo et al.). These results may warrant a reexamination of the response of miR160 and miR167 to auxin; perhaps treatment with a synthetic auxin, a higher IAA concentration, or a more extensive time course would reveal subtle fluctuations in the levels of these miRNAs in response to auxin as well. Guo et al. suggest that the slower kinetics of induction of miR164

may create a homeostatic mechanism that mediates clearance of *NAC1* mRNA after its initial induction by auxin.

These reports provide significant new information on microRNA-mediated regulation of plant development and help to paint a clearer picture of the nature of auxin homeostasis—as such, they constitute required reading for all students of plant developmental biology!

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