

MicroRNA Directs mRNA Cleavage of the Transcription Factor *NAC1* to Downregulate Auxin Signals for Arabidopsis Lateral Root Development

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Although several plant microRNAs (miRNAs) have been shown to play a role in plant development, no phenotype has yet been associated with a reduction or loss of expression of any plant miRNA. *Arabidopsis thaliana* miR164 was predicted to target five NAM/ATAF/CUC (NAC) domain-encoding mRNAs, including *NAC1*, which transduces auxin signals for lateral root emergence. Here, we show that miR164 guides the cleavage of endogenous and transgenic *NAC1* mRNA, producing 3'-specific fragments. Cleavage was blocked by *NAC1* mutations that disrupt base pairing with miR164. Compared with wild-type plants, *Arabidopsis mir164a* and *mir164b* mutant plants expressed less miR164 and more *NAC1* mRNA and produced more lateral roots. These mutant phenotypes can be complemented by expression of the appropriate *MIR164a* and *MIR164b* genomic sequences. By contrast, inducible expression of miR164 in wild-type plants led to decreased *NAC1* mRNA levels and reduced lateral root emergence. Auxin induction of miR164 was mirrored by an increase in the *NAC1* mRNA 3' fragment, which was not observed in the auxin-insensitive mutants *auxin resistant1 (axr1-12)*, *axr2-1*, and *transport inhibitor response1*. Moreover, the cleavage-resistant form of *NAC1* mRNA was unaffected by auxin treatment. Our results indicate that auxin induction of miR164 provides a homeostatic mechanism to clear *NAC1* mRNA to downregulate auxin signals.

INTRODUCTION

First discovered in *Caenorhabditis elegans*, microRNAs (miRNAs) were found recently to be expressed in several eukaryotes as well (Carrington and Ambros, 2003; Ambros, 2004). The initial cloning experiments of Reinhart et al. (2002) recovered 16 miRNAs from *Arabidopsis thaliana*, some of which were encoded by more than one genomic locus. These miRNAs were predicted to target mRNAs encoding transcription factors involved in plant development (Rhoades et al., 2002), and many such candidate mRNAs have been experimentally verified to be bona fide targets (Llave et al., 2002; Bartel, 2004). Subsequent computational analyses by three independent groups increased the number of potential *Arabidopsis* miRNAs to more than 100 (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Wang et al., 2004). The increasing biological importance of this group of small RNAs is

illustrated by the expanded list of target mRNAs, including those encoding proteins implicated in proteolysis, signaling, metabolism, ion transport, etc. These results suggest that besides controlling transcription factor mRNA abundance, miRNAs may also regulate a large number of other cellular processes (e.g., signaling pathways). In addition, direct sequencing of small RNAs from a stress-induced library has revealed several new miRNA families (Sunkar and Zhu, 2004), suggesting a role of these small RNAs in abiotic stress responses. Expression of miR393 and miR159 has been shown to be regulated by abscisic acid (Sunkar and Zhu, 2004) and gibberellin (Achard et al., 2004), respectively.

To date, the functions of plant miRNAs have been deduced from overexpression of precursor sequences encoding miRNAs and/or expression of target mRNAs that have been rendered cleavage-resistant by mutations (Bartel and Bartel, 2003; Bartel, 2004; Dugas and Bartel, 2004). Overexpression of miRNA precursors in transgenic plants leads to increased miRNA levels and decreased target mRNA levels; as expected, such transgenic plants phenocopy mutants with deficiencies of the target mRNA. Conversely, expression/overexpression of miRNA-resistant mutant mRNAs stabilizes the message and causes transgenic plants to display a phenotype similar to that obtained with target wild-type mRNA overexpression.

Further clarification of the biological role of miRNAs in plants will be aided by analysis of loss-of-function mutants that are

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directly affected in miRNA genes. Unfortunately, no complete loss-of-function mutant in any plant miRNA has yet been described. A recently characterized Arabidopsis T-DNA insertion mutant in the *MIR164b* locus (*mir164b-1*) produced 15-fold less miR164 than did the wild type, but no aerial phenotype was associated with this deficiency (Mallory et al., 2004a). This finding could be attributed to the fact that miR164 is encoded by at least three genomic loci in Arabidopsis (Reinhart et al., 2002). It should be pointed out that several Arabidopsis miRNAs are encoded by more than one genomic locus, thus rendering recovery of complete loss-of-function mutants difficult.

Predicted targets of miR164 include mRNAs encoding five members of the NAM/ATAF/CUC (NAC) domain transcription factor family (Rhoades et al., 2002). Of these, *NAC1* is involved in transmitting auxin signals for lateral root development (Xie et al., 2000, 2002) and *CUP-SHAPED COTYLEDON1* (*CUC1*)/*CUC2* are implicated in meristem development and separation of aerial organs (Aida et al., 1997), whereas the functions of *At5g07680* and *At5g61430* have not yet been defined. Recent work by two independent groups has provided evidence for miRNA-mediated regulation of *CUC1* (Mallory et al., 2004a) and *CUC2* (Laufs et al., 2004) mRNA levels in vivo. Transgenic plants overexpressing miR164 display altered floral organ numbers and fused vegetative and floral organs. By contrast, transgenic plants expressing miR164-resistant *CUC1* (Mallory et al., 2004a) or *CUC2* (Laufs et al., 2004) are defective in leaf development, and in the case of *CUC2* plants, they possess an enlarged boundary between sepals (Laufs et al., 2004). Whereas *NAC1* mRNA was cleaved at the expected site in wild-type plants (Mallory et al., 2004a), no changes in *NAC1* mRNA levels were detected in transgenic plants overexpressing miR164 (Laufs et al., 2004). The root phenotype of transgenic plants overexpressing miR164 was not investigated in these studies (Laufs et al., 2004; Mallory et al., 2004a).

We previously found that the transcription regulator NAC1 acts downstream of TRANSPORT INHIBITOR RESPONSE1 (TIR1) to transmit auxin signals promoting lateral root emergence (Xie et al., 2000) and that there is a positive correlation between *NAC1* mRNA levels and lateral root numbers. NAC1 was subsequently found to be an unstable protein, with its stability being regulated by the RING motif E3 ligase, SINAT5 (for Arabidopsis homolog of Drosophila protein SINA) (Xie et al., 2002). Because it is an unstable protein, we asked whether its mRNA stability is also regulated. Here, we confirm that *NAC1* mRNA is a target of miR164 in vivo (Rhoades et al., 2002; Mallory et al., 2004a). Analysis of transgenic plants and four *mir164* mutant alleles reveals that modest changes in miR164 levels can result in substantial changes in *NAC1* mRNA levels, and the transgenic plants and *mir164* mutants have the expected lateral root phenotypes correlating with *NAC1* mRNA abundance. Finally, we found that miR164 expression is late-auxin-responsive and that a major role of this miRNA is to clear *NAC1* mRNA to attenuate auxin signaling.

RESULTS

NAC1 mRNA Is Cleaved in Vivo

NAC1 is a transcription activator in the auxin signaling pathway for Arabidopsis lateral root development (Xie et al., 2000, 2002).

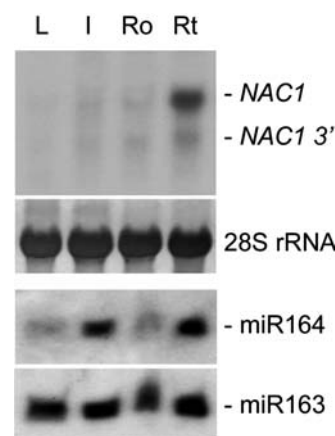


Figure 1. Expression Patterns of *NAC1* and miR164 in Wild-Type Arabidopsis RNA.

Gel blot analyses of *NAC1* mRNA and miR164 from wild-type (Col) leaves (L), inflorescences (I), 2-week-old seedling rosette leaves (Ro), and roots (Rt). A gel blot containing total RNA (10 μ g per lane) was hybridized with a *NAC1* 3'-specific fragment (nucleotides 682 to 1063; top gel). Intact *NAC1* mRNA and the *NAC1* 3' fragment resulting from miR164-guided cleavage are indicated. 28S rRNA visualized by methylene blue staining was used as a loading control (second gel). A gel blot containing low-molecular-weight RNA (30 μ g per lane) was hybridized with an end-labeled DNA oligonucleotide complementary to miR164 (third gel). The membrane was stripped and reprobed with an end-labeled DNA oligonucleotide complementary to miR163 for a loading control (bottom gel).

NAC1 mRNA accumulates mainly in roots (Figure 1). Besides the full-length *NAC1* mRNA, we consistently detected a shorter band (700 nucleotides) (Figure 1), which may be a 3' cleavage product of *NAC1* mRNA as it hybridized to a *NAC1* 3'-specific probe. Using 5' rapid amplification of cDNA ends (RACE), we amplified cDNA corresponding to this mRNA fragment. Sequence analysis of 10 independent cDNA clones produced identical results and placed the 5' end of the cleaved fragment at nucleotide 764 of the *NAC1* mRNA. This nucleotide position is located in the middle of the miR164/*NAC1* mRNA complementary region (Rhoades et al., 2002; Mallory et al., 2004a) (Figure 2A).

Plant miRNAs have recently been proposed to guide mRNAs for cleavage (Llave et al., 2002; Kasschau et al., 2003; Palatnik et al., 2003), and mRNAs for five NAC domain proteins, including *NAC1*, were predicted to be targets of miR164 (Rhoades et al., 2002; Laufs et al., 2004; Mallory et al., 2004a). RNA gel blot analysis showed that in Arabidopsis, miR164 expression levels were higher in roots and inflorescences compared with other organs (Figure 1) (Mallory et al., 2004a), whereas miR163 (Reinhart et al., 2002) accumulated to a similar level in all organs. The higher miR164 expression in roots suggests that this miRNA may target *NAC1* mRNA in vivo.

NAC1 mRNA Cleavage Is Directed by miR164

To obtain direct evidence that miR164 can mediate the cleavage of *NAC1* mRNA, we used a transient expression system involving *Agrobacterium tumefaciens* infiltration of *Nicotiana benthamiana*

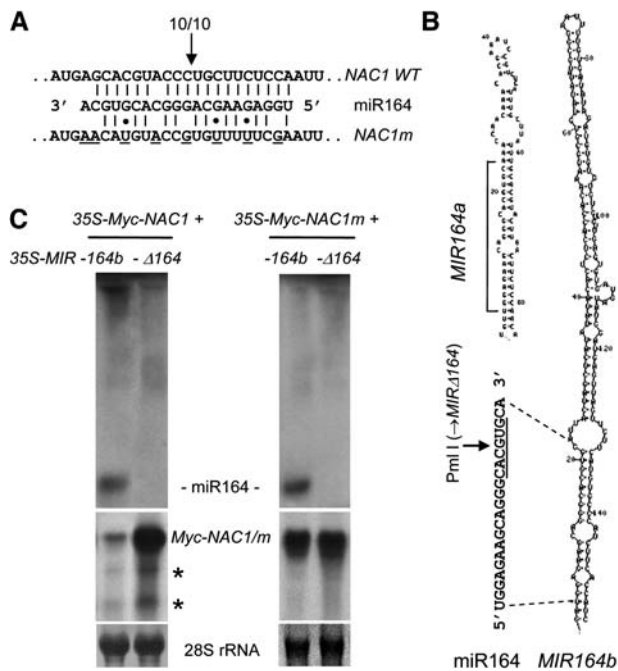


Figure 2. Determination of *NAC1* mRNA Cleavage by miR164 Using a Transient Expression System.

(A) Alignment of miR164 with *NAC1* (wild type) and *NAC1* mutant (*NAC1m*) mRNA. The *NAC1*/miR164 pair contains two mismatches in base pairing, whereas there are eight mismatches in the *NAC1* mutant (*NAC1m*/miR164). The mismatched nucleotides are underlined, and the predicted G-U base pairing is shown with dots. The arrow indicates the cleavage site between nucleotides 763 and 764 of the *NAC1* mRNA. Ten clones were sequenced, and an identical 5' end was obtained in all clones.

(B) miR164 sequence and the predicted structure of the *MIR164a* and *MIR164b* synthetic precursors. A deletion mutant, *MIRΔ164b* (contains only 5 nucleotides of miR164), was created by digestion of the precursor *MIR164b* sequence with *Pml*I to remove 16 nucleotides of the miR164 sequence.

(C) Effect of miR164 on *Myc-NAC1* (left) and *Myc-NAC1m* (right) transcripts in coinfiltration assays. Ten micrograms of low-molecular-weight RNAs (top gels) or 5 μg of total RNA (middle gels) from *N. benthamiana* leaf samples collected at 48 h after infiltration was loaded and hybridized with the appropriate probes as described for Figure 1. 28S rRNA was used as a loading control (bottom gels). The constructs used for coinfiltration are indicated above each lane. *Myc-NAC1* and *Myc-NAC1m* mRNA are indicated. Asterisks indicate additional bands presumably derived from cleavage of *Myc-NAC1* transcripts by *N. benthamiana* endogenous miRNA.

leaves (English et al., 1997). miR164 is presumably encoded by three genomic loci, *MIR164a*, *MIR164b*, and *MIR164c* (Reinhart et al., 2002; Dugas and Bartel, 2004; <http://www.sanger.ac.uk/cgi-bin/Rfam/mirna/browse.pl>). It has been shown that transcription of genomic sequences (~2 kb) containing the miRNA foldback region by a 35S promoter can produce the mature miRNA (Llave et al., 2002; Aukerman and Sakai, 2003; Chen, 2004; Parizotto et al., 2004). In our experiments, we used a 153-bp sequence derived from *MIR164b* as a synthetic precursor of miR164 (Figure 2B). As a negative control, we deleted from

the synthetic precursor sequences encoding 16 nucleotides of miR164 and designated the deletion mutant *35S-MIRΔ164* (Figure 2B). Figure 2C shows that the synthetic precursor *35S-MIR164b*, but not the deletion mutant *35S-MIRΔ164*, could produce miR164 in *N. benthamiana*. The levels of *NAC1* (*Myc-NAC1*) mRNA (Xie et al., 2002) were decreased significantly when coexpressed with *35S-MIR164b* but not *35S-MIRΔ164* (Figure 2C). This result provides direct evidence that miR164 produced from *35S-MIR164b* triggers *Myc-NAC1* mRNA cleavage. As a negative control, we introduced eight nucleotide mismatches into the miR164/*NAC1* mRNA complementary region of *NAC1* mRNA sequence without changing the encoded amino acids, and the mutant was designated *NAC1m* (Figure 2A). In contrast with *Myc-NAC1* mRNA, no cleavage was seen with *Myc-NAC1m* mRNA (Figure 2C), confirming the importance of the complementary region in miR164-mediated cleavage.

Transgenic Plants Expressing a Cleavage-Resistant Form of *NAC1* mRNA

We generated Arabidopsis transgenic plants expressing *35S-Myc-NAC1* and *35S-Myc-NAC1m*. Figure 3A shows that a 3' cleavage fragment of *Myc-NAC1* (500 nucleotides) was present in *35S-Myc-NAC1* transgenic plants but was not detected in *35S-Myc-NAC1m* transgenic plants. This observation indicates that the *Myc-NAC1m* transcript is cleavage-resistant and not a target of endogenous miR164, consistent with the transient coexpression results obtained from *N. benthamiana* (Figure 2C). In 7-d-old seedlings, no lateral root initials were apparent in the wild type, whereas short lateral roots and three to four lateral root initials were found in some *35S-Myc-NAC1* transgenic lines (data not shown). Most of the *35S-Myc-NAC1m* transgenic seedlings produced five to six lateral root initials and more short lateral roots compared with *35S-Myc-NAC1* lines (data not shown). At 12 d after germination (Figure 3B), the average number of lateral roots per centimeter of primary root for each line ($n = 20$) was 1.1 ± 0.12 , 3.8 ± 0.24 , and 4.9 ± 0.15 for the wild type (Columbia [Col]), *35S-Myc-NAC1*, and *35S-Myc-NAC1m*, respectively. Student's *t* test showed that the unit lateral root numbers differed significantly between *35S-Myc-NAC1* and *35S-Myc-NAC1m* lines ($P < 0.001$). Together with the observation that *Myc-NAC1m* was resistant to miR164-mediated cleavage, our results provide evidence that the regulation of *NAC1* mRNA levels by miR164 is an important control point for lateral root initiation. In most seedlings of transgenic lines carrying either *35S-Myc-NAC1* or *35S-Myc-NAC1m*, two lateral roots emerged at the hypocotyl/root junction and secondary lateral roots developed at a very early stage (data not shown).

Overexpression of miR164

To further confirm the regulation of *NAC1* mRNA by miR164, we introduced the synthetic precursor *MIR164b* into the 17-β-estradiol-inducible system, pX7 (Guo et al., 2003). The resulting construct, *pX7-MIR164* (*pX7-164*), was used to retransform Arabidopsis transgenic plants (Landsberg *erecta* [Ler]) expressing either *35S-Myc-NAC1* or *35S-Myc* (vector control) (Xie et al., 2002). Figure 4A shows that the inducer increased miR164 levels

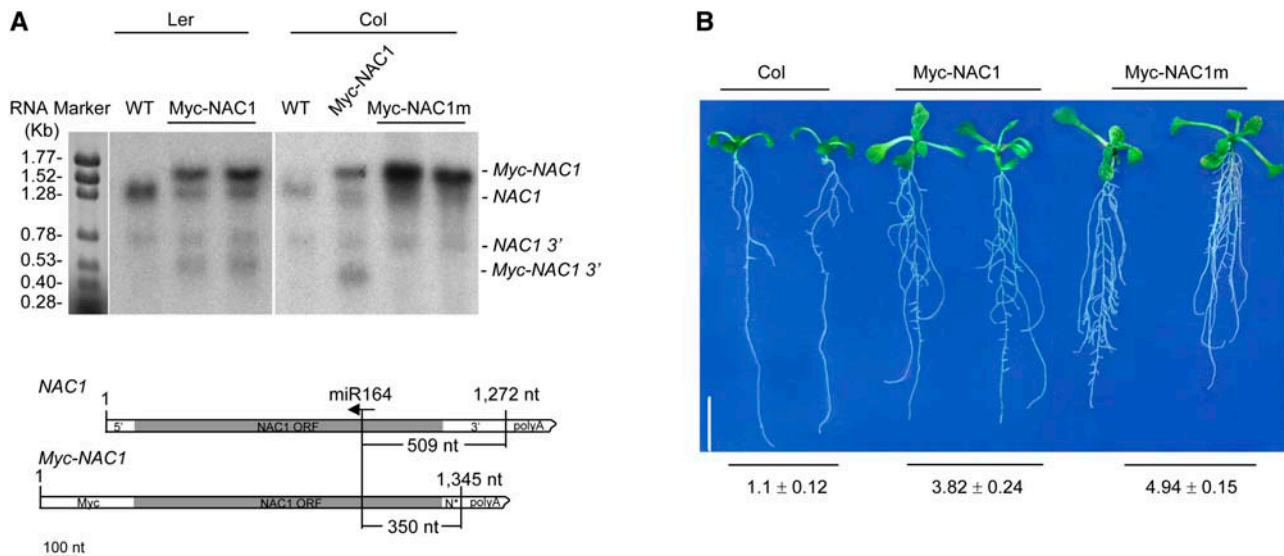


Figure 3. RNA Analysis and Phenotypes of 35S-*NAC1* Transgenic Plants.

(A) *Myc-NAC1* but not *Myc-NAC1m* transcripts were cleaved by endogenous miR164 (top). RNAs were extracted from roots of 2-week-old seedlings of the wild type (*Ler*), 35S-*Myc-NAC1* (*Ler*), the wild type (*Col*), 35S-*Myc-NAC1* (*Col*), and 35S-*Myc-NAC1m* (*Col*). Each lane contained 5 μ g of RNA, and blots were hybridized to a *NAC1* gene-specific probe as described for Figure 1. Full-length endogenous *NAC1* and *Myc-NAC1* mRNA and their respective 3' fragments resulting from miR164-guided cleavage are indicated. At bottom is a scheme of endogenous *NAC1* and *Myc-NAC1m* transcript constructs and the positions complementary to miR164.

(B) Effects of overexpression of 35S-*Myc-NAC1* and 35S-*Myc-NAC1m* on lateral root development. Wild-type (*Col*) and transgenic (*Col*) seedlings expressing 35S-*Myc-NAC1* or 35S-*Myc-NAC1m* transgene were grown on MS medium with 2% sucrose. Seedlings were photographed 12 d after germination. Lateral root initials were identified by staining whole seedlings with a mixture of toluidine blue and basic fuchsin and counted with a binocular microscope. Numbers of lateral roots per centimeter of primary root of each seedling (average \pm SE) for wild-type *Col*, 35S-*Myc-NAC1* (12 independent lines), and 35S-*Myc-NAC1m* (18 independent lines) are presented at bottom ($n = 20$). Bar = 1 cm.

by 2- to 10-fold (top panels, lanes 2, 4, and 6), indicating appropriate maturation of the synthetic precursor *MIR164b* transcript in *Arabidopsis*. Transgenic *Myc-NAC1* and endogenous *NAC1* mRNA levels were significantly reduced in inducer-treated roots (Figure 4A, middle panel, lanes 2, 4, and 6) compared with untreated controls (lanes 1, 3, and 5), although there was no detectable increase in the levels of *Myc-NAC1* mRNA 3' cleavage product. Changes in the *NAC1* mRNA levels were accompanied by the expected changes in lateral root initials (Figure 4B). Some double transgenic plant lines displayed shoot developmental defects upon inducer treatment (data not shown), presumably resulting from miR164-mediated cleavage of other *NAC* domain mRNAs (e.g., *CUC1/CUC2* mRNAs) (Laufs et al., 2004; Mallory et al., 2004a).

Because *NAC1* mRNA is expressed in lateral root initials, it could be argued that the reduction of *NAC1* mRNA levels in the induced *pX7-164* plants was attributable to the fewer number of lateral roots in the inducer-treated transgenic plants and not to the direct action of miR164. To investigate this possibility, we performed a shorter time course of induction with two independent lines of 35S-*Myc/pX7-164* plants. Figure 4C shows that 24 h of inducer treatment reduced *NAC1* mRNA levels by fourfold to fivefold, and during this period there was no decrease in lateral root initials/numbers. This result provides evidence that miR164-directed changes in *NAC1* mRNA levels precede changes in lateral root development.

Phenotypes of T-DNA Insertion Mutants in *MIR164a* and *MIR164b*

To investigate possible loss-of-function phenotypes, we obtained three T-DNA insertion mutants in *MIR164a* and one in *MIR164b* from the SALK collection of T-DNA insertion mutants. At 7 d after germination, seedlings of all four insertion mutants (*mir164a-1*, *mir164a-2*, *mir164a-3*, and *mir164b-1*) but not wild-type (*Col*) seedlings developed lateral roots (Figure 5A, top). Some mutant seedlings developed two lateral roots at the hypocotyl/root junction and produced secondary lateral roots at an early stage, similar to *Myc-NAC1* or *Myc-NAC1m* transgenic plants (data not shown). At 10 d after germination, mutant seedlings produced \sim 10-fold more lateral roots per unit of primary root than wild-type controls. Figure 5B shows the T-DNA insertion sites of these mutants, which have been verified by PCR and sequence analysis. In *mir164b-1*, the T-DNA was inserted in the loop region (+80) of the putative hairpin structure (Figures 2B and 5B) (Mallory et al., 2004a). This mutant is likely to be a miR164b null allele. For the three mutant alleles in the *MIR164a* locus, the T-DNA insertion sites are \sim 1.2 to 1.4 kb 5' of miR164a (Figure 5B). RNA gel blot analysis showed that miR164 expression was reduced two to three times not only in *mir164b-1* but also in *mir164a-1*, *mir164a-2*, and *mir164a-3* (Figure 5C). In all cases, miR163 expression was not affected (Figure 5C). These results suggest that miR164 expression was

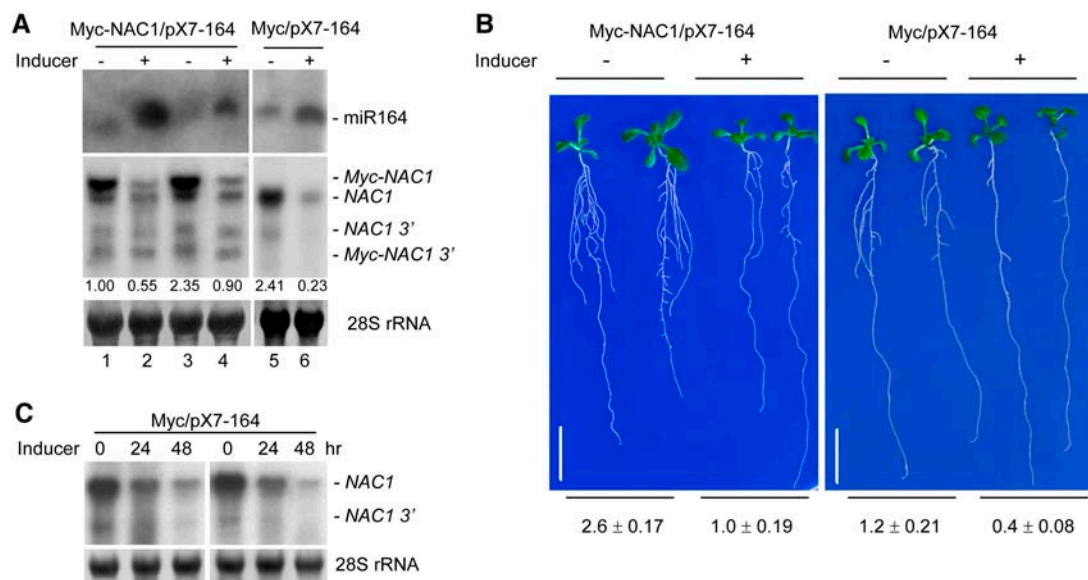


Figure 4. RNA Analysis and Phenotypes of Transgenic Plants with Inducible Expression of miR164.

(A) Induction of miR164 and effect on target *NAC1* mRNA accumulation. Seedlings containing either *35S-Myc-NAC1/pX7-164* or *35S-Myc/pX7-164* were germinated on selective medium for 6 d before being transferred to MS medium or MS + 17- β -estradiol (2 μ M). After 10 d, root samples were collected for analysis. Ten micrograms of low-molecular-weight RNAs (top gel) or 20 μ g of total RNA (middle gel) was loaded and hybridized with the appropriate probes as described for Figure 1. 28S rRNA was used as a loading control (bottom gel). miR164, full-length *Myc-NAC1*, and *NAC1* mRNAs and their derived 3' fragments are indicated. Plus and minus signs indicate with or without inducer treatment, respectively. Numbers below the middle gel indicate relative endogenous *NAC1* mRNA levels.

(B) Effect of inducible expression of miR164 on lateral root development. Representative plants from transgenic lines in **(A)** were photographed 10 d after transfer to medium with (+) or without (-) inducer. Numbers of lateral roots per centimeter of primary root (average \pm SE) were determined as described for Figure 3B. Bars = 1 cm.

(C) Effect of a shorter time course of induction on endogenous *NAC1* mRNA levels. Two independent lines of *35S-Myc/pX7-164* seedlings were germinated on selective medium for 9 d before being transferred to MS + 17- β -estradiol (2 μ M). Root samples were collected 24 and 48 h after treatment. Each lane contained 10 μ g of total RNA (top gel). Blots were hybridized with *NAC1* 3'-specific probes as described for Figure 1. 28S rRNA was used as a loading control (bottom gel).

affected in the *mir164a-1*, *mir164a-2*, and *mir164a-3* insertion mutants. The specific reduction of miR164 levels was accompanied by an increased *NAC1* mRNA level, but levels of *CUC3* mRNA, which is not targeted by miR164, remained unaltered (Figure 5C).

We introduced into the four T-DNA insertion mutants \sim 2.2 kb of the appropriate genomic sequences, including the miR164 coding region (Figure 2B). Expression of miR164 and *NAC1* was restored to wild-type levels, indicating complementation of the deficiency (Figure 5C). Phenotype analysis confirmed restoration of the wild-type root phenotype (Figure 5A, bottom).

Phenotypes of Plants with Disrupted miRNA Metabolism

The *Arabidopsis* mutant *dicer-like1* (*dcl1-9*) displays reduced miRNA accumulation (Park et al., 2002) because of a defect in miRNA biogenesis. Increased miRNA accumulation was seen in transgenic plants expressing P1/HC-Pro, a viral suppressor, but these plants phenocopy the miRNA-deficient *dcl1-9* because P1/HC-Pro interferes with miRNA functions (Kasschau et al., 2003). Figure 5D shows that miR164 abundance was reduced in *dcl1-9* but enhanced in P1/HC-Pro-overexpressing plants com-

pared with their respective wild-type controls. *NAC1* mRNA accumulation, however, increased in both *dcl1-9* and P1/HC-Pro plants relative to either wild-type control. Consistent with the higher accumulation level of *NAC1* mRNA, more lateral roots were observed in both *dcl1-9* and P1/HC-Pro plants (Figure 5E).

miR164 Clears *NAC1* mRNA during Auxin Response

As an early auxin-responsive gene, an increase in *NAC1* mRNA was evident within 30 min of 2 μ M 1-naphthalene acetic acid (NAA) treatment and reached a maximum level after 2 h (Xie et al., 2000, 2002). We found that under this condition, there was no change in miR164 levels (data not shown). However, at 10 μ M NAA, we consistently detected an increase of miR164, but not of miR163, by \sim 1.5-fold at 6 to 8 h after treatment (Figure 6A). Coincident with the miR164 increase at 6 h, there was a reduction in *NAC1* mRNA levels and a concomitant increase in the levels of *NAC1* mRNA 3' fragment (Figure 6A). This observation suggests that the reduction of *NAC1* mRNA levels after prolonged auxin treatment may result from miR164-mediated cleavage. The *NAC1* mRNA 3' fragment was unstable, as its intensity had decreased at 14 h after application of NAA.

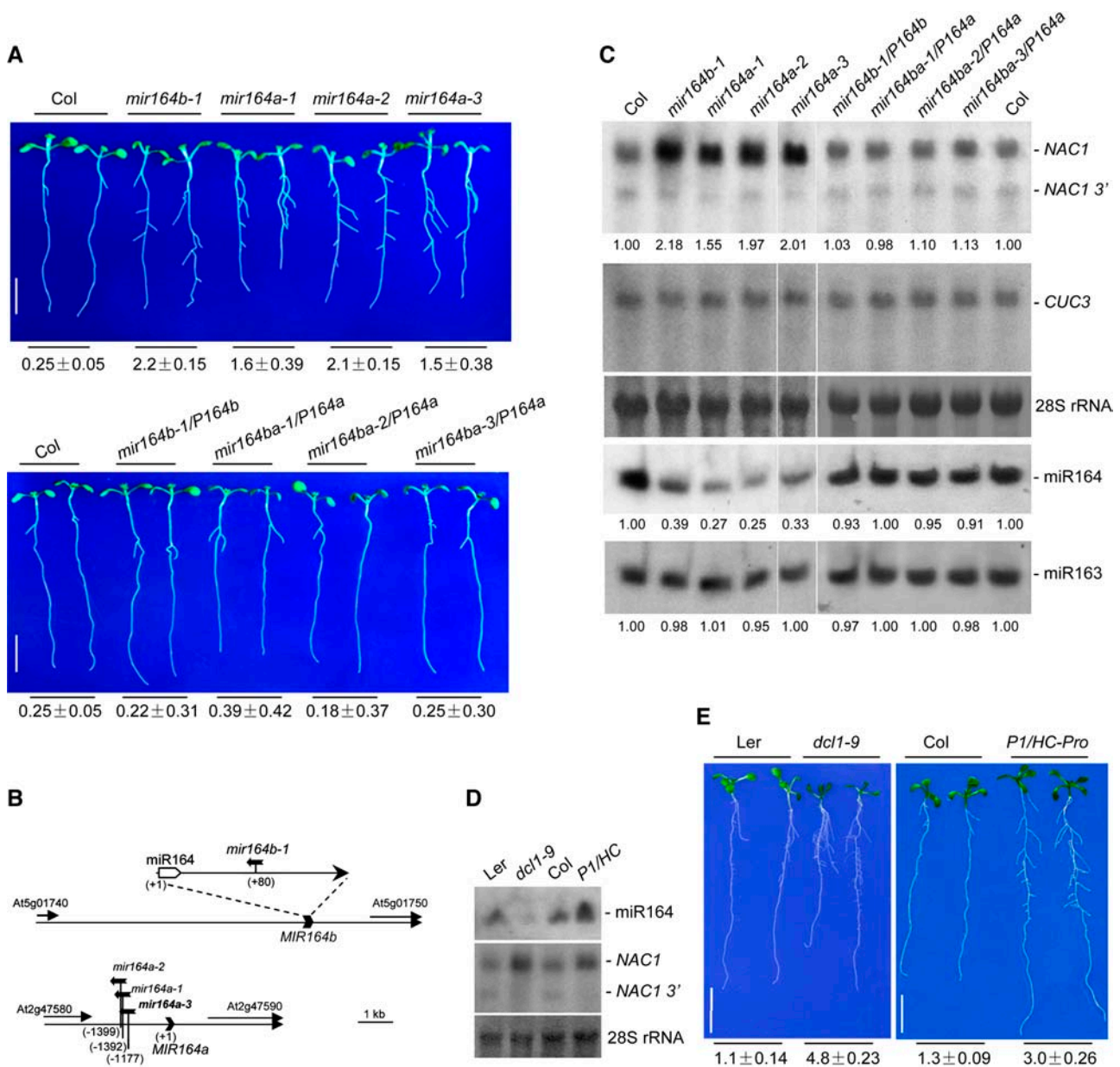


Figure 5. RNA Gel Blot Analysis and Root Phenotypes of *mir164a* and *mir164b* Mutants, *dcl1-9* Mutant, and P1/HC-Pro Transgenic Plants.

(A) Root phenotypes of wild-type Col, *mir164a*, and *mir164b* mutants (top) and of complemented lines (bottom). Seedlings were photographed 10 d after germination on MS medium with 2% sucrose. Bars = 1 cm.

(B) T-DNA insertion sites of *mir164b-1*, *mir164a-1*, *mir164a-2*, and *mir164a-3* relative to the annotated genes.

(C) RNA analysis of *mir164a* and *mir164b* mutants and complemented lines. Ten micrograms of total RNA (top gel) or low-molecular-weight RNAs (fourth gel) from roots of 10-d-old seedlings was loaded and hybridized with the appropriate probes as described for Figure 1. The filters were then stripped and rehybridized with a *CUC3* 3' gene-specific sequence (nucleotides 1273 to 1863; second gel) or with a 5' end-labeled DNA oligonucleotide complementary to miR163 (fifth gel) to serve as loading controls. 28S rRNA was used as a total RNA loading control (third gel). For each plant line, the relative accumulation levels of full-length *NAC1* mRNA, miR164, and miR163 are indicated below the lanes. Levels in wild-type Col were arbitrarily designated as 1.00.

(D) and (E) RNA analysis and root phenotypes of the *dcl1-9* mutant and P1/HC-Pro transgenic plants. *dcl1-9* (Ler) and P1/HC-Pro (Col) transgenic seedlings and their respective wild-type controls were grown on MS medium with 2% sucrose for 2 weeks.

(D) Root RNA was analyzed as described for Figure 1 using 10 μ g of low-molecular-weight RNAs (top gel) or 3 μ g of total RNA (middle gel). 28S rRNAs bands were used as loading controls (bottom).

(E) Representative plants were photographed. *dcl1-9* and P1/HC-Pro seedlings were identified by their developmental phenotypes. Bars = 1 cm. Numbers below (A) and (E) indicate the number of lateral roots per centimeter of primary root (average \pm SE) as described for Figure 3B ($n = 20$).

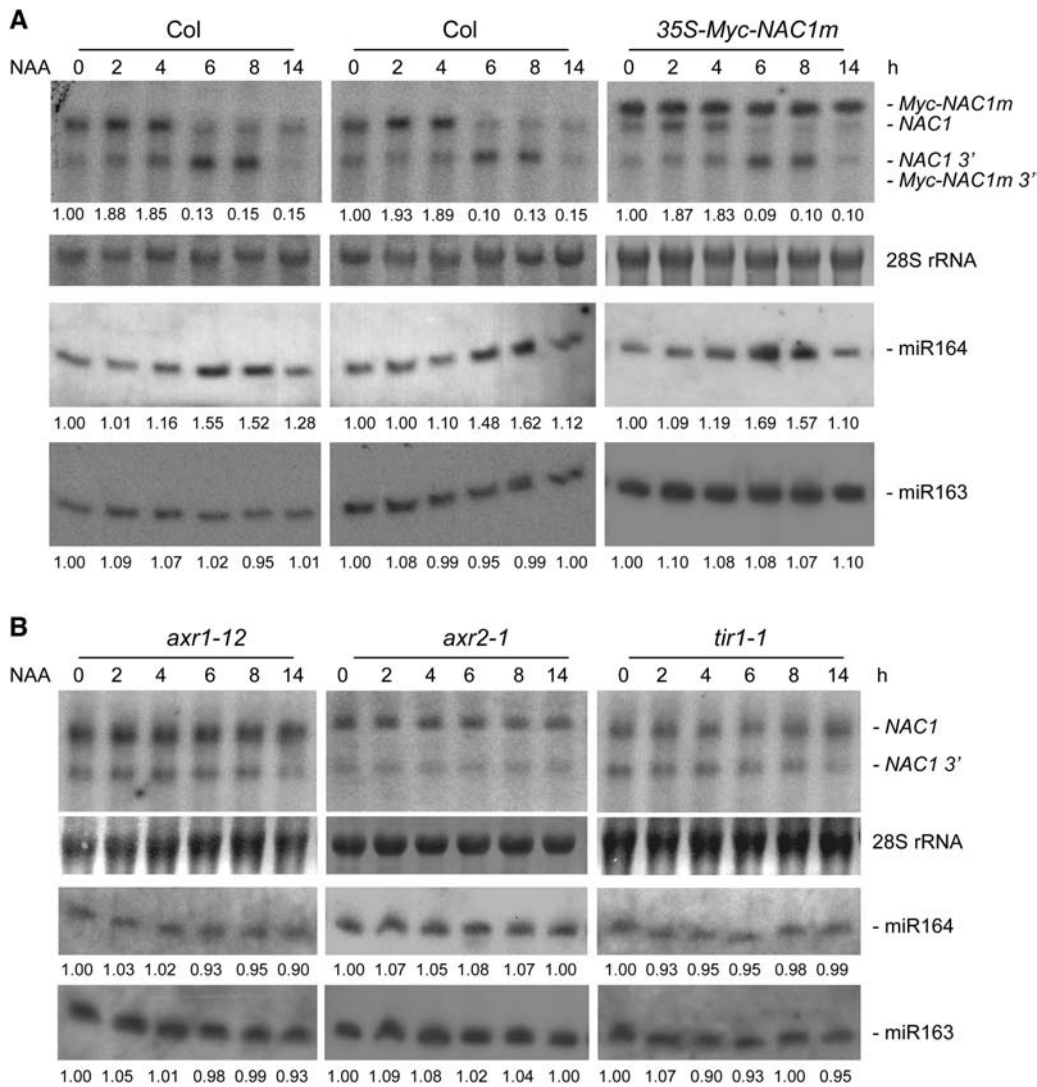


Figure 6. RNA Analysis of Wild-Type (Col) Plants, 35S-Myc-NAC1m Transgenic Plants, and *axr1-12*, *axr2-1*, and *tir1-1* Plants.

Ten-day-old seedlings were treated with NAA (10 μ M), and root samples were collected at the times indicated at top. Each lane contained 10 μ g of total RNA (top two gels) or low-molecular-weight RNAs (bottom two gels). Blots were hybridized with the appropriate probes as described for Figure 1. Stripped low-molecular-weight RNA filter was rehybridized with a miR163 probe (bottom gels). 28S rRNA was used as a loading control (second gel). Numbers indicate relative levels of *NAC1* mRNA (top gel), miR164 (third gel), and miR163 (bottom gel). Relative mRNA levels (average \pm SE) are 1.89 ± 0.03 (2 h), 1.86 ± 0.03 (4 h), 0.11 ± 0.02 (6 h), and 0.12 ± 0.02 (8 h) for *NAC1* and 1.03 ± 0.05 (2 h), 1.15 ± 0.05 (4 h), 1.57 ± 0.05 (6 h), and 1.57 ± 0.10 (8 h) for miR164. Positions of full-length endogenous *NAC1*, transgenic *Myc-NAC1* mRNA, their respective 3' fragments, and miR164 and miR163 are shown.

The specificity and reproducibility of this auxin-induced cleavage were further confirmed by analysis of transgenic plants expressing 35S-Myc-NAC1m. Figure 6A shows that the transgenic *Myc-NAC1m* mRNA was resistant to auxin-induced cleavage and the endogenous *NAC1* mRNA remained susceptible. As additional controls, we also investigated miR164 and *NAC1* mRNA levels in three *Arabidopsis* auxin-insensitive mutants, *auxin resistant1* (*axr1-12*) (del Pozo and Estelle, 1999; del Pozo et al., 2002), *axr2-1* (Timpte et al., 1994), and *tir1-1* (Gray et al., 2001),

and found no changes in either miR164 levels or *NAC1* mRNA levels with auxin treatment (Figure 6B). AXR1 and TIR1 are both implicated in the auxin-induced rapid degradation of indoleacetic acid repressors (Gray et al., 2001), whereas *axr2-1* expresses a dominant mutant of Indoleacetic Acid7 (Timpte et al., 1994). Our results confirm that the increase of miR164 levels and of miR164-mediated *NAC1* mRNA cleavage is, in part, auxin-dependent. The slower induction kinetics of miR164 suggests that it mediates the clearance of *NAC1* mRNA, which is induced faster.

DISCUSSION

miR164 Mediates *NAC1* mRNA Cleavage in Vivo

Plant miRNAs have been implicated in the control of leaf morphogenesis (Palatnik et al., 2003), leaf polarity (Kidner and Martienssen, 2004; Juarez et al., 2004; Mallory et al., 2004b), flowering time (Aukerman and Sakai, 2003; Achard et al., 2004; Chen, 2004), and flower development (Laufs et al., 2004; Mallory et al., 2004a). In contrast with animal miRNAs, most plant miRNAs have been shown to mediate cleavage of their target mRNAs (Dugas and Bartel, 2004), whereas only miRNA172 has been implicated in translational control (Aukerman and Sakai, 2003; Chen, 2004). Using coexpression in *N. benthamiana*, we show here that miR164 directs *NAC1* mRNA cleavage in vivo at a position complementary to the 10th nucleotide from the 5' end of miR164 and that disruption of the base-pairing region compromises cleavage. These results confirm and extend previous findings (Rhoades et al., 2002; Mallory et al., 2004a). It is possible that *NAC1* mRNA cleavage might be developmentally regulated in Arabidopsis, because no cleavage was detected in flowers of transgenic plants overexpressing miR164 (Laufs et al., 2004).

miR164 Negatively Regulates Lateral Root Development

By manipulating miR164 levels or expressing the cleavage-resistant *NAC1m* mRNA in vivo, we have provided several lines of evidence that this miRNA functions as a negative regulator of auxin-mediated lateral root development by controlling *NAC1* mRNA levels. (1) Plants overexpressing *NAC1m* produce more lateral roots compared with *NAC1*-overexpressing plants. (2) Mutant plants (*dcl1-9*) defective in miRNA biogenesis possess higher *NAC1* mRNA levels and develop more lateral roots. (3) Conditional overexpression of miR164 reduces *NAC1* mRNA levels as well as lateral root numbers. (4) Mutants partially affected in miR164 production accumulate higher levels of *NAC1* mRNA and produce more lateral roots. In all of these instances, there is a strict inverse correlation between changes in miR164 levels and changes in both *NAC1* mRNA levels and lateral root initials. An exception is seen in P1/Hc-Pro transgenic plants, which accumulate miR164 but in which cleavage of *NAC1* mRNA is blocked by the viral suppressor. Therefore, it is not surprising that these plants maintain high *NAC1* mRNA levels and develop more lateral roots.

We have characterized four T-DNA insertion mutants of Arabidopsis that accumulate reduced miR164 levels. All of these mutants have a single T-DNA insertion in either *MIR164a* (three alleles) or *MIR164b* (one allele), and the phenotype segregates with the T-DNA. In addition to these two loci, Arabidopsis contains a third locus, *MIR164c*, which can potentially produce miR164 (Dugas and Bartel, 2004). However, their relative contributions to miR164 expression are not clear. Nonetheless, our results show that both *MIR164a* and *MIR164b* contribute to miR164 expression in roots, as mutations in these loci reduce miR164 levels but do not eliminate expression totally. Based on the site of T-DNA disruption (Figure 5B) (Mallory et al., 2004a),

mir164b-1 is likely to be a miR164b null allele. The T-DNA insertion sites of the three *mir164a* mutant alleles are located ~1.2 kb upstream of the first nucleotide of miR164 (Figure 5B). Because of their reduced miR164 expression, we propose that transcription of this locus is affected in all three mutants. Notwithstanding the functional redundancy, a partial decrease (twofold to threefold) of miR164 level is sufficient to increase *NAC1* mRNA expression levels by approximately twofold, indicating a fine balance between the regulator and the regulated mRNA.

Recent results have shown that in both animals and plants, miRNAs are transcribed from Pol II promoters (Kurihara and Watanabe, 2004; Parizotto et al., 2004). In our complementation experiments, we used genomic fragments with ~1.9- to 2.25-kb upstream sequences. Because rescue of molecular and morphological phenotypes was seen in the complemented lines, we conclude that these genomic fragments contain the requisite signals for miR164a and miR164b expression.

Auxin Regulation of miR164

Several plant miRNAs regulate or are predicted to regulate mRNAs involved in various phytohormone signaling pathways (Rhoades et al., 2002; Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Wang et al., 2004). To date, however, only miR159 has been shown to respond to gibberellins (Achard et al., 2004) and miR393 to abscisic acid (Sunkar and Zhu, 2004). Here, we show that miR164 levels in Arabidopsis roots can be increased by another phytohormone, auxin, through a signaling pathway dependent on AXR1, AXR2, and TIR1. These signaling components are known to be required for auxin-mediated lateral root development (Timpte et al., 1994; del Pozo and Estelle, 1999; Gray et al., 2001; del Pozo et al., 2002). Time-course studies showed that the miR164 increase, which occurs late in auxin treatment (after 6 h), is rather modest, being only 1.5-fold. Nevertheless, there is a clear and concomitant effect on *NAC1* mRNA, whereas cleavage-resistant *NAC1m* mRNA is unaffected (Figure 6A). Consistent with the results obtained with the miR164 mutants, these results show that cleavage of *NAC1* mRNA requires only a modest increase in its regulator. *NAC1* has been shown to be expressed early (1 to 2 h) in pericycle cells during auxin-induced lateral root initiation (Xie et al., 2000, 2002). The modest auxin-induced increase of miR164 might result from restricted expression in these specialized cells but at a later developmental time. This issue remains to be resolved by future in situ hybridization experiments.

NAC1 is a transcriptional activator for auxin-induced lateral root initiation (Xie et al., 2000) that has been shown to be unstable (Xie et al., 2002). The *NAC1* instability is attributable to its polyubiquitination by the SINAT5 E3 ligase, which targets it for degradation by 26S proteasomes (Xie et al., 2002). Our work here extends the signal desensitization to *NAC1* mRNA levels, which, together with targeted *NAC1* degradation, ensures a more rapid signal suppression. The induction of miR164, at least in part, by auxin suggests an autoregulatory loop by which the miRNA mediates the clearance of *NAC1* mRNA to attenuate and terminate auxin signaling. A corollary of this observation is that we expect many mRNA targets of miRNAs to encode unstable

proteins such as NAC1, because unstable mRNAs are less likely to encode stable proteins.

Among the five NAC domain mRNAs targeted by miR164, *CUC1* and *CUC2* are involved in cotyledon separation and meristem formation (Aida et al., 1997; Furutani et al., 2004). Expression of these two genes is repressed by auxin, and a localized increase in auxin concentration has been shown to phenocopy *cuc1 cuc2* double mutant plants (Furutani et al., 2004). Transgenic plants overexpressing miR164 also have a similar phenotype (Laufs et al., 2004; Mallory et al., 2004a). It is possible that the *CUC1/CUC2* repression is brought about, at least in part, by an auxin-induced increase in miR164 expression, as shown here. Future work should be directed toward understanding the mechanism of miRNA164 regulation by phytohormones.

METHODS

Plasmid Construction

Genomic sequence (153 bp) containing the *MIR164b* foldback was used as a synthetic precursor sequence (Figure 2B). The sequence was amplified from *Arabidopsis thaliana* genomic DNA by PCR using two primers, 164b5' (5'-GATGGAGAAGCAGGGCACGTGCATTAC-3') and 164b3' (5'-GATGGTGAAGATGGGCACATGAAGAAC-3'), and cloned into pGEM-T Easy Vector (Promega, Madison, WI), giving pT-MIR164b, which was verified by sequencing. A *NcoI*/Klenow-*SacI* fragment from pT-MIR164b containing the *MIR164b* sequence was then cloned into the *SmaI* and *SacI* sites in pCAMBIA-1300 (AF234296) between the 35S promoter and the NOS 3' poly(A) addition signal. The resulting construct was named *35S-MIR164b*. The deletion mutant *35S-MIRΔ164* was created by digesting *35S-MIR164b* with *Bam*HI/Klenow+*PmlI* followed by self-ligation to remove sequences encoding 16 of the 21 nucleotides of the miR164 sequence (Figure 2B). A *SphI*/Klenow-*SpeI* fragment from pT-MIR164b containing the *MIR164b* sequence was also cloned into pX7-GFP (Guo et al., 2003) digested with *XhoI*/Klenow+*SpeI* to give *pX7-164*. For complementation of the three *mir164a* mutants, we used the construct *p164a-164a-NOS*, which contains a 5' upstream fragment (−1911 to +1, with +1 being the first nucleotide of *mir164*) of *MIR164a*, the synthetic *MIR164a* precursor sequence, and the NOS poly(A) addition sequences. The synthetic *MIR164a* precursor contains 84 bp, which includes the foldback region of miR164 (Figure 2B). For complementation of the *mir164b-1* mutant, we used the construct *p164b-164b-NOS*, which contains a 5' upstream fragment of *MIR164b* (−2255 to +1, with +1 being the first nucleotide of *mir164b*), the synthetic *MIR164b* precursor (Figure 2B), and the NOS poly(A) addition sequences.

Site-directed mutagenesis of the *NAC1* cDNA in the nucleotide sequence of the miR164 complementary site was performed with two primers in opposite orientations: *NAC1m5'* (5'-CATCATCAATGAACATGTACCGTGTTCGAATTTGTACAGAACCAAACC-3') and *NAC1m3'* (5'-CTGTGACAAATTCGAAAACACGGTACATGTTTCATTGATGATGTA-GTGATG-3'). *NAC1* cDNA intermediate plasmid pBS-*NAC1* (Xie et al., 2000) was used as template, using the QuickChange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The resulting DNA was digested with *DpnI* to eliminate the methylated, nonmutated original template DNA before transformation of *Escherichia coli* DH5 α . Correct mutagenesis was verified by sequencing. A *HincII-XhoI* (586 bp) fragment from pBS-*NAC1m*, which contains the mutations, was cloned into *35S-Myc-NAC1* (Xie et al., 2000) digested with *SpeI*/Klenow+*XhoI* to replace the corresponding original wild-type sequence. The resulting clone was designated *35S-Myc-NAC1m*.

Plant Materials and Transformation

Arabidopsis (Ler) plants expressing either *35S-Myc-NAC1* or *35S-Myc* (vector control) (Xie et al., 2002) were used for retransformation with *pX7-MIR164* (Guo et al., 2003). Wild-type *Arabidopsis* (Col) was used for *35S-Myc-NAC1* and *35S-Myc-NAC1m* transformation. Transgenic plants were generated by the floral dip method (Clough and Bent, 1998). Double transgenic plants (*Myc-NAC1/pX7-164* and *Myc/pX7-164*) were selected on MS medium (Sigma-Aldrich, St. Louis, MO) containing 10 μ g/mL Basta and 20 μ g/mL hygromycin B. For chemical-induced expression of miRNA, 6-d-old T2 seedlings germinated on selective medium were transferred to inductive medium containing 2 μ M 17- β -estradiol. *Myc-NAC1* and *Myc-NAC1m* transgenic *Arabidopsis* (Col) plants were selected on MS medium containing 10 μ g/mL Basta. Twelve *Myc-NAC1* and 18 *Myc-NAC1m* T2 single-copy lines were selected for the population study of lateral root phenotype.

The T-DNA insertion mutants *mir164b-1* (SALK_136105), *mir164a-1* (SALK_061177), *mir164a-2* (SALK_068270), and *mir164a-3* (SALK_047619) were obtained from the ABRC. The SALK_136105 line obtained from the ABRC was homozygous with respect to kanamycin resistance, and the single-copy T-DNA was inserted into the loop of the predicted hairpin structure (Mallory et al., 2004a) (Figure 2B). Lines from SALK_061177, SALK_068270, and SALK-047619 showed a 3:1 segregation of kanamycin resistance, indicating a single T-DNA insertion in each. The T-DNA insertion positions of all *mir164* mutants were confirmed by PCR using primers LBa1 (SALK T-DNA primer, 5'-TGGT-TCACGTAGTGGGCCATCG-3') and P164b (5'-GGGTAGCATGTTCC-ATGGGTGAGCATGCTGTCTCC-3') for *mir164b-1* and LBa1 and P164a (5'-GCCTTCTTCCATATTTGATCAGATAACTGAAGACC-3') for *mir164a-1*, *mir164a-2*, and *mir164a-3*. Homozygous mutant lines were selected for RNA and phenotypic analysis.

The mutants *dcl1-9* (Park et al., 2002), *axr1-12* (del Pozo and Estelle, 1999), *axr2-1* (Timpote et al., 1994), and *tir1-1* (Gray et al., 2001) and P1/Hc-Pro transgenic plants (Kasschau et al., 2003) have been described.

Plant Growth Conditions, Auxin Treatment, and Observation of Root Phenotypes

Arabidopsis seeds were surface sterilized with 20% bleach and 0.01% Triton X-100 and washed three times with sterile water. Sterilized seeds were suspended in 0.15% agarose and plated on MS medium. Plates were vernalized in darkness for 2 d at 4°C and then transferred to a tissue culture room at 22°C under a 16-h-light/8-h-dark photoperiod. For observation of root phenotypes, seedlings were grown on vertical MS agar medium containing 2% sucrose. Lateral root initials were identified by staining whole seedlings with a mixture of toluidine blue and basic fuchsin and counted with a binocular microscope. For auxin treatment, seedlings were grown vertically on MS agar medium for 12 d, then incubated with 10 μ M NAA. Root samples were collected at various times for RNA analysis.

Potted plants were grown in growth chambers at 22°C and 75% humidity under a 16-h-light/8-h-dark photoperiod.

Agrobacterium tumefaciens Infiltration in *Nicotiana benthamiana*

The constructs *35S-MIR164b*, *35S-MIRΔ164*, *35S-Myc-NAC1*, and *35S-Myc-NAC1m* were transformed into *A. tumefaciens* strain EHA105 by electroporation and selected on Luria-Bertani medium containing rifampicin at 10 μ g/mL and kanamycin at 50 μ g/mL (for *35S-MIR164b* and *35S-MIRΔ164*) or spectinomycin at 100 μ g/mL (for *35S-Myc-NAC1* and *35S-Myc-NAC1m*). *Agrobacterium* cells were infiltrated into leaves of *N. benthamiana* as described (English et al., 1997). For coinfiltration experiments, equal volumes of an *Agrobacterium* culture containing *35S-MIR164b* or *35S-MIRΔ164* (OD₆₀₀ = 1.75) and *35S-Myc-NAC1* or

35S-*Myc-NAC1m* (OD₆₀₀ = 0.25) were mixed before infiltration into *N. benthamiana* leaves.

RNA Isolation and Analysis

Total RNA was isolated from plant tissues by LiCl precipitation (Verwoerd et al., 1989). For RNA gel blot analysis, total RNA was separated on a 1.2% agarose gel containing 6% formaldehyde and transferred to Hybond-N⁺ membranes. To specifically detect *NAC1* transcripts, blots were hybridized with a *Bam*HI-*Sal*I fragment from pBS-*NAC1* encoding the *NAC1* C-terminal domain labeled with [α -³²P]dCTP using a Ready-primed labeling kit (Amersham International, Buckinghamshire, UK). To specifically detect *CUC3* transcripts, the membrane was stripped and reprobbed with a PCR fragment encoding the *CUC3* C-terminal domain. Low-molecular-weight RNAs were precipitated from the LiCl supernatant fraction of total RNA extraction with three volumes of ethanol. Low-molecular-weight RNAs were separated by electrophoresis on denaturing 17% polyacrylamide gels, and miRNA gel blot hybridizations were performed as described (Reinhart et al., 2002). DNA oligonucleotides complementary to miR164 or miR163 were end-labeled using T4 polynucleotide kinase (Roche Applied Science, Penzberg, Germany) and used for hybridizations. Signal intensity was measured using a PhosphorImager (Bio-Rad, Hercules, CA).

Mapping of miRNA-Guided Cleavage Site

The FirstChoice RLM-RACE kit (Ambion, Austin, TX) was used for RLM-RACE assay according to the manufacturer's instructions. Briefly, total RNAs were extracted from roots of wild-type *Arabidopsis* (Col) seedlings, and poly(A) mRNA was purified using an Oligotex mRNA mini kit (Qiagen, Valencia, CA). Poly(A) mRNA was directly ligated to the RLM-RACE 5' RACE RNA Oligo adaptor (45 nucleotides) from the FirstChoice RLM-RACE kit (Ambion). The oligo(dT) (15-mer) primer was used to prime cDNA synthesis with reverse transcriptase. The resulting cDNA was used for the first round of nested PCR using the 5' RACE Outer Primer together with a *NAC1* gene-specific primer (5'-GCAATTC³CAACAGTGCT-TGG-3') complementary to nucleotides 1040 to 1060 of the *NAC1* mRNA sequence. The 5' RACE Inner Primer and the *NAC1* gene-specific primer were used for the second round of nested PCR. Gel-purified PCR products were cloned into pGEM-T Easy vector (Promega) for sequencing.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AAF21437 (*NAC1*) and AF234296 (pCAMBIA-1300).

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MicroRNA Directs mRNA Cleavage of the Transcription Factor *NAC1* to Downregulate Auxin Signals for Arabidopsis Lateral Root Development

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