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RESEARCH ARTICLE

A Gain-of-Function Mutation in *IAA28* Suppresses Lateral Root Development

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The phytohormone auxin is important in many aspects of plant development. We have isolated an auxin-resistant Arabidopsis mutant, *iaa28-1*, that is severely defective in lateral root formation and that has diminished adult size and decreased apical dominance. The *iaa28-1* mutant is resistant to inhibition of root elongation by auxin, cytokinin, and ethylene, but it responds normally to other phytohormones. We identified the gene defective in the *iaa28-1* mutant by using a map-based positional approach and found it to encode a previously uncharacterized member of the *Aux*/*IAA* gene family. *IAA28* is preferentially expressed in roots and inflorescence stems, and in contrast to other *Aux*/*IAA* genes, *IAA28* transcription is not induced by exogenous auxin. Studies of the gain-of-function *iaa28-1* mutant suggest that IAA28 normally represses transcription, perhaps of genes that promote lateral root initiation in response to auxin signals.

INTRODUCTION

Auxins are plant growth hormones implicated in numerous developmental processes, including lateral root initiation, vascular tissue differentiation, establishment of apical dominance, and tropic responses (Davies, 1995). Many auxin responses result from changes in cell expansion and division, but these responses can vary in different tissues. For example, auxin promotes cell division in root pericycle cells, which leads to lateral root formation (Laskowski et al., 1995), but inhibits cell division in lateral meristems of the shoot, resulting in apical dominance (Hillman, 1984). The most common naturally occurring auxin is indoleacetic acid (IAA). Despite the importance of IAA in plant growth and development, the molecular details of auxin action remain largely unknown.

Auxin rapidly and specifically alters transcript levels of numerous genes (Abel and Theologis, 1996), and many auxin effects may be mediated through changes in gene expression. Although numerous genes have been identified based on strong transcriptional responses to auxin, only a small number of these genes have well-understood functions. For instance, the Arabidopsis *ACS4* gene, which encodes an ethylene biosynthetic enzyme, is rapidly induced by auxin (Abel et al., 1995a), which correlates with increased ethylene biosynthesis in response to auxin (Yang and Hoffman, 1984). The functions of the *SAUR* (for small auxin up RNA) genes, a family isolated on the basis of auxin-responsive transcription (McClure et al., 1989), are only beginning to be elucidated, but the observation that a maize SAUR protein binds calmodulin suggests a role for calcium in auxin signal transduction (Yang and Poovaiah, 2000).

Aux/IAA family members were originally identified in pea because of their strong and rapid transcriptional induction in response to auxin (Theologis et al., 1985). On the basis of auxin-induced transcription and sequence homology, *Aux/IAA* genes have also been isolated in several other species (Walker and Key, 1982; Ainley et al., 1988; Conner et al., 1990; Yamamoto et al., 1992; Abel et al., 1994). In Arabidopsis, nearly 20 *Aux/IAA* genes have been described (Abel et al., 1995b; Kim et al., 1997). Analysis of 14 of these transcripts has revealed differing developmental expression as well as varied profiles of induction by exogenous auxin, ranging from strong increases in transcript levels within minutes to weak increases after several hours (Abel et al., 1995b).

Many *Aux/IAA* genes are rapidly induced not only by auxin but also by the translational inhibitor cycloheximide (Theologis et al., 1985; Abel et al., 1995b), suggesting that *Aux/IAA* transcripts are unstable or that *Aux/IAA* transcription is normally repressed by short-lived proteins. The observation that some Aux/IAA proteins are extremely short-lived in vivo (Abel et al., 1994) suggests that the Aux/IAA

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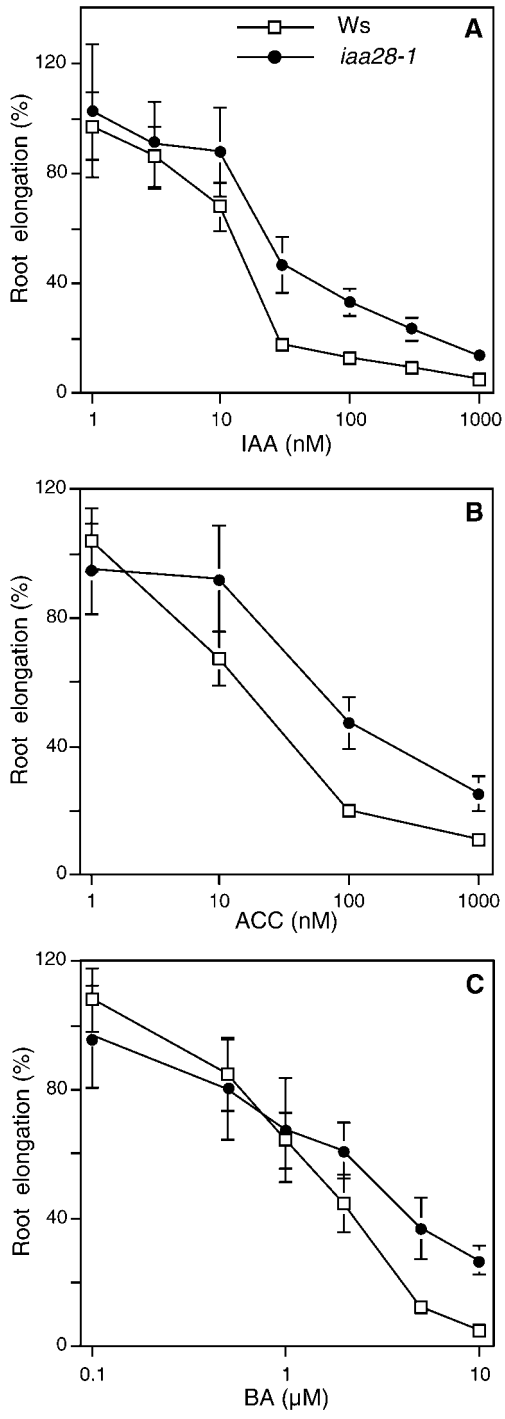

Figure 1. *iaa28-1* Mutants Are Resistant to Root Elongation Inhibition by Several Phytohormones.

After 8 days of growth under yellow-filtered light at 22°C on medium supplemented with various concentrations of IAA **(A)**, aminocyclocarboxylic acid (ACC) **(B)**, or BA **(C)**, seedlings were removed from the agar, and the length of the longest root was recorded. All results were standardized against growth on unsupplemented medium. Error bars represent ±SD ($n \geq 22$). Ws, Wassilewskija.

proteins themselves might regulate *Aux/IAA* transcription in response to auxin.

Aux/IAA proteins share four domains of homology separated by variable regions (Ainley et al., 1988; Conner et al., 1990; Oeller et al., 1993). Domains III and IV are dimerization domains that are conserved not only among the Aux/IAA proteins (Kim et al., 1997) but also among most auxin response factor (ARF) proteins (Guilfoyle et al., 1998b). Unlike Aux/IAA proteins, ARFs contain a DNA binding domain (Guilfoyle et al., 1998b) and bind to auxin-responsive elements (AuxREs) found in the promoters of some *Aux/IAA* genes and other auxin-responsive genes (Ulmasov et al., 1999b). Both Aux/IAA proteins and ARFs can regulate the expression of reporter genes fused to AuxRE-containing promoters in transient assays (Ulmasov et al., 1997b, 1999a). The interactions of ARFs with ARFs, ARFs with Aux/IAA proteins, or Aux/IAA proteins with Aux/IAA proteins may regulate auxin-responsive transcription (Guilfoyle et al., 1998a; Morgan et al., 1999). However, the number of potential binding partners coupled with differing regional expression and induction profiles suggest a complicated network of interactions that remains to be elucidated.

Several gain-of-function mutants in Arabidopsis *Aux/IAA* genes have been identified. These mutants have pleiotropic phenotypes, some of which are consistent with increased auxin sensitivity and others with decreased auxin sensitivity. For example, the *iaa7/axr2-1* (Wilson et al., 1990) and *iaa3/shy2-2* (Tian and Reed, 1999) mutants have reduced apical dominance, which may reflect a decreased auxin response. Conversely, the *iaa17/axr3-1* mutant has increased apical dominance, suggesting an increased auxin response (Leyser et al., 1996). Interestingly, several of these mutants also show altered expression of auxin-inducible genes or reporter constructs (Timpte et al., 1994; Abel et al., 1995b; Leyser et al., 1996), consistent with Aux/IAA proteins regulating auxin-induced transcription.

Several Aux/IAA proteins are rapidly turned over in vivo (Abel et al., 1994). Because all reported gain-of-function *Aux/IAA* mutations map to domain II (Rouse et al., 1998; Tatematsu et al., 1999; Tian and Reed, 1999; Nagpal et al., 2000), this class of mutations may stabilize the encoded proteins. The hypothesis that modulating Aux/IAA protein stability can alter auxin responses is supported by the observations that fusion of domain II to a reporter protein confers instability to the reporter and that changes analogous to those found in the *iaa17/axr3-1* gain-of-function mutant stabilize the protein (Worley et al., 2000).

Here, we report the cloning and characterization of *IAA28*, a new member of the Arabidopsis *Aux/IAA* gene family. Plants with an *iaa28* gain-of-function mutation are resistant to several phytohormones and have a novel adult phenotype marked by multiple morphological alterations, including a profound defect in lateral root initiation. *IAA28* is strongly and specifically expressed in roots and inflorescence stems. *IAA28* transcription is repressed by auxin, and functional experiments suggest that IAA28 represses auxin-induced transcription, perhaps of genes that promote lateral root initiation.

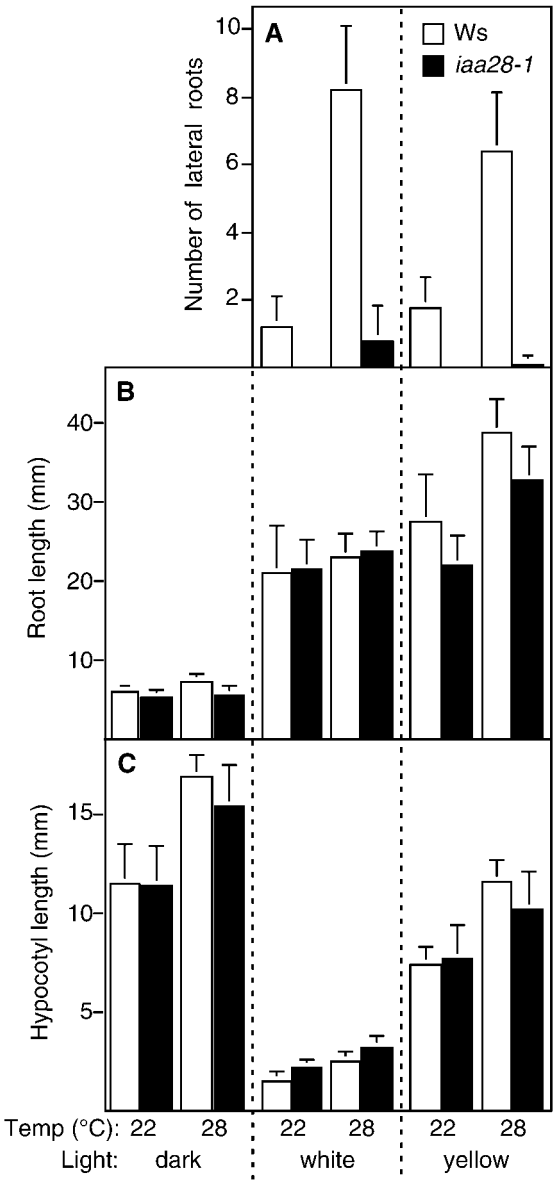

Figure 2. *iaa28-1* Root and Hypocotyl Phenotypes under Various Environmental Conditions.

Lateral roots **(A)** of plants ($n = 24$) grown under the indicated environmental conditions were counted after 8 days of growth on unsupplemented medium. Roots **(B)** and hypocotyls **(C)** were measured after 5 days (1 day in the light, 4 days in the dark) for dark-grown plants and after 8 days for plants grown under white or yellow-filtered light. Error bars represent ±SD ($n \geq 15$).

RESULTS

iaa28-1 Roots Are Resistant to Several Phytohormones and Are Defective in Lateral Root Formation

As part of a project to identify components controlling IAA homeostasis (Bartel and Fink, 1995; Davies et al., 1999; Lasswell et al., 2000), we isolated an Arabidopsis mutant that was resistant to inhibition of root elongation by exogenous IAA-alanine, a potential IAA storage form (Hangarter and Good, 1981). Analysis of this mutant in subsequent generations indicated that it was resistant not only to auxin conjugates but also to a range of exogenous free IAA concentrations (Figure 1A), suggesting that the defective gene acted in auxin response rather than metabolism.

Mutants that respond abnormally to one phytohormone often respond abnormally to others as well, reflecting the complex interacting network of hormone response pathways. *iaa28-1* roots are resistant not only to auxin but also to the ethylene precursor aminocyclocarboxylic acid and the synthetic cytokinin benzyladenine (BA) (Figures 1B and 1C). In contrast, *iaa28-1* roots display normal sensitivity to the phytohormones abscisic acid, methyl jasmonate, and epibrassinolide, and *iaa28-1* hypocotyls respond normally to aminocyclocarboxylic acid over a range of concentrations (data not shown).

iaa28-1 plants have fewer lateral roots than do wild-type plants grown under the same conditions (Figure 2A). The production of lateral roots is important in the long-term development of a mature root system, and both endogenous and exogenous auxins can promote lateral root initiation. Mutants such as *rty* and *sur2* that accumulate high levels of free IAA have short, highly branched roots (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995; Lehman et al., 1996; Delarue et al., 1998), reflecting the inhibition of root elongation and the promotion of lateral root initiation by auxin. In addition, Arabidopsis plants grown at high temperature (28 versus 22°C) accumulate free IAA (Gray et al., 1998), which correlates with increased hypocotyl elongation (Gray et al., 1998) (Figure 2C), root elongation (Figure 2B), and lateral root formation (Figure 2A). Although *iaa28-1* roots and hypocotyls elongate almost normally in response to high temperature (Figures 2B and 2C), these plants still have at least 10-fold fewer lateral roots than do similarly grown wild-type plants (Figure 2A). Therefore, it is likely that *iaa28-1* roots are resistant to the stimulatory effect of endogenous IAA on lateral root initiation.

Because *iaa28-1* is resistant to root elongation inhibition by exogenous auxin (Figure 1A) and is defective in lateral root induction (Figure 2A) in conditions that increase endogenous auxin (Gray et al., 1998), we examined the promotion of lateral root initiation by exogenous auxins. As shown in Figure 3A, *iaa28-1* does not initiate normal numbers of lateral roots in response to either IAA or indole-3-butyric acid, a naturally occurring auxin that is used commercially because

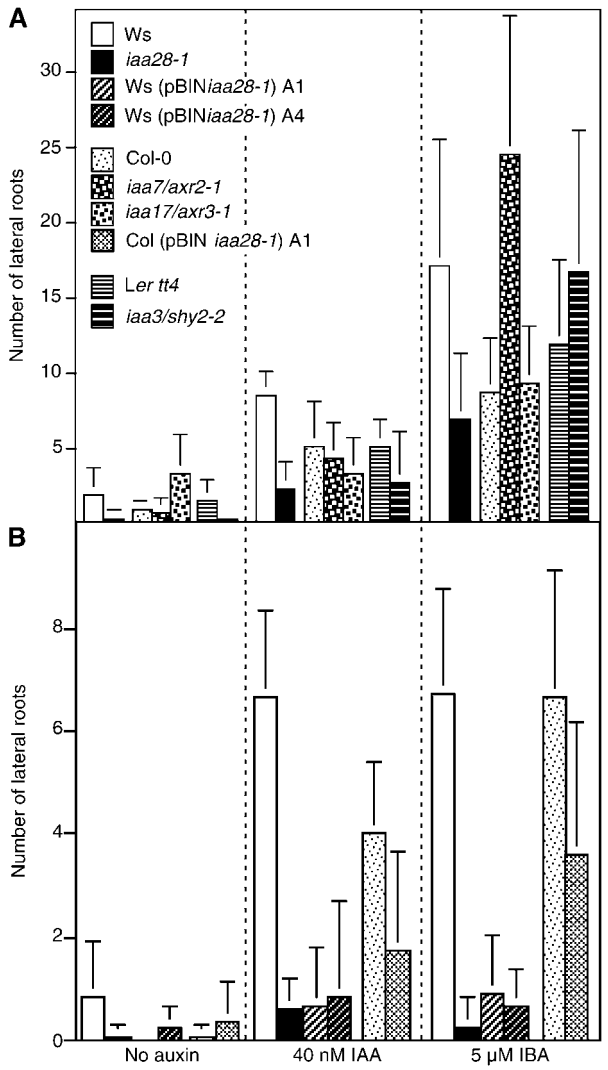

Figure 3. Lateral Root Induction in *Aux/IAA* Mutants by Exogenous Auxins.

Lateral roots were counted 4 days after transfer of 4-day-old seedlings to the indicated medium.

(A) *Aux/IAA* gene mutants are grouped with their corresponding parental wild type.

(B) Wild-type plants homozygous for a genomic *iaa28-1* phenocopy construct (pBIN*iaa28-1*) are grouped with their corresponding wild type. L*er*, Landsberg *erecta*; IBA, indole-3-butyric acid. Error bars represent ±SD ($n \geq 8$).

of its efficacy in promoting lateral root initiation (Hartmann et al., 1990; Zolman et al., 2000). However, *iaa28-1* is not completely insensitive to auxins in this assay, because the mutant plants do form more lateral roots upon treatment. In comparison, gain-of-function *iaa7/axr2* (Wilson et al., 1990), *iaa17/axr3* (Leyser et al., 1996), and *iaa3/shy2* (Tian and Reed, 1999) mutants, which are also resistant to the inhibitory effects of IAA on root elongation, have at least as many lateral roots as do the wild type after treatment with indole-3-butyric acid (Figure 3A).

iaa28-1 mutants also have defects in root hair development. *iaa28-1* seedlings have fewer root hairs than do the wild type (data not shown), and this phenotype is enhanced when the seedlings are grown in conditions expected to increase endogenous IAA levels, such as yellow-filtered light (Stasinopoulos and Hangarter, 1990) or high temperature (Gray et al., 1998).

Aside from the defects in lateral root and root hair formation described above, *iaa28-1* seedlings are relatively normal. We noted small but reproducible defects in root elongation in *iaa28-1* seedlings grown under yellow-filtered light (Figure 2B), but we have not determined whether the observed difference in growth under white and yellow-filtered light reflects the decreased fluence of light (approximately twofold) or the slowed photochemical breakdown of indolic compounds under the yellow filters (Stasinopoulos and Hangarter, 1990).

Mature *iaa28-1* Plants Exhibit Diverse Morphological Aberrations

The morphology of mature *iaa28-1* plants suggests important roles for IAA28 in development. For example, the lateral root defect of *iaa28-1* seedlings (Figure 2A) becomes more pronounced in mature plants. *iaa28-1* plants grown hydroponically for 4 weeks only occasionally develop a single lateral root, whereas wild-type plants develop a highly branched root system under the same conditions (Figure 4A). This developmental defect results in poorly anchored plants that frequently become dislodged when grown in soil.

The aerial portions of *iaa28-1* adult plants also are malformed. After the transition to flowering, the mutant is shorter (Figure 5A) and develops more stems than do the wild type (Figures 4A and 5B). These later inflorescence stems are often dramatically shorter than are the primary stems (Figure 5C). The inflorescence internodes are short, particularly on secondary inflorescences (data not shown). This shortening sometimes results in a circular tuft of siliques at the stem apex. Flowers of the mutant appear normal, but siliques are constricted and smaller than those of the wild type. The decrease in both silique size and number results in lower seed yield in the *iaa28-1* mutant (data not shown). Although *iaa28-1* seedling leaves are normal in number and appearance, leaves of mature plants tend to be darker green than are those of the wild type.

IAA28 Is an *Aux/IAA* Gene

We used a map-based positional approach to identify the gene defective in the *iaa28-1* mutant. We initially mapped *iaa28-1* between the simple sequence length polymorphism markers nga139 and nga76 (Bell and Ecker, 1994) on chro-

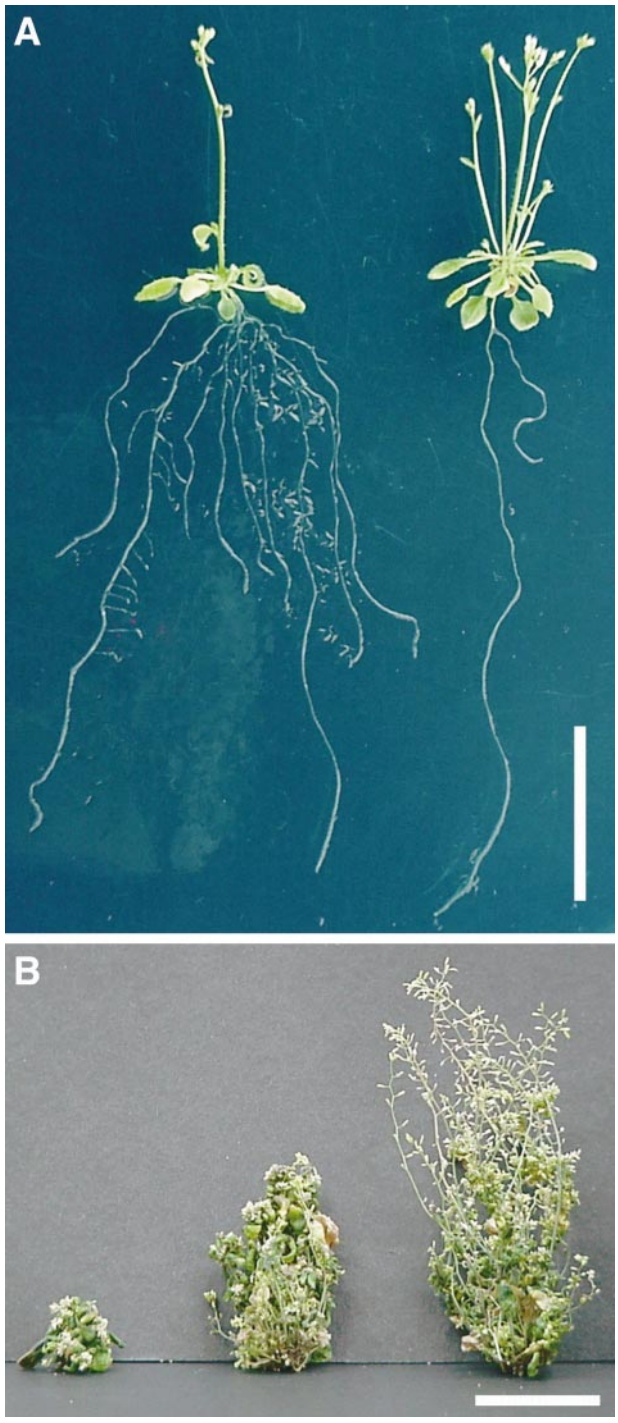

Figure 4. Morphology of *iaa28-1* Gain-of-Function Mutants.

(A) Four-week-old Ws (at left) and *iaa28-1* (at right) plants grown hydroponically.

(B) Ws plants expressing the *iaa28-1* cDNA from the cauliflower mosaic virus 35S promoter were grown in soil for 2 months. Sixty-three of 83 primary transformants overexpressing the *iaa28-1* cDNA were infertile dwarfs with phenotypes within the range of the three plants shown. Bars = 2.5 cm.

mosome 5 (Figure 6A). Using additional markers developed from available genomic sequence in the area, we localized *iaa28-1* to a 55-kb region corresponding to the overlap interval of the bacterial artificial chromosomes F18A17 and T1N24, which were being sequenced by the Cold Spring Harbor Sequencing Consortium (http://www.cshl.org/arabweb/ and http://genome.wustl.edu/gsc/). A previously uncharacterized member of the *Aux/IAA* gene family, which apparently encoded a protein 27 to 46% identical to other Aux/IAA proteins, was found in this interval. Because mutations in several other *Aux/IAA* genes confer auxin resistance and pronounced morphological alterations (Wilson et al., 1990; Leyser et al., 1996; Tian and Reed, 1999), we sequenced this gene from *iaa28-1* mutant and wild-type (Wassilewskija [Ws]) genomic DNA. This analysis revealed a C-to-T base pair change in the mutant, consistent with an ethyl methanesulfonate–induced mutation. To determine the splicing pattern of the gene, we isolated and sequenced a full-length wild-type cDNA (GenBank accession number AF149816), which revealed that the computer-derived annotation of *IAA28* (T1N24.24; GenBank accession number AF149413) was incomplete because it ended prematurely after the second exon. In addition, we found that proline 53 in the conserved second domain of IAA28 was replaced with a leucine residue in the mutant protein (Figure 6B). This alteration is similar in nature and location to gain-of-function mutations reported in the *iaa3/shy2* (Tian and Reed, 1999), *iaa7/axr2* (Nagpal et al., 2000), *iaa17/axr3* (Rouse et al., 1998), and *iaa19/msg2* (Tatematsu et al., 1999) mutants (Figure 6B).

Because analogous domain II mutations in other *Aux/IAA* genes are gain-of-function mutations and result in semidominant or dominant phenotypes (Rouse et al., 1998; Tatematsu et al., 1999; Tian and Reed, 1999; Nagpal et al., 2000), we examined the inheritance pattern of *iaa28-1*. A screen of F2 progeny from a heterozygous *IAA28/iaa28-1* parent showed that 70 of 225 plants (27.5%) exhibited the characteristic mutant aerial phenotype, consistent with a single, fully penetrant, recessive mutation. However, *IAA28/iaa28-1* heterozygotes were slightly more auxin-resistant than were wild-type plants after 8 days of growth on medium containing 100 nM IAA (Figure 7A), indicating a semidominant mutation. Similarly, *IAA28/iaa28-1* heterozygotes had fewer lateral roots than did the wild type after 13 days growth on unsupplemented medium (Figure 7B). The semidominant root phenotypes conferred by the *iaa28-1* mutation suggested that the lesion resulted in a gain of function.

To test directly whether the *iaa28-1* mutation is a gain-of-function allele, we expressed the mutant gene in wild-type plants and assayed for phenocopy of mutant characteristics. We modified a genomic fragment, including the promoter region, to contain the *iaa28-1* base change (see Methods). Approximately half (8 of 17) of the independently derived transgenic plants containing the mutant genomic construct (pBIN*iaa28-1*) exhibited phenotypes characteristic of the *iaa28-1* mutant, including reduced stature, increased numbers of secondary inflorescence stems, and resistance

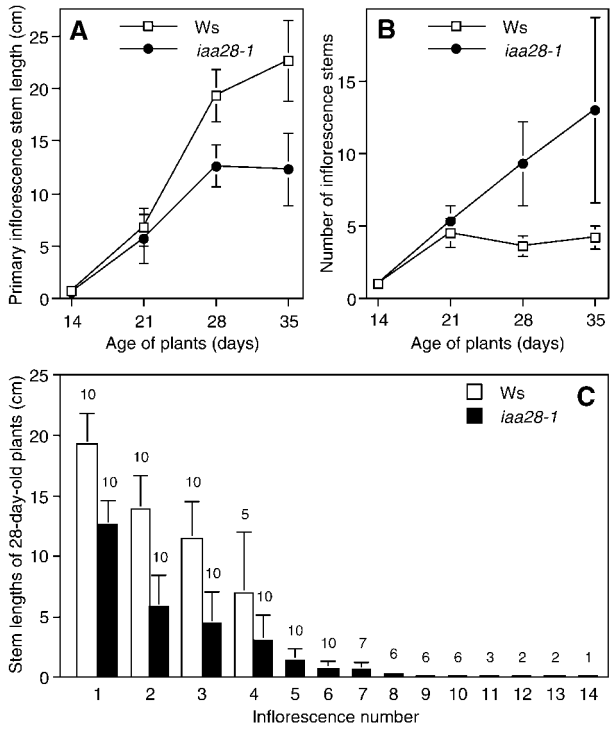

Figure 5. The *iaa28-1* Mutant Has Decreased Apical Dominance and Reduced Stature.

Wild-type (Ws) and *iaa28-1* inflorescence stems were counted and measured in 10 plants of each line grown in soil under continuous illumination. Error bars represent ±SD.

(A) *iaa28-1* primary inflorescence stems are shorter than wild-type stems.

(B) *iaa28-1* develops more inflorescence stems than does the wild type.

(C) Later *iaa28-1* inflorescence stems are shorter than wild-type stems. Numbers above the error bars indicate the numbers of plants with that number of inflorescence stems.

to root elongation inhibition by auxin (data not shown). In addition, like the original mutant, these phenocopy lines had far fewer lateral roots than did the wild type, even when induced with exogenous auxin (Figure 3B). Because we were able to recapitulate the mutant phenotype by transforming wild-type plants with mutant genomic DNA, we conclude that *iaa28-1* is a gain-of-function mutation and that the base change identified in the *IAA28* gene is responsible for the *iaa28-1* mutant phenotype.

We also introduced wild-type and mutant *IAA28* cDNAs driven by the highly expressed 35S promoter from cauliflower mosaic virus into wild-type plants. Whereas overexpression of the wild-type cDNA caused no discernible morphological effects (data not shown), most of the 35S–*iaa28-1* plants (63 of 83) had more extreme phenotypes than did the original *iaa28-1* mutant and were completely infertile as heterozygotes (Figure 4B). These infertile plants were small with very wrinkled leaves and shortened petioles, unlike the original mutant. Some of the 35S–*iaa28-1* transgenic lines (11 of 83) remained fertile and had aerial phenotypes similar to those of the *iaa28-1* mutant, including reductions of inflorescence and internode length (data not shown). However, some of these mutant overexpressor lines had agravitropic roots, unlike the original mutant (data not shown). In addition, several of these 35S–*iaa28-1* lines were partially deetiolated when grown for extended periods in the dark, whereas the *iaa28-1* mutant etiolated normally (data not shown). The severe defects caused by overexpression of the mutant, but not the wild-type, cDNA are consistent with the gain-of-function nature of the mutation. Because the qualitative differences between the original mutant and the 35S-driven mutant cDNA transformants in gravitropism, etiolation, fertility, and leaf morphology may reflect differences in the tissue specificity or strength of the *IAA28* and 35S promoters, we examined *IAA28* expression in wild-type plants.

IAA28 Expression Is Highly Organ Specific

We used RNA gel blot analysis to assay *IAA28* mRNA levels in various organs of wild-type plants. *IAA28* expression was very high in roots, significant in inflorescence stems, and minor in leaves, siliques, and flowers (Figures 8A and 8B). These sites of expression are consistent with the sites of phenotypic alterations in the *iaa28-1* mutant. To examine *IAA28* expression within organs, we transformed wild-type plants with an *IAA28* promoter–β-glucuronidase (GUS) fusion. As shown in Figures 8C to 8F, incubation of light-grown seedlings with the GUS substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) resulted in intense root staining. The staining extended from the elongation zone to the root–hypocotyl junction, with the most pronounced expression in the root hair differentiation zone. No staining was detected in the tips of primary (Figure 8D) or lateral (Figure 8F) roots, lateral root primordia (Figure 8E), hypocotyls, cotyledons, or leaf primordia, even after extended incubation at higher substrate concentrations (Figure 8C and data not shown). *IAA28* promoter–GUS staining was more intense in seedlings grown in white light compared with those grown under yellow filters, and dark-grown seedlings required prolonged incubation at higher concentrations of substrate for visible staining (data not shown). *IAA28* promoter–GUS seedlings grown at 28°C, which increases endogenous IAA levels (Gray et al., 1998), had less GUS staining than did seedlings grown at 22°C (data not shown), suggesting that IAA may repress *IAA28* transcription.

IAA28 Transcript Levels Are Not Induced by Auxin

Aux/IAA genes were originally identified because of their strong and rapid transcriptional induction in response to auxin (Theologis et al., 1985). Subsequent analysis of sev-

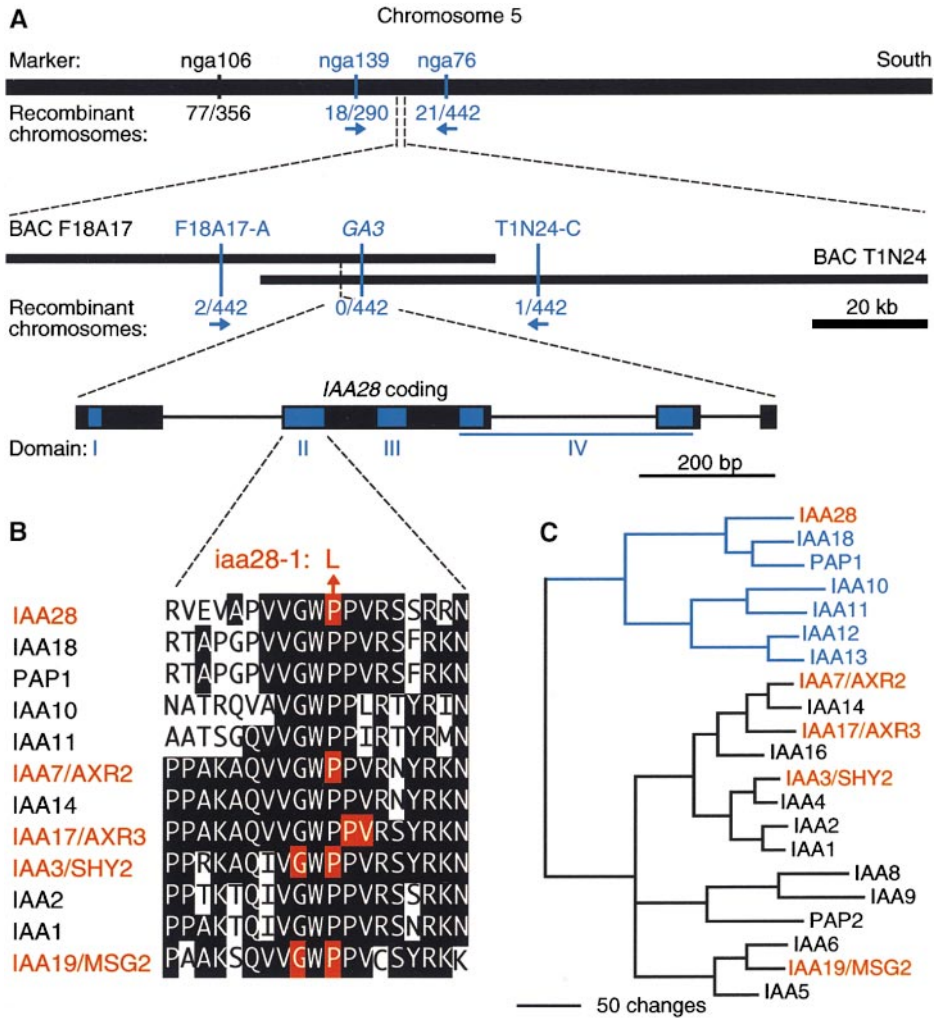

Figure 6. Positional Cloning of *IAA28*.

(A) The *iaa28-1* mutation was initially mapped between nga139 and nga76 on chromosome 5. The number of recombinant chromosomes scored is indicated beneath each marker assayed. Additional markers were made that narrowed the *iaa28-1* location to 55 kb. Sequencing of a new *Aux/IAA* gene (*IAA28*) in the F18A17-A to T1N24-C interval revealed a C-to-T transition that changes a conserved proline to a leucine. *IAA28* exons are indicated by thick rectangles, and introns are indicated by thin lines. The positions of the four Aux/IAA domains are indicated by roman numerals below blue rectangles. BAC, bacterial artificial chromosome.

(B) All reported *Aux/IAA* gene gain-of-function mutants have mutations in conserved domain II. Domain II of IAA28 is aligned with domain II of several other Arabidopsis Aux/IAA proteins, including those for which gain-of-function mutations have been reported. Reported mutated residues from each protein are highlighted in red.

(C) Phylogenetic tree of Arabidopsis Aux/IAA proteins. Sequences were aligned using MegAlign (DNAStar, Madison, WI) by using the Clustal method with PAM250 residue weights, and the phylogenetic tree was generated using PAUP 4.0b3a (Swofford, 2000) by using the bootstrap method. The IAA28 clade is highlighted in blue, and proteins in which gain-of-function mutations have been identified are shown in red. IAA1 to IAA15 were described by Abel et al. (1995b), and IAA16 to IAA19 were described by Kim et al. (1997). PAP1 (GenBank accession number AF088281) and PAP2 (GenBank accession number AF087936) are unpublished. IAA4 and IAA5 are also known as AtAux2-11 and AtAux2-27 (Conner et al., 1990), respectively.

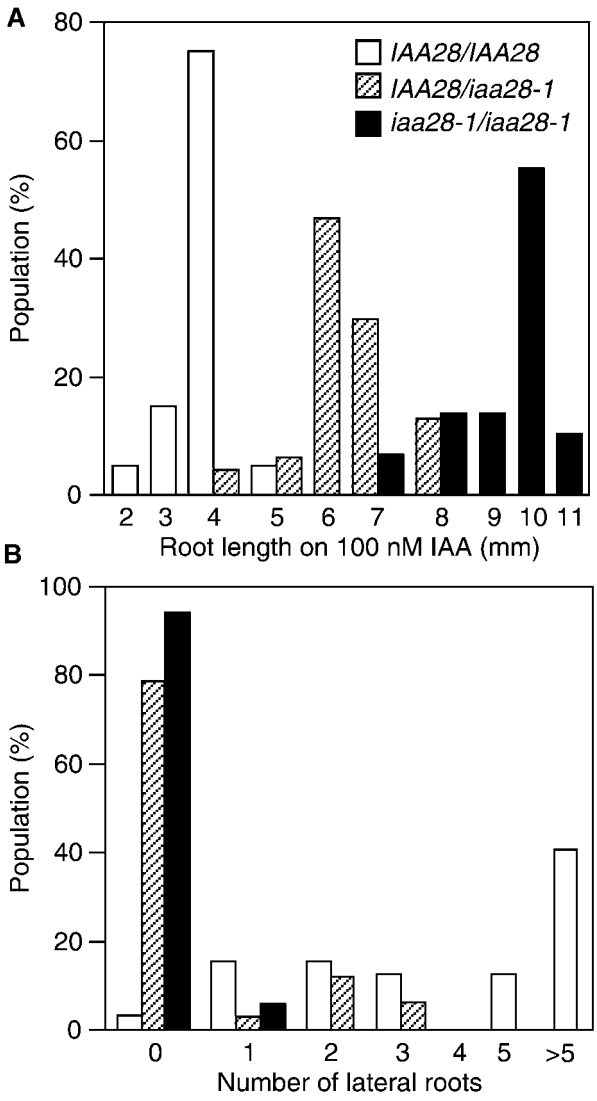

Figure 7. *iaa28-1* Root Phenotypes Are Semidominant.

(A) Seeds from an *IAA28/iaa28-1* heterozygote were plated on medium containing 100 nM IAA. After 8 days, seedlings were removed from the agar, and the length of the longest root was recorded (see Methods). Seedlings were then genotyped using polymerase chain reaction (PCR) (n = 20 for *IAA28/IAA28*, 47 for *IAA28/iaa28-1*, and 29 for *iaa28-1/iaa28-1*).

(B) Seeds from an *IAA28/iaa28-1* heterozygote were plated on unsupplemented medium, and lateral roots were counted after 13 days (see Methods). Seedlings were then genotyped using PCR (n = 31 for *IAA28/IAA28*, 33 for *IAA28/iaa28-1*, and 18 for *iaa28-1/iaa28-1*).

eral of these genes identified by sequence homology has shown that *Aux/IAA* genes can be grouped based on their auxin induction kinetics. Some of these genes respond to auxin with large increases in transcript levels within minutes, whereas others respond more weakly and slowly (Abel et al., 1995b). To determine whether *IAA28* expression responds to auxin similarly to other *Aux/IAA* genes, we assayed levels of *IAA28* mRNA after auxin treatment in both the wild-type (Ws) and *iaa28-1* backgrounds. In contrast to the *IAA1* transcript, which is strongly induced by auxin, we found that *IAA28* mRNA levels actually decreased slightly in response to auxin in both wild-type and mutant backgrounds (Figure 9). This auxin repression is unique among characterized *Aux/IAA* genes (Abel et al., 1995b).

IAA28 Is a Transcriptional Repressor of Auxin-Induced Gene Expression

Because *Aux/IAA* genes are thought to encode transcriptional modulators that regulate auxin-induced gene expression, we examined the expression of a reporter gene driven by a synthetic AuxRE in the *iaa28-1* mutant. The BA–GUS construct contains multiple AuxREs driving GUS expression and is rapidly and strongly induced by auxin in the root elongation zone and in the shoot apex (Oono et al., 1998). After crossing this BA–GUS construct into the *iaa28-1* mutant, we compared GUS expression in wild-type BA–GUS plants and plants homozygous for both *iaa28-1* and the BA–GUS construct. Untreated *iaa28-1* seedlings had reduced root staining (Figure 10C) compared with wild-type seedlings (Figure 10A). After auxin treatment, the BA–GUS construct was only weakly induced in the *iaa28-1* root elongation zone (Figure 10D) compared with the robust wild-type induction (Figure 10B). This decrease in auxin-induced transcription is apparently tissue specific, because BA–GUS staining in wild-type and mutant shoot apices was indistinguishable (data not shown). This tissue specificity might explain why the *IAA1* message is still auxin induced in the *iaa28-1* mutant at the whole plant level (Figure 9). Because *iaa28-1* is a gain-of-function mutation, this finding suggests that IAA28 normally acts (directly or indirectly) as a transcriptional repressor.

DISCUSSION

iaa28-1 Is a Gain-of-Function Mutation in an *Aux/IAA* Gene

The gene defective in the *iaa28-1* mutant is a new member of the Arabidopsis *Aux/IAA* gene family. Arabidopsis contains nearly 20 *Aux/IAA* genes (Abel et al., 1995b; Kim et al., 1997). It has been noted (Abel et al., 1995b) that Aux/IAA proteins can be divided into two phylogenetic clades (Figure 6C). IAA28 is the first member of its clade with a reported

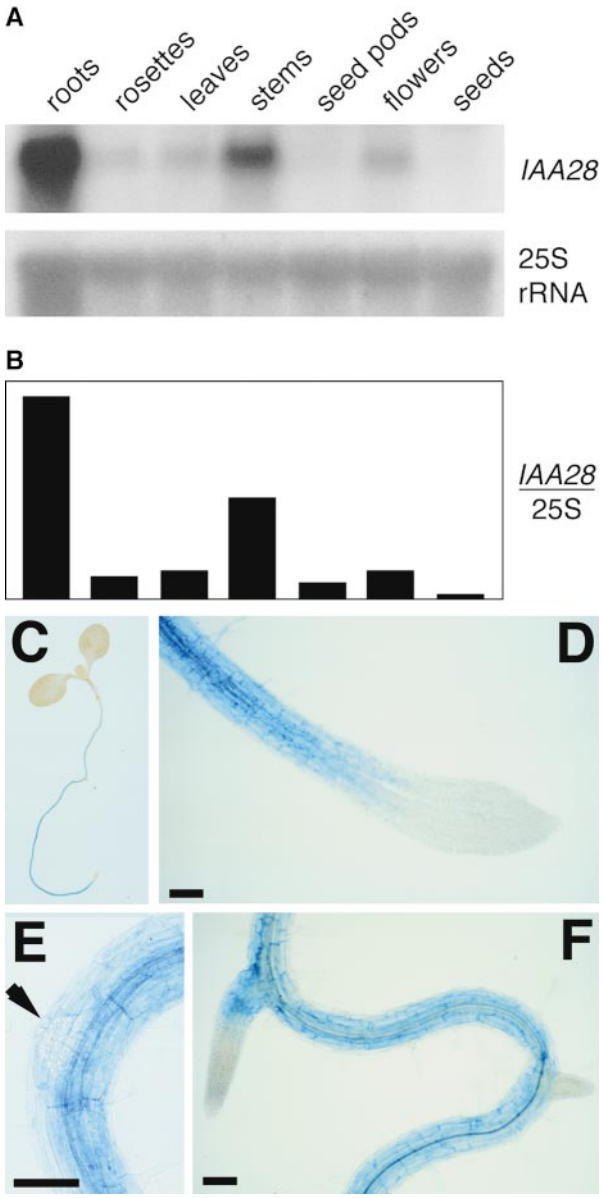

Figure 8. Specificity of *IAA28* Expression.

(A) Total RNA (9.5 μg) isolated from 14-day-old roots and aerial tissues (rosettes), 29-day-old leaves, inflorescence stems, siliques (seed pods), and flowers; and dry seed was separated on a denaturing gel, transferred to a nylon membrane, and probed with an antisense *IAA28* RNA probe to determine *IAA28* mRNA levels, with a 25S rDNA probe as a loading control.

(B) A phosphorimager was used to quantify the signal in each band. The graph represents the *IAA28* signal divided by the 25S signal expressed in arbitrary units.

(C) to **(F)** Transgenic seedlings homozygous for a 3.1-kb *IAA28* promoter–GUS fusion grown for 8 days at 22°C under white light were stained in 0.1 mg/mL X-Gluc for 2.5 hr at 37°C.

(C) Seedling expression is limited to roots.

(D) Staining is absent from primary root tips.

(E) Staining is absent from lateral root primordia (arrowhead).

(F) Staining is absent from lateral root tips.

Bars in **(D)** to **(F)** = 0.1 mm.

mutation; the other Aux/IAA proteins in which mutations have been described, IAA3/SHY2 (Tian and Reed, 1999), IAA7/AXR2 (Nagpal et al., 2000), IAA17/AXR3 (Rouse et al., 1998), and IAA19/MSG2 (Tatematsu et al., 1999), fall into the larger clade.

Aux/IAA proteins are short-lived nuclear proteins (Abel et al., 1994) that transcriptionally regulate auxin-induced genes (Guilfoyle et al., 1998a; Morgan et al., 1999). This regulation may involve homodimer or heterodimer formation between Aux/IAA proteins (Kim et al., 1997), between Aux/IAA proteins and ARF transcription factors (Ulmasov et al., 1997b), or between ARFs (Ulmasov et al., 1997a). These interactions occur through the conserved domains III and IV of Aux/IAA proteins (Ainley et al., 1988; Conner et al., 1990; Oeller et al., 1993), which are also present in most ARFs (Guilfoyle et al., 1998b). Because all gain-of-function mutations in *Aux/IAA* genes isolated to date are clustered in a conserved region (Figure 6B), it has been suggested that domain II may normally act to confer instability and that these domain II mutations might stabilize the normally short-lived proteins (Rouse et al., 1998), which is consistent with the rapid in vivo turnover of several Aux/IAA proteins (Abel et al., 1994). In support of this hypothesis, domain II confers instability to a reporter protein, and domain II mutations can stabilize this fusion protein (Worley et al., 2000). Therefore, it is likely that the domain II mutation that we identified in *iaa28-1* (Figure 6B) stabilizes the encoded protein, resulting in gain-of-function defects. Consistent with the possibility that the lesion results in a gain of function, the mutation confers semidominant root defects (Figure 7), and the mutant gene driven by its own promoter can phenocopy root and shoot defects of the *iaa28-1* mutant when introduced into wild-type plants (Figure 3B and data not shown).

IAA28 Expression

IAA28 mRNA is concentrated in the roots and inflorescence stems (Figure 8). In contrast, the other *Aux/IAA* genes whose expression patterns have been reported are more ubiquitously expressed (Abel et al., 1995b). For example, both *IAA3/SHY2* and *IAA7/AXR2* are expressed strongly in stems and flowers, moderately in leaves, and weakly in roots (Abel et al., 1995b). The *IAA28* expression pattern reinforces the specific roles for IAA28 in lateral root formation, apical dominance, and stem elongation suggested by the *iaa28-1* mutant phenotypes.

Analysis of plants transformed with an *IAA28* promoter–GUS fusion revealed developmental and environmental regulation

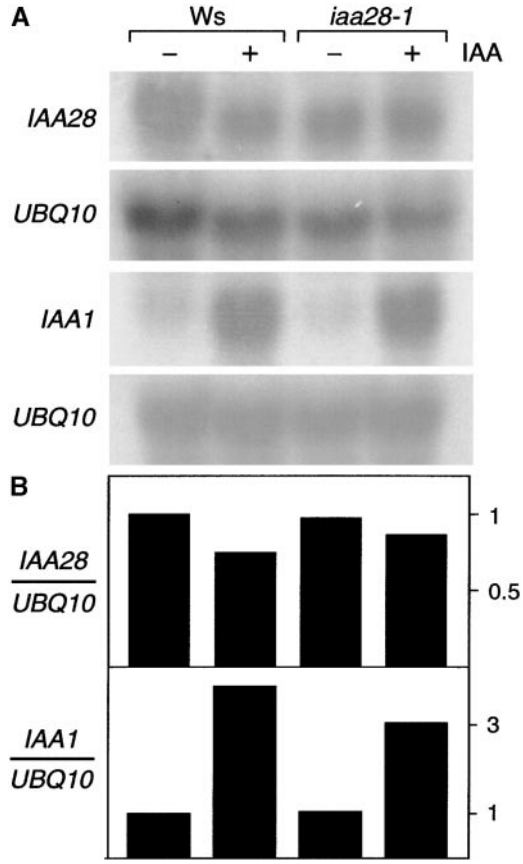

Figure 9. *IAA28* mRNA Levels in Response to Auxin.

(A) Total RNA (9.5 μg) isolated from IAA- (+) or mock-treated (−) 8-day-old wild type (Ws) and *iaa28-1* seedlings was separated on a denaturing gel, transferred to a nylon membrane, and probed with antisense *IAA28*, *IAA1*, and *UBQ10* (loading control) RNA probes.

(B) A phosphorimager was used to quantify the signal in each band. The graphs represent the *IAA28* or *IAA1* signal divided by the *UBQ10* signal expressed in arbitrary units.

of *IAA28* expression. This fusion is strongly expressed from the distal root elongation zone to the root–hypocotyl junction, with most intense staining in the root hair initiation zone (Figure 8C). Staining was not detected in seedling shoots, in the tips of primary and lateral roots, and in lateral root primordia (Figures 8C to 8F). These sites of seedling expression are consistent with the root-specific defects in *iaa28-1* seedlings (Figure 2) and with a model in which IAA28 inhibits lateral root formation.

IAA28 promoter–GUS staining was less intense in roots of seedlings grown under yellow-filtered light than in seedlings grown in white light, and it was less intense in seedlings grown at 28 versus 22°C (data not shown). Yellow filters slow the breakdown of indolic compounds (Stasinopoulos and Hangarter, 1990), and plants grown in yellow light may contain more auxin than do plants grown in white light. In addition, Arabidopsis plants grown at 28°C have more auxin (Gray et al., 1998) and lateral roots (Figure 2A) than do plants grown at 22°C. This repression of *IAA28* promoter–GUS expression under two conditions that may promote higher auxin levels suggests that auxin represses *IAA28* expression. Intriguingly, *IAA28* mRNA levels also were downregulated by exogenous auxin treatment (Figure 9), in contrast to the increased *IAA1* to *IAA14* mRNA levels found after seedling IAA treatment (Abel et al., 1995b).

Gain-of-Function Mutations in *Aux/IAA* Genes Reveal Overlapping and Distinct Roles in Development

Like other gain-of-function *Aux/IAA* mutants, *iaa28-1* plants have pronounced morphological alterations and are resistant to several phytohormones. Several gain-of-function mutations in Arabidopsis *Aux/IAA* genes confer IAA-resistant root elongation, including *iaa28-1* (Figure 1A), *iaa3/shy2* (Tian and Reed, 1999), *iaa7/axr2* (Wilson et al., 1990), and *iaa17/axr3* (Leyser et al., 1996). The resistance of the *iaa28-1* mutant to other phytohormones probably results from interactions between hormone response pathways. *iaa28-1* is resistant to root elongation inhibition by the ethylene precursor aminocyclocarboxylic acid (Figure 1B). Similarly, mutations in both the AUX1 auxin import carrier (Bennett et al., 1996) and the EIR1 auxin efflux carrier (Luschnig et al., 1998) have been isolated based on their ethylene-resistant root phenotypes (Pickett et al., 1990; Roman et al., 1995). In addition, other gain-of-function *Aux/IAA* mutants, including *iaa3/shy2* (Tian and Reed, 1999), *iaa7/axr2* (Wilson et al., 1990), and *iaa17/axr3* (Leyser et al., 1996), have ethylene-resistant roots. The resistance of *iaa28-1* and other auxin-resistant mutants to ethylene may reflect physiological interactions between auxin and ethylene, such as the inhibition of auxin transport by ethylene and the stimulation of ethylene biosynthesis by auxin (reviewed in Hobbie, 1998).

Interdependence has also been reported for auxin and cytokinin responses. The auxin-resistant *axr1* and *aux1* mutants have cytokinin-resistant roots (Timpte et al., 1995). Both *iaa28-1* (Figure 1C) and *iaa3/shy2-2* (Tian and Reed, 1999) are cytokinin resistant. *iaa17/axr3-1* mutants are not only strongly resistant to both auxin and cytokinin, but the phenotypic defects of gain-of-function *iaa17/axr3* mutants are partially rescued by treatment with exogenous cytokinin (Leyser et al., 1996). Both auxin and cytokinin are required to grow plant cells in tissue culture (Skoog and Miller, 1957), and it is thought that the ratio of auxin to cytokinin, rather than absolute levels of these hormones, controls plant development (Eklöf et al., 1997).

Lateral root induction is essential for the development of a mature root system. *iaa28-1* mutants did not initiate normal numbers of lateral roots even when treated with exogenous auxins (Figure 3) or when grown at 28°C (Figure 2A), a temperature that increases endogenous auxin levels (Gray et al.,

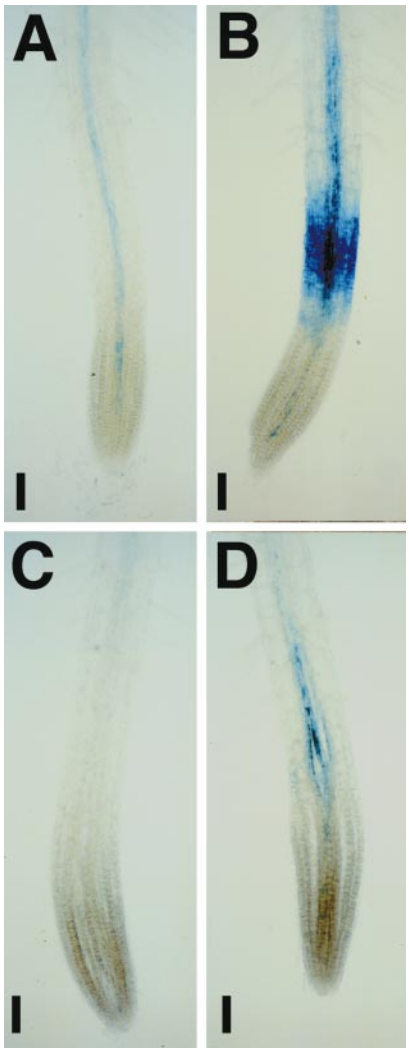

Figure 10. BA–GUS in the Wild Type and *iaa28-1*.

Eight-day-old BA–GUS (**[A]** and **[B]**) and BA–GUS *iaa28-1* homozygotes (**[C]** and **[D]**) were treated with 10 μM IAA for 2.5 hr (**[B]** and **[D]**) or mock treated for the same time (**[A]** and **[C]**) before staining in 0.5 mg/mL X-Gluc for 24 hr at 37°C. Staining is decreased in the roots of the BA–GUS *iaa28-1* homozygotes both with and without auxin treatment. Bars = 0.1 mm.

1998). This lateral root defect persisted in mature plants (Figure 4A). Because *iaa28-1* is a gain-of-function mutation, the lateral root defect suggests that one function of IAA28 is to repress lateral root formation. Observation of *iaa28-1* roots using light microscopy did not reveal additional unemerged lateral root primordia (data not shown), indicating an early block in lateral root formation. It will be interesting to examine markers that report the early cell divisions that initiate lateral root primordia formation (Ferreira et al., 1994; Smith and Fedoroff, 1995) in the *iaa28-1* mutant to determine more precisely the position of the block in the mutant.

Like *iaa28-1*, *alf4* mutants form few, if any, lateral roots and are resistant to the stimulatory effect of IAA on lateral root formation (Celenza et al., 1995). In contrast to *iaa28-1*, *alf4* primary root elongation is inhibited by auxin, as in the wild type (Celenza et al., 1995). Examining the expression of *ALF4* in *iaa28-1* and of *IAA28* in *alf4* and other mutants that lack lateral roots might determine the order of action of these genes in lateral root formation.

Other *Aux/IAA* mutants also have root defects. For instance, *iaa17/axr3-1* mutants have short primary roots and develop adventitious roots (Leyser et al., 1996), *iaa3/shy2* gain-of-function mutants have short roots with more root hairs (Tian and Reed, 1999), and *iaa7/axr2-1* mutants have roots of normal length with fewer root hairs (Wilson et al., 1990). *iaa28-1* is unique among described *Aux/IAA* mutants in that its lateral root defects were not fully rescued by exogenous auxin (Figure 3) and the mutant roots had a normal gravity response (data not shown).

Gain-of-function mutations in different *Aux/IAA* genes cause distinct adult phenotypes. *iaa28-1* mutants have reduced apical dominance (Figure 5B), short inflorescence stems (Figures 5A and 5C), and relatively normal leaves. *iaa7/axr2-1* mutants are dwarfs with severely wrinkled leaves, reduced apical dominance, and agravitropic shoots (Wilson et al., 1990). *iaa17/axr3-1* mutants have small, flat, dark green leaves and a single, unbranched inflorescence stem, suggesting exaggerated apical dominance (Leyser et al., 1996). Conversely, *iaa3/shy2-2* mutants are small with tiny hyponastic leaves and several short inflorescence stems (Kim et al., 1996; Reed et al., 1998; Tian and Reed, 1999). The overlapping yet distinct root and shoot phenotypes of gain-of-function *Aux/IAA* mutations suggest that *Aux/IAA* genes play only partially redundant roles in several auxin-mediated responses.

We cannot determine whether any of the *iaa28-1* shoot defects result indirectly from the paucity of lateral roots in the mutant. However, the *axr4 aux1* double mutant has an extreme reduction in lateral roots but a wild-type shoot (Hobbie and Estelle, 1995), suggesting that a lack of lateral roots does not necessarily influence shoot development. Moreover, *IAA28* mRNA is significantly expressed in stems (Figure 8A), which suggests a direct role for the IAA28 protein in shoot development.

Because gain-of-function mutations in *IAA28* and *IAA17/AXR3* have opposing apical dominance and lateral root phenotypes, we attempted to isolate an *iaa28-1 axr3-1* double mutant to determine whether the mutations would suppress one another. Surprisingly, we found that the double heterozygote is a more severe dwarf than either single homozygous mutant and that the homozygous double mutants (as well as some plants heterozygous for one mutation and homozygous for the other) die as young seedlings (L.E. Rogg and B. Bartel, unpublished data). Because of the complications presented by gain-of-function mutations, detailed

analysis of functional redundancy among the *Aux/IAA* genes awaits double mutant construction among loss-of-function alleles of the various family members.

Overexpression of the mutant *iaa28-1* cDNA results in striking defects more severe than those of the original mutant. In particular, most 35S–*iaa28-1* plants had severely wrinkled leaves and shortened petioles (Figure 4B), like those of the *iaa7/axr2-1* mutant (Wilson et al., 1990). Interestingly, whereas the original *iaa28-1* mutant had normal root gravitropism (data not shown), root gravitropism was disrupted in some of the 35S–*iaa28-1* lines that remained fertile (the only lines that could be assayed). *iaa7/axr2* (Wilson et al., 1990), *iaa17/axr3* (Leyser et al., 1996), and *iaa3/shy2* (Tian and Reed, 1999) gain-of-function mutants all show root gravitropism defects, suggesting that gravity response differences among the *Aux/IAA* mutants may result from differences in the expression patterns of the corresponding genes rather than functional differences in the encoded proteins. Consistent with this hypothesis, the *IAA28*-GUS reporter was strongly expressed in roots but was not expressed in the root tip cells that sense and respond to gravity (Figure 8D). Similarly, the differences in leaf morphology between the *iaa28-1* mutant and the 35S–*iaa28-1* transformant may reflect the very low level of *IAA28* expression in wild-type leaves (Figures 8A and 8C).

Although dark-grown *iaa28-1* mutants etiolated normally, overexpression of the mutant *iaa28-1* cDNA caused partial deetiolation (data not shown). The gain-of-function *iaa3/shy2* mutant exhibits altered dark responses, including leaf development and inappropriate expression of light-induced genes in the dark (Kim et al., 1996, 1998). Similarly, gain-of-function *iaa7/axr2* and *iaa17/axr3* mutants deetiolate when grown in the dark (Nagpal et al., 2000). Like the gravitropism and leaf morphology differences cited above, these alterations in dark responses can be explained by expression differences. The *IAA28* promoter–GUS fusion was very weakly expressed in dark-grown seedlings, and we were unable to detect the *IAA28* mRNA in dark-grown seedlings by using RNA gel blot analysis (data not shown). In contrast, *IAA1* to *IAA14* are all significantly expressed in dark-grown seedlings (Abel et al., 1995b), and *IAA4* promoter–reporter gene fusions are expressed in both dark- and light-grown seedlings (Wyatt et al., 1993).

IAA28 Is a Transcriptional Repressor of Auxin-Induced Gene Expression

Certain Aux/IAA proteins appear to repress transcription from auxin-inducible promoters, whereas others induce transcription. For example, basal levels of several *Aux/IAA* genes are lower in *iaa7/axr2* gain-of-function mutants than in wild-type plants (Abel et al., 1995b), suggesting that IAA7/AXR2 normally represses these genes. In contrast, a reporter gene fused to an auxin-inducible promoter is ectopically expressed in roots even in the absence of auxin treatment in gain-of-function *iaa17/axr3* mutants (Leyser et al., 1996), suggesting that IAA17/AXR3 can activate transcription. The BA–GUS construct contains a synthetic array of AuxREs that strongly promote GUS expression in the shoot apex and the root elongation zone in response to auxin treatment (Oono et al., 1998). BA–GUS expression in the *iaa28-1* mutant was notably reduced in the root (Figure 10) but not in the shoot apex (data not shown). This root-specific decrease was seen in both untreated and auxin-treated plants, consistent with the possibility that the *iaa28-1* mutant is less responsive to endogenous and exogenous auxin. Because *iaa28-1* is a gain-of-function mutation, IAA28 may normally act to repress auxin-inducible gene expression.

Interestingly, *IAA28* mRNA levels decrease slightly in response to auxin treatment (Figure 9). To date, all other *Aux/IAA* genes have been shown to be auxin induced to various extents (Abel et al., 1995b); thus, this finding adds still another layer of complexity to the emerging model of Aux/IAA expression and function.

The combined genetic and molecular evidence suggests that IAA28 acts to transcriptionally repress auxin-induced genes required for lateral root initiation. The gain-of-function nature of the *iaa28-1* mutation suggests that the iaa28-1 protein may effectively reduce the concentrations of its normal binding partners, which may include other Aux/IAA proteins or ARFs. A subset of these proteins may act to transcriptionally activate genes necessary for lateral root induction when not bound to IAA28. The *iaa28-1* mutant is a valuable tool for the future identification of the activators, partners, and targets of IAA28 action and the molecular characterization of auxin responses.

METHODS

Plant Growth Conditions

Arabidopsis accessions used were Columbia (Col-0), Wassilewskija (Ws), and Landsberg *erecta* (L*er*) *tt4*. Seed were surface-sterilized and grown on plant nutrient medium containing 0.5% sucrose (PNS) (Haughn and Somerville, 1986) solidified with 0.6% agar. The medium was supplemented with phytohormones from 0.01, 1, 10, or 100 mM stocks in ethanol or with 15 μg/mL kanamycin from a 25 mg/mL stock in water. Plates were sealed with gas-permeable Leukopor surgical tape (Beiersdorf, Inc., Norwalk, CT) and incubated for 8 days at 22°C under continuous yellow-filtered light to slow the breakdown of indolic compounds (Stasinopoulos and Hangarter, 1990), unless noted otherwise. Plants transferred to soil (Metromix 200; Scotts, Marysville, OH) were grown at 22 to 25°C under continuous illumination with Sylvania (Danvers, MA) Cool White fluorescent bulbs.

Isolation and Phenotypic Characterization of the *iaa28* Mutant

The *iaa28-1* mutant was isolated in a screen for indoleacetic acid (IAA)–alanine–resistant mutants (Davies et al., 1999; Lasswell et al.,

2000) from the M2 progeny of ethyl methanesulfonate–mutagenized (Normanly et al., 1997) Ws seed. Phenotypic characterization was performed on *iaa28-1* plants that had been backcrossed to Ws three or four times to remove unlinked mutations. Hormone resistance (Figure 1) was quantified through root elongation assays. Seed were sown on PNS supplemented with various concentrations of phytohormones. After 8 days of growth at 22°C under yellow-filtered light, the longest root of each seedling was measured. Root length data were standardized against lengths on unsupplemented medium. Roots and hypocotyls of dark-grown seedlings were measured after 1 day in light followed by 4 days in darkness. Root hairs were examined using light microscopy with Nomarski optics.

Lateral roots of 8-day-old seedlings grown under different environmental conditions (Figure 2A) were counted with a dissecting microscope. A lateral root was scored if a visible primordium had formed, even if it had not yet emerged from the primary root. The effect of exogenous auxin (Figure 3) was determined by transferring 4-day-old seedlings grown on unsupplemented medium to either hormone-supplemented medium or unsupplemented medium and counting lateral roots after 4 additional days of growth.

To examine long-term root development (Figure 4A), *iaa28-1* and Ws plants were grown hydroponically (Arteca and Arteca, 2000). Seed were germinated and grown on solid PNS for 8 days and then transferred to floats in Magenta vessels (Sigma) containing plant nutrient medium, without sucrose, diluted sixfold with water. Plants were grown at 22°C in constant white light, and medium was changed every 7 days.

To examine aerial adult phenotypes (Figure 5), Ws and *iaa28-1* seed were germinated and grown on PNS for 8 days before transfer to soil. Plants were examined and measured once a week from 2 weeks after sowing until 5 weeks of age.

To examine the inheritance of the *iaa28-1* root defects (Figure 7), F2 seeds from the fourth backcross were plated on PNS and incubated vertically for 13 days at 22°C under white light, or were plated on PNS containing 100 nM IAA and incubated for 8 days at 22°C under yellow-filtered light. Plants were removed from the media, and roots were scored for lateral roots or measured as described above. DNA was then isolated from each plant individually, and the genotype (*IAA28*/*IAA28*, *IAA28*/*iaa28-1*, or *iaa28-1*/*iaa28-1*) was scored by digesting the product of polymerase chain reaction (PCR) amplification with the primers 5′-CAGGTTCTCTTAATCTCTTCTCATGAATG-3′ and 5′-GATCTTAACGTACAATTCCTTCTCTTCATC-3′ with HaeIII. The 320-bp product has one or no HaeIII sites in *IAA28* or *iaa28-1*, respectively.

Cloning the *IAA28* Gene

F2 seedlings from an outcross of *iaa28-1* to Col-0 were screened for resistance to 40 μM IAA-alanine or 200 nM IAA for mapping. DNA was isolated (Celenza et al., 1995) from resistant plants and screened with PCR-based polymorphic markers (Bell and Ecker, 1994). Additional markers were developed by PCR amplifying and sequencing genomic DNA fragments from Ws, comparing these sequences with those in the database for Col-0, and identifying polymorphisms that altered product size or restriction enzyme sites. New markers developed include F18A17-A (amplification with the primers 5′-GAGGGAGTAGTAGATTTGAGAC-3′ and 5′-GTGATATGAAGCGGTTCAGTAC-3′ yields a 602-bp product with two ScrfI sites in Col-0 and one in Ws), *GA3* (amplification with the primers 5′-CGGAGGCTATCATGTCCCTGC-3′ and 5′-CCCAACGCTTCTTATCCATGTTGC-3′ yields a 156-bp product in Col-0 and a 144-bp product in Ws or L*er*), and T1N24-C (amplification with the primers 5′-CCTGCCAAGATCATACACGTGG-3′ and 5′-GGATCAAACCTCTTGTGGTTCTC-3′ yields a 1185-bp product with two EaeI sites in Ws and an uncut Col-0 product). These markers were used to map the mutation to a 55-kb interval that contained an uncharacterized *Aux/IAA* gene. Two primers (5′-GGGATTGTAGTTCCTTGCCAACC-3′ and 5′-CCATCGAACTGATGATTTTGGCC-3′) flanking this gene were used to PCR-amplify DNA prepared from Ws and the *iaa28-1* mutant, yielding a 1181-bp product. Amplification products were purified by sequential ethanol, polyethylene glycol, and ethanol precipitations (Ausubel et al., 1995) and sequenced directly using an automated DNA sequencer (Lone Star Laboratories, Inc., Houston, TX) with the primers used for amplification.

To make the *iaa28-1* phenocopy construct, a 7.8-kb HindIII fragment containing the *IAA28* gene and promoter region was subcloned from bacterial artificial chromosome T1N24 (Choi et al., 1995) into the HindIII site of pBluescript KS+ (Stratagene, La Jolla, CA). The *iaa28-1* mutation was created in this construct by using oligonucleotide-directed mutagenesis (Ausubel et al., 1995) with the primer 5′-GATGATCTCACCGGCAGCCATCCCACCACTG-3′ (the altered residue is underlined), which introduces the *iaa28-1* mutation and destroys HaeIII and AciI sites. A 6.1-kb HindIII-KpnI fragment from this construct (containing *iaa28-1*, 3.1 kb of the 5′ untranslated region, and 1.8 kb of the 3′ untranslated region) was ligated into the HindIII and KpnI sites of the plant transformation vector pBIN19 (Bevan, 1984) to give pBIN*iaa28-1*. This construct was introduced into *Agrobacterium tumefaciens* GV3101 by electroporation (Ausubel et al., 1995), and the resultant strain was used to transform wild-type Col-0 and Ws plants by using the floral dip method (Clough and Bent, 1998). T1 seed were screened on medium containing 15 μg/mL kanamycin, and resistant seedlings were transferred to soil and allowed to set seed. Homozygous lines were selected by examining the kanamycin resistance of T3 seedlings.

A full-length *IAA28* cDNA was isolated by hybridizing a 1181-bp genomic PCR product that spans the *IAA28* coding region to a pSPORT-based Col-0 cDNA library (S. LeClere and B. Bartel, unpublished data). This cDNA (GenBank accession number AF149816) was sequenced with vector-derived and internal primers. The *iaa28-1* mutation was introduced into the cDNA as described for the genomic clone. The 887-bp inserts from the *IAA28* and *iaa28-1* cDNAs were excised with SalI and NotI and ligated into XhoI-NotI-digested 35SpBARN (S. LeClere and B. Bartel, unpublished data), a binary vector modified from p1′Barbi (Mengiste et al., 1997) to contain a multiple cloning site flanked by the cauliflower mosaic virus 35S promoter and the nopaline synthase (*nos*) terminator. The overexpression constructs (35S–*IAA28* and 35S–*iaa28-1*) were introduced into Col-0 and Ws plants by Agrobacterium-mediated transformation (Clough and Bent, 1998). At ~1 and 2 weeks after germination, T1 seedlings were screened for herbicide resistance by spraying with Finale herbicide (AgrEvo Environmental Health, Montvale, NJ) diluted in water to 0.26 mg/mL glufosinate ammonium.

IAA28 Expression

Total RNA was isolated from Col-0 plants as described previously (Davies et al., 1999), electrophoresed on a 1% agarose gel containing 0.37 M formaldehyde (Ausubel et al., 1995), and transferred to a Bright-Star Plus nylon membrane (Ambion, Austin, TX). A ^{32}P-labeled antisense *IAA28* RNA probe (Riboprobe in vitro transcription system;

Promega, Madison, WI) was hybridized in NorthernMax Prehyb/Hyb buffer (Ambion) overnight at 65°C and washed at high stringency as recommended by the manufacturer. The *IAA28* RNA probe was made by linearizing the cDNA plasmid with EcoRI before RNA synthesis using Sp6 RNA polymerase (Riboprobe in vitro transcription system; Promega). A 25S rDNA probe ^{32}P labeled using random 12-mer oligonucleotides (Ausubel et al., 1995) was used to confirm equal loading of the lanes.

For the auxin induction experiments, Ws and *iaa28-1* seed were plated on sterile filter paper atop agar-solidified PNS in 150-mm Petri dishes. After 8 days under white light at 22°C, the filter paper and seedlings were moved to ~40 mL of liquid plant nutrient medium without sucrose with or without 10 μM IAA for 2 hr. Total RNA, blots, and the *IAA28* probe were prepared as described above. The *IAA1* RNA probe was made by linearizing a cDNA plasmid (expressed sequence tag E6D11T7) with HindIII before RNA synthesis by using T3 RNA polymerase. The *UBQ10* probe used as a loading control was made by subcloning the EcoRI-NotI fragment from expressed sequence tag 193N23T7 into pBluescript KS+ digested with the same enzymes. The resultant plasmid was linearized with EcoRV before RNA synthesis using T7 RNA polymerase.

To construct the *IAA28* promoter–GUS translational fusion, a BamHI site was introduced immediately after the *IAA28* initiation codon by using oligonucleotide-directed mutagenesis with the primer 5′-CTTAGCTCCAATCTCTTTTGGGATCCCATTTTTCTAAGTTTGTG-3′ (the altered residues are underlined). The 3.1-kb promoter fragment was excised using HindIII and BamHI and ligated into pBI101 (Jefferson et al., 1987) cut with the same enzymes. The construct was transformed into Ws, and lines homozygous for the construct were selected as described above. β-Glucuronidase (GUS) activity was histochemically localized with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) as described previously (Bartel and Fink, 1994).

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REFERENCES

Abel, S., and Theologis, A. (1996). Early genes and auxin action. Plant Physiol. **111,** 9–17.

Abel, S., Oeller, P.W., and Theologis, A. (1994). Early auxin-induced genes encode short-lived nuclear proteins. Proc. Natl. Acad. Sci. USA **91,** 326–330.

Abel, S., Nguyen, M.D., Chow, W., and Theologis, A. (1995a). *ACS4*, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. J. Biol. Chem. **270,** 19093–19099.

Abel, S., Nguyen, M.D., and Theologis, A. (1995b). The *PS-IAA4/5*-like family of early auxin-inducible mRNAs in *Arabidopsis thaliana*. J. Mol. Biol. **251,** 533–549.

Ainley, W.M., Walker, J.C., Nagao, R.T., and Key, J.L. (1988). Sequence and characterization of two auxin-regulated genes from soybean. J. Biol. Chem. **263,** 10658–10666.

Arteca, R.N., and Arteca, J.M. (2000). A novel method for growing *Arabidopsis thaliana* plants hydroponically. Physiol. Plant. **108,** 188–193.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1995). Short Protocols in Molecular Biology, 3rd ed. (New York: John Wiley and Sons).

Bartel, B., and Fink, G.R. (1994). Differential regulation of an auxin-producing nitrilase gene family in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **91,** 6649–6653.

Bartel, B., and Fink, G.R. (1995). ILR1, an amidohydrolase that releases active indole-3-acetic acid from conjugates. Science **268,** 1745–1748.

Bell, C.J., and Ecker, J.R. (1994). Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genomics **19,** 137–144.

Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schultz, B., and Feldmann, K.A. (1996). *Arabidopsis AUX1* gene: A permease-like regulator of root gravitropism. Science **273,** 948–950.

Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. **12,** 8711–8721.

Boerjan, W., Cervera, M.-T., Delarue, M., Beeckman, T., Dewitte, W., Bellini, C., Caboche, M., Van Onckelen, H., Van Montagu, M., and Inzé, D. (1995). *superroot*, a recessive mutation in Arabidopsis, confers auxin overproduction. Plant Cell **7,** 1405–1419.

Celenza, J.L., Grisafi, P.L., and Fink, G.R. (1995). A pathway for lateral root formation in *Arabidopsis thaliana*. Genes Dev. **9,** 2131–2142.

Choi, S., Creelman, R.A., Mullet, J.E., and Wing, R.A. (1995). Construction and characterization of a bacterial artificial chromosome library of *Arabidopsis thaliana*. Weeds World **2,** 17–20.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16,** 735–743.

Conner, T.W., Goekjian, V.H., LaFayette, P.R., and Key, J.L. (1990). Structure and expression of two auxin-inducible genes from *Arabidopsis*. Plant Mol. Biol. **15,** 623–632.

Davies, P.J. (1995). Introduction. In Plant Hormones, P.J. Davies, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1–38.

Davies, R.T., Goetz, D.H., Lasswell, J., Anderson, M.N., and Bartel, B. (1999). *IAR3* encodes an auxin conjugate hydrolase from Arabidopsis. Plant Cell **11,** 365–376.

Delarue, M., Prinsen, E., Van Onckelen, H., Caboche, M., and Bellini, C. (1998). *Sur2* mutations of *Arabidopsis thaliana* define a new locus involved in the control of auxin homeostasis. Plant J. **14,** 603–611.

Eklöf, S., Åstot, C., Blackwell, J., Moritz, T., Olsson, O., and Sandberg, G. (1997). Auxin-cytokinin interactions in wild-type and transgenic tobacco. Plant Cell Physiol. **38,** 225–235.

Ferreira, P.C.G., Hemerly, A.S., de Almeida Engler, J., Van Montagu, M., Engler, G., and Inzé, D. (1994). Developmental expression of the Arabidopsis cyclin gene *cyc1At*. Plant Cell **6,** 1763–1774.

Gray, W.M., Östin, A., Sandberg, G., Romano, C.P., and Estelle, M. (1998). High temperature promotes auxin-mediated hypocotyl elongation in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95,** 7197–7202.

Guilfoyle, T., Hagen, G., Ulmasov, T., and Murfett, J. (1998a). How does auxin turn on genes? Plant Physiol. **118,** 341–347.

Guilfoyle, T.J., Ulmasov, T., and Hagen, G. (1998b). The ARF family of transcriptional activators and their role in plant hormone-responsive transcription. Cell. Mol. Life Sci. **54,** 619–627.

Hangarter, R.P., and Good, N.E. (1981). Evidence that IAA conjugates are slow-release sources of free IAA in plant tissues. Plant Physiol. **68,** 1424–1427.

Hartmann, H.T., Kester, D.E., and Davies, F.T. (1990). Anatomical and physiological basis of propagation by cuttings. In Plant Propagation, Principles and Practices (Englewood Cliffs, NJ: Prentice Hall), pp. 199–245.

Haughn, G.W., and Somerville, C. (1986). Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol. Gen. Genet. **204,** 430–434.

Hillman, J.R. (1984). Apical dominance. In Advanced Plant Physiology, M.B. Wilkins, ed (London: Pitman Publishing), pp. 127–148.

Hobbie, L.J. (1998). Auxin: Molecular genetic approaches in *Arabidopsis*. Plant Physiol. Biochem. **36,** 91–102.

Hobbie, L., and Estelle, M. (1995). The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. Plant J. **7,** 211–220.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6,** 3901–3907.

Kim, B.C., Soh, M.S., Kang, B.J., Furuya, M., and Nam, H.G. (1996). Two dominant photomorphogenic mutations of *Arabidopsis thaliana* identified as suppressor mutations of *hy2*. Plant J. **9,** 441–456.

Kim, B.C., Soh, M.S., Hong, S.H., Furuya, M., and Nam, H.G. (1998). Photomorphogenic development of the *Arabidopsis shy2–1D* mutation and its interaction with phytochromes in darkness. Plant J. **15,** 61–68.

Kim, J., Harter, K., and Theologis, A. (1997). Protein–protein interactions among the Aux/IAA proteins. Proc. Natl. Acad. Sci. USA **94,** 11786–11791.

King, J.J., Stimart, D.P., Fisher, R.H., and Bleecker, A.B. (1995). A mutation altering auxin homeostasis and plant morphology in Arabidopsis. Plant Cell **7,** 2023–2037.

Laskowski, M.J., Williams, M.E., Nusbaum, H.C., and Sussex, I.M. (1995). Formation of lateral root meristems is a two-stage process. Development **121,** 3303–3310.

Lasswell, J., Rogg, L.E., Nelson, D.C., Rongey, C., and Bartel, B. (2000). Cloning and characterization of *IAR1*, a gene required for auxin conjugate sensitivity in Arabidopsis. Plant Cell **12,** 2395–2408.

Lehman, A., Black, R., and Ecker, J.R. (1996). *HOOKLESS1*, an ethylene response gene, is required for differential cell elongation in the Arabidopsis hypocotyl. Cell **85,** 183–194.

Leyser, H.M.O., Pickett, F.B., Dharmasiri, S., and Estelle, M. (1996). Mutations in the *AXR3* gene of *Arabidopsis* result in altered auxin response including ectopic expression from the *SAUR-AC1* promoter. Plant J. **10,** 403–413.

Luschnig, C., Gaxiola, R.A., Grisafi, P., and Fink, G.R. (1998). EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. Genes Dev. **12,** 2175–2187.

McClure, B.A., Hagen, G., Brown, C.S., Gee, M.A., and Guilfoyle, T.J. (1989). Transcription, organization, and sequence of an auxin-regulated gene cluster in soybean. Plant Cell **1,** 229–239.

Mengiste, T., Amedeo, P., and Paszkowski, J. (1997). High-efficiency transformation of *Arabidopsis thaliana* with a selectable marker gene regulated by the T-DNA 1 promoter. Plant J. **12,** 945–948.

Morgan, K.E., Zarembinski, T.I., Theologis, A., and Abel, S. (1999). Biochemical characterization of recombinant polypeptides corresponding to the predicted baa fold in Aux/IAA proteins. FEBS Lett. **454,** 283–287.

Nagpal, P.L., Walker, L.M., Young, J.C., Sonawala, A., Timpte, C., Estelle, M., and Reed, J.W. (2000). *AXR2* encodes a member of the Aux/IAA protein family. Plant Physiol. **123,** 563–574.

Normanly, J., Grisafi, P., Fink, G.R., and Bartel, B. (1997). Arabidopsis mutants resistant to the auxin effects of indole-3-acetonitrile are defective in the nitrilase encoded by the *NIT1* gene. Plant Cell **9,** 1781–1790.

Oeller, P.W., Keller, J.A., Parks, J.E., Silbert, J.E., and Theologis, A. (1993). Structural characterization of the early indoleacetic acid-inducible genes, *PS-IAA4/5* and *PS-IAA6*, of pea (*Pisum sativum* L.). J. Mol. Biol. **233,** 789–798.

Oono, Y., Chen, Q.G., Overvoorde, P.J., Köhler, C., and Theologis, A. (1998). *age* mutants of Arabidopsis exhibit altered auxin-regulated gene expression. Plant Cell **10,** 1649–1662.

Pickett, F.B., Wilson, A.K., and Estelle, M. (1990). The *aux1* mutation of *Arabidopsis* confers both auxin and ethylene resistance. Plant Physiol. **94,** 1462–1466.

Reed, J.W., Elumalai, R.P., and Chory, J. (1998). Suppressors of an *Arabidopsis thaliana phyB* mutation identify genes that control light signaling and hypocotyl elongation. Genetics **148,** 1295–1310.

Roman, G., Lubarsky, B., Kieber, J.J., Rothenberg, M., and Ecker, J.R. (1995). Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: Five novel mutant loci integrated into a stress response pathway. Genetics **139,** 1393–1409.

Rouse, D., Mackay, P., Stirnberg, P., Estelle, M., and Leyser, O. (1998). Changes in auxin response from mutations in an *AUX/IAA* gene. Science **279,** 1371–1373.

Skoog, F., and Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. **11,** 118–131.

Smith, D.L., and Fedoroff, N.V. (1995). *LRP1*, a gene expressed in lateral and adventitious root primordia of Arabidopsis. Plant Cell **7,** 735–745.

Stasinopoulos, T.C., and Hangarter, R.P. (1990). Preventing photochemistry in culture media by long-pass light filters alters growth of cultured tissues. Plant Physiol. **93,** 1365–1369.

Swofford, D.L. (2000). PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). (Sunderland, MA: Sinauer Associates).

Tatematsu, K., Watahiki, M.K., and Yamamoto, K.T. (1999). Evidence for a dominant mutation of IAA19 that disrupts hypocotyl growth curvature responses and alters auxin sensitivity. 10th International Conference on Arabidopsis Research (Melbourne, Australia), Abstract No. 8-39.

Theologis, A., Huynh, T.V., and Davis, R.W. (1985). Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. J. Mol. Biol. **183,** 53–68.

Tian, Q., and Reed, J.W. (1999). Control of auxin-regulated root development by the *Arabidopsis thaliana SHY2/IAA3* gene. Development **126,** 711–721.

Timpte, C., Wilson, A.K., and Estelle, M. (1994). The *axr2-1* mutation of *Arabidopsis thaliana* is a gain-of-function mutation that disrupts an early step in auxin response. Genetics **138,** 1239–1249.

Timpte, C., Lincoln, C., Pickett, F.B., Turner, J., and Estelle, M. (1995). The *AXR1* and *AUX1* genes of *Arabidopsis* function in separate auxin-response pathways. Plant J. **8,** 561–569.

Ulmasov, T., Hagen, G., and Guilfoyle, T.J. (1997a). ARF1, a transcription factor that binds auxin response elements. Science **276,** 1865–1868.

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T.J. (1997b). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell **9,** 1963–1971.

Ulmasov, T., Hagen, G., and Guilfoyle, T.J. (1999a). Activation and repression of transcription by auxin-response factors. Proc. Natl. Acad. Sci. USA **96,** 5844–5849.

Ulmasov, T., Hagen, G., and Guilfoyle, T.J. (1999b). Dimerization and DNA binding of auxin response factors. Plant J. **19,** 309–319.

Walker, J.C., and Key, J.L. (1982). Isolation of cloned cDNAs to auxin-responsive poly(A)$^+$ RNAs of elongating soybean hypocotyl. Proc. Natl. Acad. Sci. USA **79,** 7185–7189.

Wilson, A.K., Pickett, F.B., Turner, J.C., and Estelle, M. (1990). A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene, and abscisic acid. Mol. Gen. Genet. **222,** 377–383.

Worley, C.K., Zenser, N., Ramos, J., Rouse, D., Leyser, O., Theologis, A., and Callis, J. (2000). Degradation of Aux/IAA proteins is essential for normal auxin signalling. Plant J. **21,** 553–562.

Wyatt, R.E., Ainley, W.M., Nagao, R.T., Conner, T.W., and Key, J.L. (1993). Expression of the *Arabidopsis AtAux2-11* auxin-responsive gene in transgenic plants. Plant Mol. Biol. **22,** 731–749.

Yamamoto, K.T., Mori, H., and Imaseki, H. (1992). cDNA cloning of indole-3-acetic acid-regulated genes: Aux22 and SAUR from mung bean (*Vigna radiata*) hypocotyl tissue. Plant Cell Physiol. **33,** 93–97.

Yang, S.F., and Hoffman, N.E. (1984). Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. **35,** 155–189.

Yang, T., and Poovaiah, B.W. (2000). Molecular and biochemical evidence for the involvement of calcium/calmodulin in auxin action. J. Biol. Chem. **275,** 3137–3143.

Zolman, B.K., Yoder, A., and Bartel, B. (2000). Genetic analysis of indole-3-butyric acid responses in *Arabidopsis thaliana* reveals four mutant classes. Genetics **156,** 1323–1337.