

age Mutants of Arabidopsis Exhibit Altered Auxin-Regulated Gene Expression

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An Arabidopsis transgenic line was constructed expressing β -glucuronidase (*GUS*) via the auxin-responsive domains (*AuxRDs*) *A* and *B* (*BA-GUS*) of the *PS-IAA4/5* gene in an indoleacetic acid (IAA)-dependent fashion. *GUS* expression was preferentially enhanced in the root elongation zone after treatment of young seedlings with 10^{-7} M IAA. Expression of the *BA-GUS* gene in the *axr1*, *axr4*, and *aux1* mutants required 10- to 100-fold higher auxin concentration than that in the wild-type background. *GUS* expression was nil in the *axr2* and *axr3* mutants. The transgene was used to isolate mutants exhibiting altered auxin-responsive gene expression (*age*). Two mutants, *age1* and *age2*, were isolated and characterized. *age1* showed enhanced sensitivity to IAA, with strong *GUS* expression localized in the root elongation zone in the presence of 10^{-8} M IAA. In contrast, *age2* exhibited ectopic *GUS* expression associated with the root vascular tissue, even in the absence of exogenous IAA. Morphological and molecular analyses indicated that the *age1* and *age2* alleles are involved in the regulation of gene expression in response to IAA.

INTRODUCTION

The plant growth hormone auxin, typified by indoleacetic acid (IAA), has been implicated in regulating many developmental and cellular processes by altering basic patterns of gene expression (Went and Thimann, 1937; Estelle, 1992; Hobbie and Estelle, 1994; Klee and Romano, 1994; Kende and Zeevaart, 1997). The signal transduction pathway(s) leading to auxin-mediated gene activation is not known. The lack of functionally defined auxin receptors and simple phenotypic traits that are specifically associated with auxin action have hindered the elucidation of the auxin signaling apparatus responsible for gene activation using biochemical and genetic approaches.

Auxin-responsive promoter elements of early genes, that is, genes that are among the first to be transcriptionally activated by auxin, provide specific molecular probes for accessing and exploring the auxin signaling apparatus in reverse (Guilfoyle, 1986; Theologis, 1986; Key, 1989; Ballas et al., 1993, 1995; Guilfoyle et al., 1993; Takahashi et al., 1995; Abel et al., 1996; Abel and Theologis, 1996). Recently, the use of transgenic plants containing reporter genes under the control of defined promoter elements have proven useful for identifying mutations that affect specific signal transduc-

tion pathways (Susek et al., 1993; Bowling et al., 1994; Jackson et al., 1995; Li et al., 1995; Martin et al., 1997). In this study, we used the auxin-responsive domains (*AuxRDs*) *A* and *B* of the early *PS-IAA4/5* gene (Ballas et al., 1993, 1995) to construct an Arabidopsis transgenic line expressing the β -glucuronidase (*GUS*) reporter in an IAA- and tissue-dependent manner. Screening a mutagenized population of plants containing this transgene led to the isolation of two mutations, *age1* and *age2* (for auxin-responsive gene expression), that exhibited altered patterns of IAA-induced gene expression and novel morphological phenotypes.

RESULTS

AuxRDs A and B of PS-IAA4/5 Mediate Auxin-Induced Gene Expression in Arabidopsis

The auxin-responsive region (*AuxRR*) of the *PS-IAA4/5* promoter is a 183-bp segment extending from positions –318 to –135 (Ballas et al., 1993, 1995). This region contains two auxin-responsive domains (*AuxRDs A* and *B*) defined by linker scanning mutagenesis that mediate auxin-induced gene expression (Ballas et al., 1993, 1995). To test the ability of these domains to function in Arabidopsis, we used Agrobacterium-mediated transformation to introduce the *GUS* gene under the control of the *AuxRR* or combinations of the *AuxRDs A* and *B*, as shown in Figure 1. The activity of the constructs was tested in both the Nossen (No-0) and

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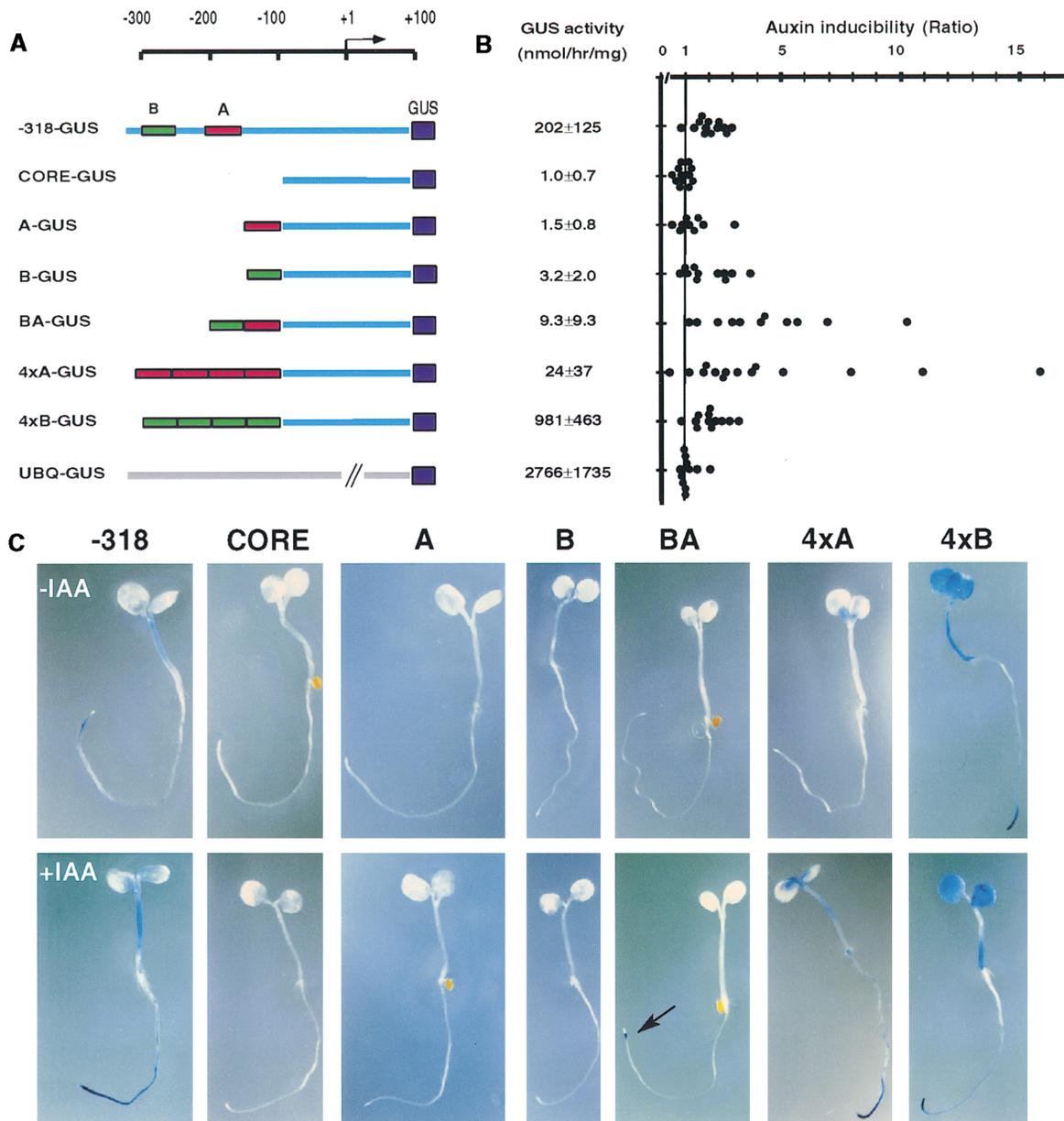


Figure 1. Promoter-*GUS* Constructs and *GUS* Activity in Transgenic Arabidopsis Plants.

(A) Schematic representation of the *AuxRD-GUS* fusions. The transcription start site (+1) is shown, and the lengths of the promoter elements are indicated by the numbers over the constructs. CORE, nonauxin responsive segment spanning positions -92 to +96 of *PS-IAA4/5* gene; UBQ, UBQ3 promoter.

(B) The mean *GUS* activity observed in seedlings analyzed without auxin treatment. The degree of auxin inducibility mediated by each promoter-*GUS* construct is shown as the ratio of *GUS* activity in seedlings treated with 2×10^{-5} M IAA relative to *GUS* activity observed in untreated seedlings. Each point is representative of seedlings from a single line.

(C) Typical *GUS* staining patterns seen in No-0 plants harboring the indicated promoter-*GUS* fusions. The specific lines shown contain -318-*GUS* (line 37), *CORE-GUS* (line 53), *A-GUS* (line 5.1), *B-GUS* (line 20), *BA-GUS* (line 1.3), *4xA-GUS* (line 30.4), and *4xB-GUS* (line 13.2). The seedlings were treated without (-IAA) or with (+IAA) 2×10^{-5} M IAA for 6 hr followed by staining with X-gluc.

Columbia (Col-0) *Arabidopsis* ecotypes, and similar levels and patterns of *GUS* expression were observed (data not shown).

Arabidopsis lines containing four copies of the *AuxRD A* ($4\times A$ -*GUS*) or one copy of the *AuxRD A* plus *AuxRD B* (*BA-GUS*) showed low levels of *GUS* expression in the absence of IAA; however, in the presence of IAA, both lines showed a two- to 15-fold increase in the level of *GUS* expression (Figure 1B). Histochemical staining of 5-day-old light-grown seedlings from the *BA-GUS* lines showed that the majority of IAA-induced *GUS* expression was localized in the elongation zone of the root, although weak IAA-induced *GUS* staining also was seen in the petioles of the cotyledons, the hypocotyl, and the root vascular tissue (Figure 1C). In the roots, the *GUS* staining pattern was strongest in the atricoblast cell files, whereas the tricoblast cell file exhibited a lower level of *GUS* staining (Figures 2A and 2B). The enhanced levels of *GUS* staining in cell files that failed to form root hairs are similar to those reported for the expression of *GL2*, a non-auxin-regulated gene encoding a homeobox protein that appears to repress root hair differentiation (Masucci et al., 1996). Genetic analysis of *Arabidopsis* mutants with altered patterns of root hair formation in combination with auxin- and ethylene-related mutations suggests that the signaling pathways of these growth regulators act to promote root hair outgrowth at a relatively late stage of differentiation (Masucci et al., 1996). The significance of the expression of *BA-GUS* in cell files that fail to form root hairs is not known.

The auxin-induced increase in *GUS* activity seen in the $4\times A$ -*GUS* lines appeared as variable patterns of staining in the cotyledon petioles, the hypocotyl, root elongation zone, and the root vascular tissue extending beyond the elongation zone (Figure 1C). $4\times B$ -*GUS* lines showed a pattern of spatially unregulated *GUS* staining that is independent of IAA treatment (Figure 1C). These results suggest that specific cells are more or less competent to respond to auxin by mediating gene expression through specific auxin-responsive domains. For example, whereas the *BA-GUS* and $4\times A$ -*GUS* lines showed similar levels of *GUS* expression with and without auxin treatment, the cells capable of mediating auxin-induced *GUS* expression from these different domains were distinct. This differential regulation could also be seen when comparing the levels and distribution of *GUS* expression in the $4\times B$ -*GUS* lines with the *BA-GUS* lines. Both *AuxRDs A* and *B* functioned to mediate the amplitude of the auxin response, whereas the combination of the two domains mediated the response in a specific set of cells in the root tip (Figure 1C). It is clear from these experiments and those performed with transgenic tobacco that the roots of light-grown plants contain the highest proportion of cells that are able to sustain auxin-induced expression from the *AuxRDs A* and *B* (Ballas et al., 1993).

Plants containing single copies of the *A* or *B* domain (*A-GUS* and *B-GUS*, respectively) showed low levels of *GUS* activity in the absence of auxin, but the addition of IAA resulted in small increases in *GUS* expression. Histochemical staining

of seedlings from these lines showed diffuse and variable staining patterns (Figure 1C). In contrast, plants containing the entire *AuxRR* (-318 -*GUS*) showed high levels of *GUS* expression in the absence of auxin that was enhanced two-fold in response to IAA. Again, histochemical analysis revealed that without auxin, seedlings displayed low levels of *GUS* activity throughout the plant and that IAA treatment enhanced *GUS* expression in the root tip (Figure 1C). Finally, control lines containing the non-auxin-responsive core region (positions -92 to $+96$) of the *PS-IAA4/5* promoter (*CORE-GUS*) exhibited low levels of *GUS* activity that do not change in response to IAA (Figure 1).

Selection of the BA3 Reporter Line

After examining the strength and consistency of *GUS* staining patterns of eight different *BA-GUS* lines, we chose one line, designated WT/BA3, for detailed characterization. The similarity in size and shape of the WT/BA3 pollen grains with those from nontransgenic plants suggests that WT/BA3 is a diploid (Altmann et al., 1994). The WT/BA3 line is homozygous for the reporter gene, as determined by *GUS* staining and resistance to kanamycin. Table 1 shows the effect of various growth regulators on *GUS* expression in the root elongation zone of the WT/BA3 line. *GUS* expression was strongest in the presence of IAA, 1-naphthaleneacetic acid (NAA), or 2,4-D. Indole-3-butyric acid (IBA), a less active form of auxin, mediated a weaker response. The inactive auxin analog 2,3-dichlorophenoxyacetic acid (2,3-D), the IAA transport inhibitors *p*-chlorophenoxyisobutyric acid (PCIB) and 2,3,5-triiodobenzoic acid (TIBA), and IAA metabolic precursors indole and tryptophan were unable to induce *GUS* expression after 6 hr of treatment. Fusicoccin (FC), a fungal toxin that induces proton secretion in various plant tissues, also had no effect on the levels of *GUS* activity. Finally, the plant hormones gibberellic acid (GA), abscisic acid (ABA), benzyladenine (BA), salicylic acid (SA), and ethylene or its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), failed to enhance *GUS* expression.

To confirm that the *AuxRDs A* and *B* were responsible for the observed pattern of *GUS* expression, we introduced a second reporter gene, that encoding the green fluorescent protein (GFP), into the WT/BA3 line under the control of one copy of the *AuxRD A* plus one copy of *AuxRD B* (*BA-GFP*). *BA-GFP* expression was similar to that of *BA-GUS*; that is, it was restricted in the root elongation zone and was inducible by auxin (Figure 2C). The limited fluorescence intensity, however, precluded the use of this reporter for identifying mutants displaying altered auxin-induced gene expression.

DNA gel blot analysis using *GUS* and neomycin phosphotransferase DNA as probes showed that WT/BA3 contains two copies of the T-DNA insert as right border/right border inverted repeats (data not shown). To define the location of the transgene in the genome, a DNA fragment was amplified by thermal asymmetric interlaced polymerase chain reaction

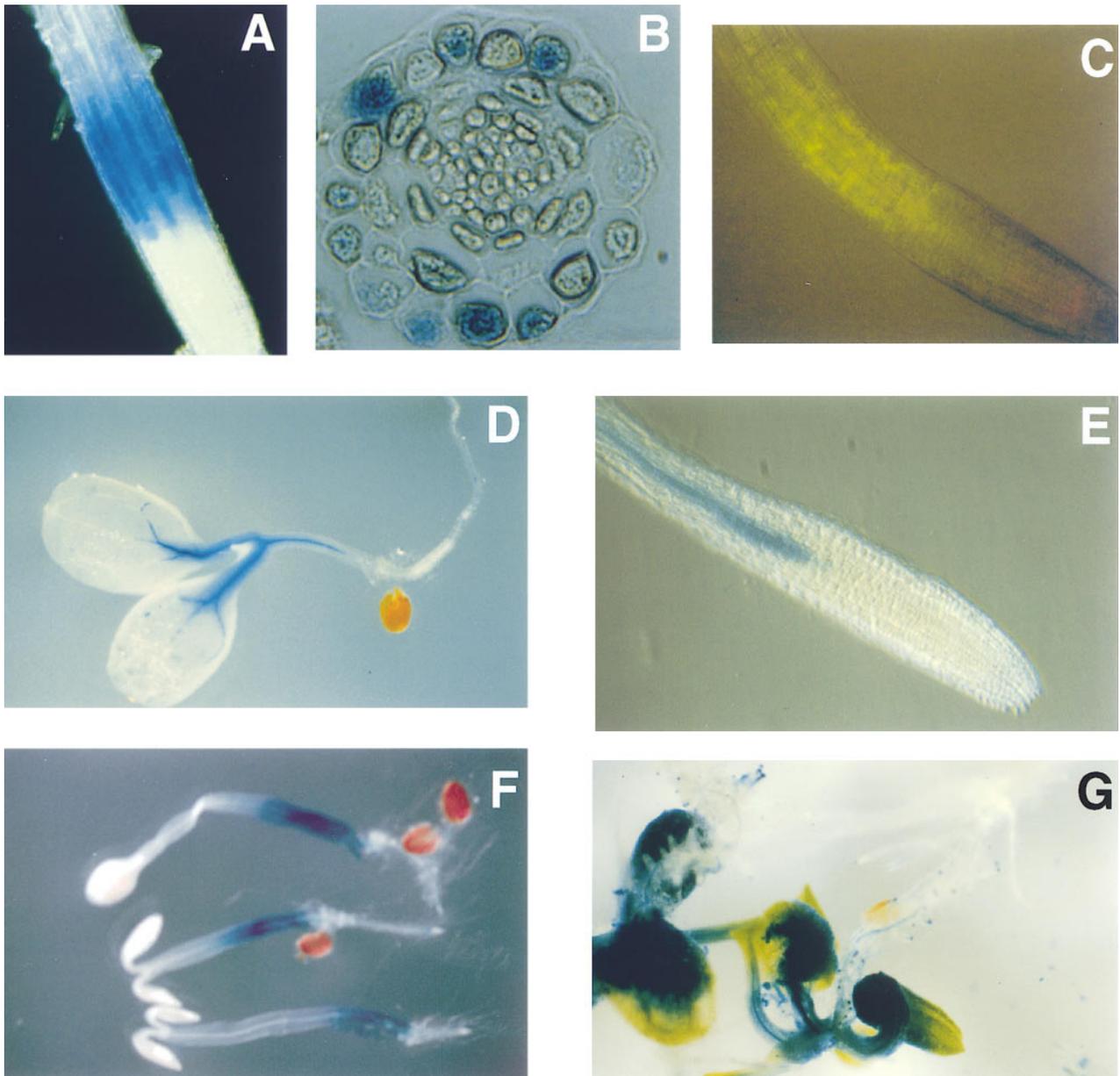


Figure 2. Details of Histochemical Localization of *GUS* or *GFP* Reporter Gene Expression.

(A) Dark-field image of *GUS* staining in a 5-day-old WT/BA3 root after treatment with 10^{-7} M IAA for 6 hr. The *GUS* staining reflects the expression of *GUS* in the elongation zone of the root tip.

(B) Cross-section through the apical region of a WT/BA3 root treated with 10^{-7} M IAA for 6 hr and then stained for *GUS* activity. The *GUS* staining pattern shows that the BA promoter mediates high levels of expression in the atricoblast cell file.

(C) Fluorescent image of the root tip from a 5-day-old wild-type seedling harboring the *BA-GFP* transgene treated with 2×10^{-5} M IAA for 24 hr.

(D) Bright-field image of a 5-day-old *axr2*/BA3 root seedling stained for *GUS* activity without being treated with IAA.

(E) Bright-field image of a 5-day-old *axr2*/BA3 seedling stained for *GUS* activity after being treated with 10^{-7} M IAA for 6 hr. Note the lack of staining in the elongation zone that is seen in WT/BA3 plants.

(F) Bright-field image of 5-day-old *hls3*/BA3 seedlings stained for *GUS* activity after being treated with 10^{-7} M IAA for 6 hr.

(G) Bright-field image of a 2-week-old *hls3*/BA3 seedling stained for *GUS* activity after being treated with 10^{-7} M IAA for 6 hr. Note the *GUS* staining in the emerging primordia of newly forming lateral roots.

(TAIL-PCR) and was sequenced (Liu et al., 1995). One side of the amplified fragment contained 120 bp that were identical to the pBin19 T-DNA sequence, whereas the second fragment of flanking sequence did not have identity to any entries in the databases. PCR amplification using primers within this novel region and a DNA template from a yeast artificial chromosome (YAC) library showed that this fragment is present on YAC clones yUP12G6, yUP23E10, and yUP24B8, demonstrating that the *BA-GUS* transgene is located near the KG17 marker in the middle of chromosome 3 (see <http://cbil.humgen.upenn.edu/~atgc/physical-mapping/physmaps.html>).

The sensitivity of the *BA-GUS* reporter gene to exogenous auxin was determined by examining GUS staining in seedlings treated with 0 to 10^{-4} M auxin (Figure 3). In the WT/BA3 seedlings, treatment with 10^{-7} M IAA resulted in a dramatic increase in GUS activity. Based on the intensity of staining, maximal expression of the transgene occurred at 10^{-4} M IAA (Figure 3), and expression was inhibited at 10^{-3} M IAA (data not shown). Several Arabidopsis mutants with reduced auxin sensitivity have been identified and characterized. To determine the tissue specificity of the *BA-GUS* transgene in these mutant backgrounds, we crossed the WT/BA3 line with *axr1-12* (Lincoln et al., 1990), *axr2-1* (Timpte et al., 1995), *axr3-1* (Leyser et al., 1996), *axr4-3* (Hobbie and Estelle, 1995), *aux1-7* (Pickett et al., 1990), and *hls3-12* (Lehman et al., 1996). F_3 seedlings homozygous for the mutant allele and the *BA-GUS* transgene were treated with 0 to 10^{-4} M auxin, and the GUS staining pattern was determined. The results from these experiments showed that *axr1/BA3*, *axr4/BA3*, and *aux1/BA3* require 10- to 100-fold higher concentration of auxin compared with the wild-type background to show GUS staining in the root elongation zone (Figure 3). These results are consistent with previous observations showing that these mutants are less sensitive to treatment with exogenous levels of auxin.

In contrast, whereas *axr2/BA3* and *axr3/BA3* failed to show this cell-specific GUS staining in the root elongation zone even at the highest levels of auxin tested (Figure 3), both lines showed GUS staining patterns that were significantly different from those of WT/BA3 plants. Specifically, the *axr2/BA3* seedlings showed auxin-induced *GUS* expression in the vascular tissue of the elongation zone, suggesting that the dominant *axr2* mutation may specifically decrease the ability of the cells outside of the vascular tissue to respond to exogenously applied auxin (Figures 2D and 2E). The *axr3/BA3* seedlings exhibited auxin-independent expression associated with the vascular tissue throughout the root, with the highest levels observed where the root bends (Figure 3). The lack of GUS staining in the root elongation zone in the *axr2/BA3* and *axr3/BA3* lines (Figure 3) was not due to the suppression of the *BA-GUS* gene because plants resulting from backcrosses to the nontransgenic Col-0 exhibited a normal *GUS* expression pattern (data not shown).

Disruption of *HLS3*, which is an allele of *ALF1*, *SUR1*, and *RTY1*, results in a dramatic increase in the levels of IAA and

the proliferation of lateral roots (Lehman et al., 1996). Interestingly, BA-mediated *GUS* expression in 5-day-old *hls3/BA3* seedlings was restricted to the lower part of the hypocotyl (Figure 2F). Such intense staining was never observed in the WT/BA3 lines even after treatment with high levels of auxin (data not shown). Furthermore, when *hls3/BA3* seedlings were examined after 2 weeks, during which time the number of lateral roots increased, GUS staining was observed in the emerging lateral root primordia and the leaves (Figure 2G). Because the *hls3* phenotype appears to be caused by an increase in IAA (Lehman et al., 1996), the high level of *GUS* expression in the hypocotyl suggests that this region may generate or accumulate high quantities of auxin. Alternatively, this increase in *GUS* expression could be mediated by an increase in sensitivity to auxin.

Isolation of *age* Mutants

The strong and reproducible auxin-induced *GUS* expression in the WT/BA3 line suggests that it can be used for identifying mutations with altered auxin-induced gene expression patterns. Using *GUS* expression in the root tip of the WT/BA3 line as a marker, we initiated a screen to isolate mutants that showed altered auxin-induced gene expression (Figure 4A). To identify putative mutants, we screened M_2

Table 1. Effect of Various Growth Regulators on *GUS* Expression in the Root Elongation Zone of the WT/BA3 Line

Treatment	GUS Activity ^a
Control	–
20 μ M IAA	+++
20 μ M IBA	+
20 μ M NAA	+++
20 μ M 2,4-D	+++
20 μ M 2,3-D	–
20 μ M PCIB	–
20 μ M TIBA	–
20 μ M FC	–
20 μ M indole	–
20 μ M tryptophan	–
20 μ M GA	–
20 μ M ABA	–
20 μ M BA	–
20 μ M SA	–
20 μ M ACC	–
20 μ L/L C ₂ H ₄	–
Air	–

^aBatches of five to 10 seedlings were analyzed in at least two independent experiments. GUS activity in the root elongation zone of 5-day-old light-grown seedlings was assessed visually as strong (+++), weak (+), or undetectable (–).

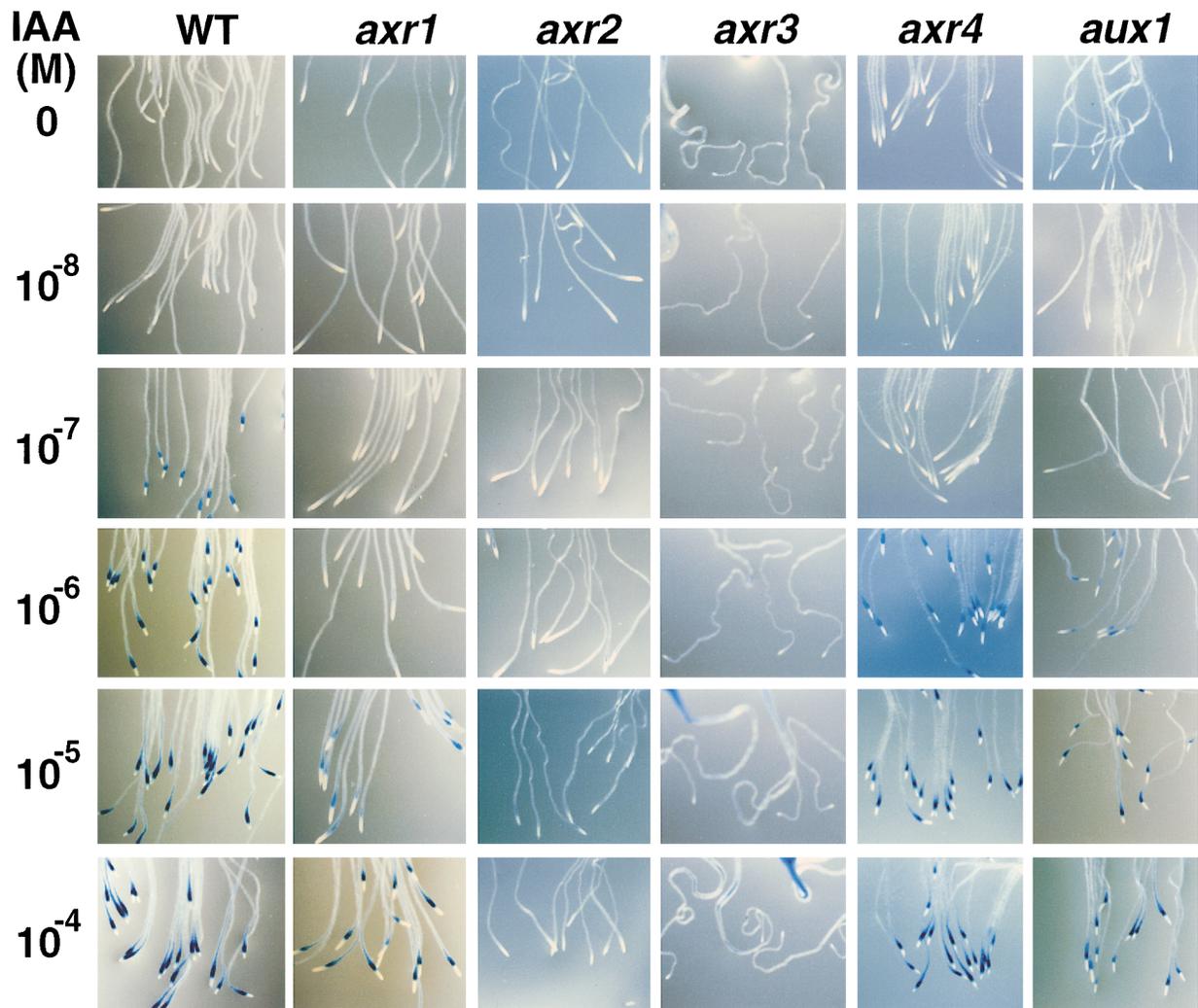


Figure 3. Histochemical Analysis of GUS Activity in the Root Tip of *BA-GUS* Plants after Treatment with 0 to 10^{-4} M IAA.

Homozygous seedlings containing the *BA-GUS* gene in wild-type (WT), *axr1-12* (*axr1*), *axr2-1* (*axr2*), *axr3-1* (*axr3*), *axr4-2* (*axr4*), or *aux1-7* (*aux1*) backgrounds were examined.

seedlings of an ethyl methanesulfonate–mutagenized population by using a nondestructive GUS staining assay (Figures 4B to 4D). Briefly, roots of 10- to 14-day-old seedlings were incubated with 0.5 to 1×10^{-8} M IAA for 6 hr and then stained with GUS for 15 hr (Figures 4C and 4D). Because WT/*BA3* seedlings failed to show any GUS activity when treated with this level of IAA (Figure 3), seedlings showing GUS staining after this treatment were selected as candidates for further analysis. An example of this screen is shown in Figure 4D. Of the 125,000 seedlings tested, 142 mutant candidates were identified. They were transplanted to soil and allowed to set seeds. Auxin-induced GUS activity was assayed in M_3 progeny of 65 of these putative mutants

to determine whether the phenotype was heritable. Forty-one of the lines showed GUS activity. Two homozygous lines, 43-1c and 12-2b, showing reproducible altered auxin-regulated gene expression were chosen for further characterization. These lines were named *age1* and *age2*, respectively.

***age* Mutants Have Altered Morphology**

In addition to the altered expression of *BA-GUS*, the *age1* and *age2* mutations showed distinct, tightly linked morphological characteristics that include defects in root and leaf

morphology. When wild-type *Arabidopsis* seedlings were grown on plates containing high concentrations of agar and maintained at a 45° angle, the roots exhibited a wavy growth pattern (Figure 5B) that appeared to be caused by the periodic change in the orientation of the root tip generated by the twisting of epidermal cells (Okada and Shimura, 1990). In contrast, when the *age1* seedlings were grown under these conditions, there was a marked decrease in the number of times the root tip underwent reorientation, even though there were no significant differences in the length of the roots (Figure 5C). In addition, the cotyledons and leaves of *age1* seedlings appeared pale when compared with the cotyledons and leaves of wild-type plants (Figures 5B to 5D). When mature, *age1* plants were shorter, exhibited a bushy growth habit, had pale, epinastic rosette leaves, and showed reduced fertility (Figure 5E). In addition, the *age1* plants bolted later, and the inflorescence stem was thinner than that of the wild type (data not shown).

Although mature *age2* plants also showed a short, bushy growth habit, the seedlings developed normally, the leaves expanded properly, and there was no change in the wavy root growth pattern (Figure 5E). Furthermore, *age2* plants

flowered at the same time as did wild-type plants and had a similar inflorescence stem. Unlike previously described auxin-response mutants, neither of the *age* mutations disrupted the root gravitropic response, nor did they lead to increased auxin resistance in a root growth inhibition assay (data not shown).

Figure 6 shows that whereas both mutations exhibited inducible *GUS* expression with 10^{-8} M IAA (compare wild type with *age1* and *age2*), the tissue specificity of expression was distinct. In *age1* plants, *GUS* expression induced by 10^{-8} M IAA was present in the root elongation zone, whereas the *age2* mutant lacked staining in this region of the seedling (Figure 6A). In addition to this localized increase in *GUS* activity, *age1* plants showed an increase in *GUS* staining in the cotyledon petioles, the hypocotyl, and the upper portion of the root vascular tissue (Figure 6B). Although the *age2* plants showed staining in similar parts of the seedling, *GUS* activity was much stronger in the vascular tissue of the upper part of the root and the lower part of the hypocotyl (Figure 6B). Furthermore, although *GUS* staining in the root elongation zone of *age2* seedlings required the same level of IAA as wild-type plants, the staining pattern associated with

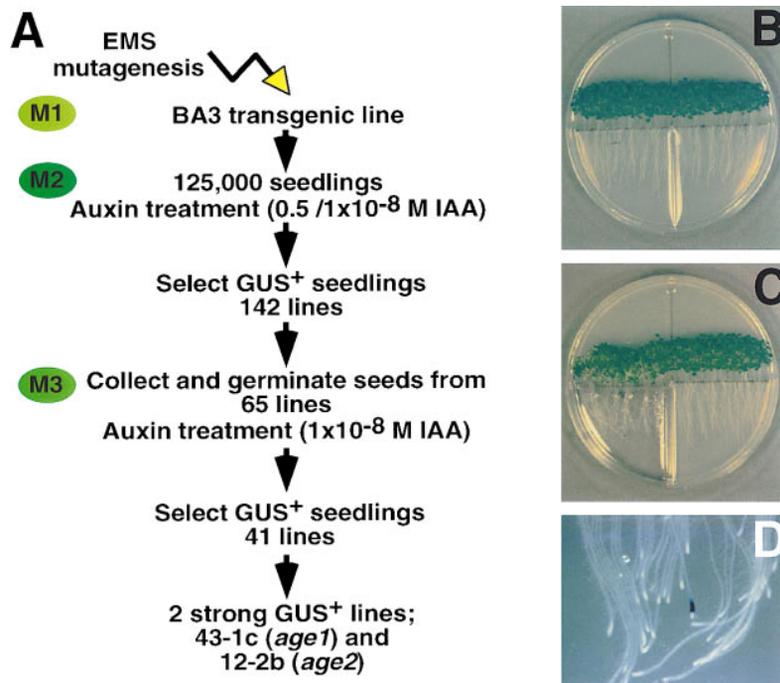


Figure 4. Outline of the Mutant Screen.

(A) Steps in the mutant screen. EMS, ethyl methanesulfonate.

(B) Two-week-old seedlings grown vertically in a four-celled Falcon 1009 Petri plate.

(C) Two-week-old seedlings with the agar removed from cell 2 so that the roots can be treated with auxin incubation solution.

(D) Simulation of the screening. A WT/BA3 seedling treated with 2×10^{-5} M IAA for 6 hr and stained for *GUS* activity was placed among the roots of seedlings incubated without IAA.

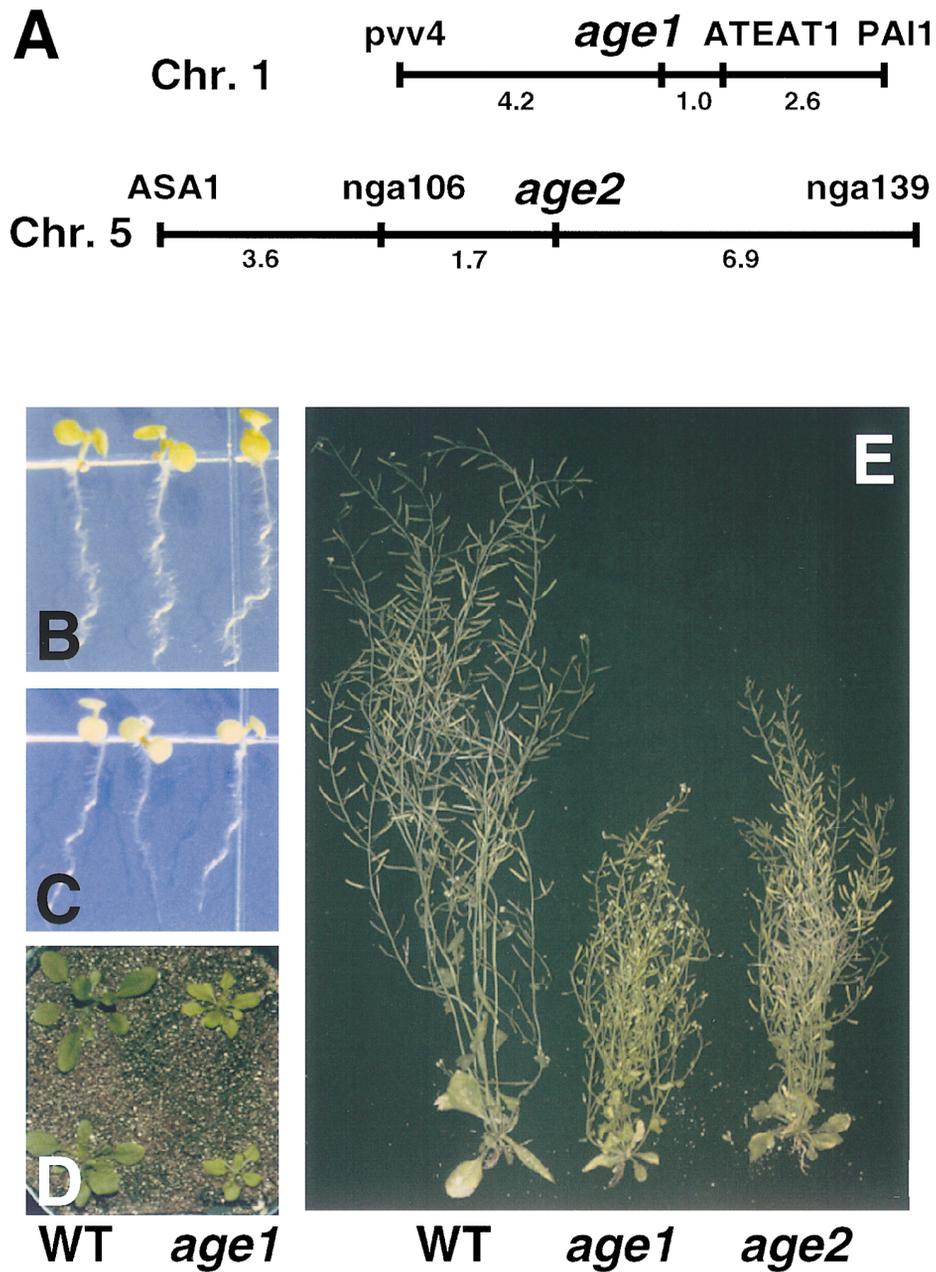


Figure 5. Characterization of *age1* and *age2*.

(A) Schematic representation of the map location of the *age1* and *age2* loci. The genetic distances, in centimorgans, to the loci indicated were determined by analyzing 190 lines for the *pvv4* marker, 195 lines for the *ATEAT1* marker, 114 lines for the *PAI1* marker, 43 lines for the *ASA1* marker, and 88 lines each for the *nga106* and *nga139* markers. Chr., chromosome.

(B) to **(E)** Five-day-old wild-type (WT) and *age1* seedlings (**[B]** and **[C]**, respectively) grown on 1.6% agar plates inclined at 45°. **(D)** shows 4-week-old wild-type (left) and *age1* (right) plants. **(E)** shows mature plants: wild type (left), *age1* (center), and *age2* (right). A comparison of **(B)** and **(C)** shows the decreased number of bends in the roots of wild-type versus *age1* plants. **(D)** shows the decreased size and pale nature of *age1* plants relative to wild-type plants. **(E)** shows the morphological phenotype of mature wild-type, *age1*, and *age2* plants.

the vascular tissue was independent of auxin treatment. Treatment of WT/BA3 plants with high concentrations of IAA occasionally induced a similar pattern of GUS staining associated with the vascular tissue.

Genetic Analysis of *age* Mutations

Backcrosses of *age1* plants with unmutagenized WT/BA3 plants were used to determine the nature of this mutation. The resulting F₁ plants showed reduced but detectable GUS staining in the root tip with 10⁻⁸ M IAA, suggesting that the *age1* mutation may be semidominant. We observed the segregation 82:49:29 (nonstained-weakly stained-strongly stained) regarding GUS staining in F₂ seedlings. In contrast, all of the F₁ plants had wild-type-like morphology, and the selfed F₂ progeny showed a 3:1 distribution of wild-type-to-mutant phenotype, as expected for a recessive mutation. To clarify the GUS staining pattern, we performed an additional self-cross. Analysis of F₃ seedlings from 127 F₂ lines showed 34 lines yielding seedlings with no staining (wild-type homozygotes), 64 lines producing a mixture of staining and nonstaining seedlings (heterozygotes), and 29 lines yielding seedlings that all showed GUS staining (mutant homozygotes). All 64 heterozygous lines showed some seedlings with weak blue staining. We observed <10 percent stained F₃ seedlings in eight lines for which we quantitated *GUS* expression. The 29 homozygous lines exhibiting GUS staining also possessed the *age1* morphological characteristics, whereas the other 98 lines showed wild-type-like morphology.

These data indicate that *age1* is a single, recessive mutation. The leakiness of *GUS* expression in the heterozygotes may be due to the inability of the wild-type protein at one-half of its concentration to effectively suppress *GUS* expression. The same amount of wild-type protein is sufficient to confer the wild-type phenotype in the heterozygous plants. Mapping of the *age1* mutation by analyzing simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequence (CAPS) markers on 195 F₂ lines resulting from crosses with the Landsberg *erecta* (*Ler*) ecotype shows that the mutation falls between the *pvv4* and *ATEAT1* markers on chromosome 1 (Figure 5A).

All F₁ seedlings from multiple lines obtained by backcrossing the *age2* mutation with WT/BA3 plants showed a *GUS* expression pattern similar to that observed in wild-type seedlings. In addition, the F₂ populations showed strong GUS staining in the vascular tissue in one-quarter of the progeny. Analysis of GUS activity in 156 F₃ lines exhibiting the morphological phenotype associated with the *age2* mutation also showed ectopic expression of *GUS*, suggesting that the morphological phenotype and the pattern of *GUS* expression are tightly linked. Taken together, these data indicate that the *age2* mutation is also a single recessive mutation. Mapping of the *age2* mutation by analyzing SSLP and CAPS markers on 114 F₂ lines resulting from crosses with the *Ler* ecotype showed that the mutation falls between

nga106 and *nga139* on chromosome 5 (Figure 5A). Because the *BA-GUS* gene is integrated in chromosome 3, this excludes the possibility that the *age1* and *age2* mutations are caused by mutations in the transgene.

Altered Steady State RNA Levels in *age* Plants

Arabidopsis has at least 25 members belonging to the *Aux/IAA* gene family that show complex, overlapping patterns of expression during plant development (Abel et al., 1995; Kim et al., 1997). The majority of these family members are rapidly (4 to 30 min) induced by exogenous IAA in wild-type *Arabidopsis*, and the expression of each of these genes is altered in the auxin-resistant mutant lines *axr1*, *axr2*, and *aux1* (Abel et al., 1995). Although the pea *PS-IAA4/5* promoter has been studied extensively (Ballas et al., 1993, 1995), the promoters of only two of the *Arabidopsis* genes, *IAA-4* and *IAA-5*, which correspond to *AtAux2-11* and *AtAux2-27*, respectively (Conner et al., 1990), have been sequenced. The elements responsible for their auxin-regulated gene expression have not been defined thoroughly.

To analyze the effect of the *age* mutations on the steady state levels of IAA mRNAs, we isolated RNA from *age1* or *age2* plants that were homozygous for both *BA-GUS* and *BA-GFP*. Figure 7 shows the results of the RNA gel blot analysis. In both wild-type and *age1* plants, the steady state RNA levels of *GUS*, *GFP*, *IAA-1*, *IAA-4*, and *IAA-5* increased after treatment with 10⁻⁷ M IAA (Figure 7A). Consistent with previous reports, neither *IAA-10* nor *EF-1 α* showed any response to exogenous auxin (Abel et al., 1995). The apparent discrepancy between the increased level of GUS or GFP activity seen in the *age1* roots treated with 10⁻⁸ M IAA and the lack of response in *GUS* or *GFP* transcript levels is likely due to the limited number of cells showing increased BA-driven expression. Although the concentration of IAA required to detect an increase in auxin-induced RNA levels remained constant in both the wild type and the *age1* mutant, the amplitude of the response differed in the two backgrounds. Specifically, there is a decrease in *GFP*, *IAA-1*, *IAA-4*, and *IAA-5* mRNA levels in the *age1* plants relative to the wild-type plants after treatment with 10⁻⁷ to 10⁻⁴ M IAA (Figure 7A). The failure to observe a decrease in *GUS* mRNA levels in the *age1* mutant may be due to one of the following reasons: (1) the expression of *GUS* mRNA may be higher due to a positional effect on the *BA3-GUS* transgene; (2) the expression of the various *Aux/IAA* genes tested may be lower in the mutant because of their differential tissue-specific expression; or (3) the mutation may preferentially affect *GUS* mRNA/protein stability or *GUS* mRNA translatability.

In *age2* seedlings that have not been treated with IAA, the levels of *GUS*, *GFP*, *IAA-1*, and *IAA-12* RNAs were higher than in the wild type (Figure 7B). The *age2* mutation had no effect on the steady state levels of *IAA-4* (Figure 7B), which is known to respond to exogenous auxin (Conner et al., 1990; Abel et al., 1995). Similarly, the *age2* mutation had no effect

on the levels of *IAA-10*, *EF-1 α* , or *GST-5*, a glutathione S-transferase gene that is not auxin regulated (Watahiki et al., 1995; Figure 7B).

DISCUSSION

A novel screen to identify mutants that exhibit altered auxin-regulated gene expression has been performed. The basis for this approach was the observation that the extensively characterized *AuxRDs* from the pea *PS-IAA4/5* promoter (Ballas et al., 1993, 1995) can mediate auxin-inducible gene expression of two reporter genes, *GUS* and *GFP*, in transgenic Arabidopsis. The expression characteristics conferred by various *AuxRD* combinations in Arabidopsis are similar to those obtained in previous studies using transient expression in pea protoplasts (Ballas et al., 1993, 1995). Both analyses suggest that the *AuxRD A* contains the major auxin-

responsive element(s) (*AuxRE[s]*) and that *AuxRD A* and *AuxRD B* act cooperatively to activate early gene expression in response to auxin (Figure 1; Ballas et al., 1995). A second feature that facilitated the screen was the cell-specific expression of *BA-GUS* upon auxin treatment. Finally, the non-destructive nature of the root assay allowed the facile identification of putative mutants and their subsequent rescue for setting seeds.

The pattern of *BA-GUS* expression observed in the *axr1*, *aux1*, and *axr4* mutants (Timpte et al., 1995) is consistent with the hypothesis that there are multiple pathways mediating auxin-regulated gene expression. Although we have not examined *BA-GUS* expression in double mutants, it is known that *AXR1* functions in a signaling pathway that is independent of either *AUX1* or *AXR4* (Hobbie and Estelle, 1995; Timpte et al., 1995). Because auxin-induced *BA-GUS* expression is altered in each of these mutants (Figure 3), multiple, distinct auxin signaling pathways affect *BA*-directed gene expression.

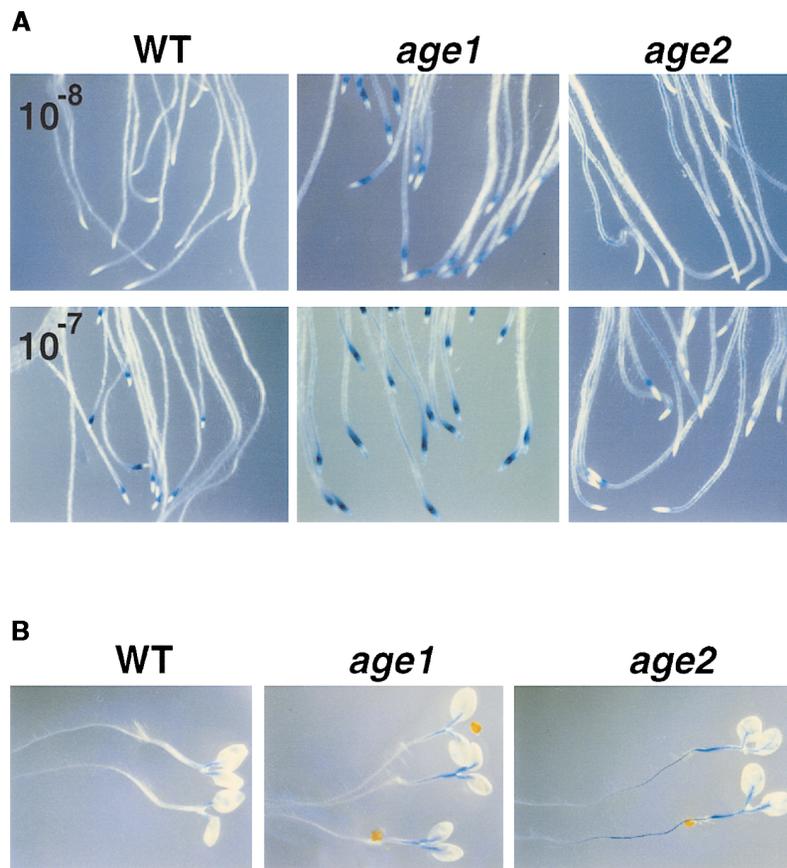


Figure 6. Histochemical Staining of WT/BA3, *age1*/BA3, and *age2*/BA3 Plants.

(A) GUS staining patterns in the roots of 5-day-old seedlings after a 6-hr treatment with either 10⁻⁸ or 10⁻⁷ M IAA. WT, wild type.

(B) GUS staining patterns in the aerial portion of the seedlings that had not been treated with IAA.

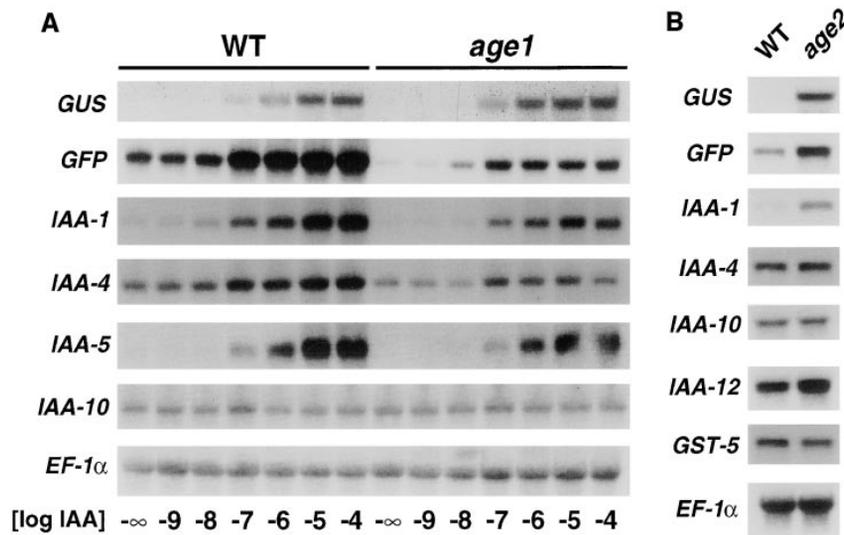


Figure 7. RNA Gel Blot Analysis of Wild Type, *age1*, or *age2* Plants That Were Homozygous for Both *BA-GUS* and *BA8-GFP*.

(A) Total RNA was isolated from 5-day-old wild-type (WT) or *age1* seedlings that had been treated with 0 to 10^{-4} M IAA. Twenty micrograms of RNA was separated by electrophoresis, blotted to a Nytran membrane, and probed with radiolabeled gene-specific probes for the indicated cDNAs.

(B) Twenty micrograms of RNA isolated from the roots of 5-day-old untreated *age2* seedlings was analyzed as described for **(A)**.

Interestingly, the ectopic *BA-GUS* expression associated with the root vascular tissue in the *axr3* background is similar to that seen in seedlings expressing a *SAUR-AC1* promoter—*GUS* fusion (Leyser et al., 1996). Because *SAUR-AC1* has been identified as an early auxin response gene (Gil et al., 1994), the *axr3* mutation may lie in a signaling pathway that also mediates *BA-GUS* expression. The recent identification of *AXR3* as *IAA-17*, a member of the *Aux/IAA* gene family (Rouse et al., 1998), raises the possibility that the *Aux/IAA* gene products may be involved in auto-regulating early auxin gene expression (Kim et al., 1997).

Mutants related to heat shock, light-regulated gene expression, circadian rhythm, systemic acquired resistance, and carbon sensing have been obtained by using screens that identify altered expression of promoter-reporter gene constructs under different selective conditions (Susek et al., 1993; Bowling et al., 1994; Jackson et al., 1995; Li et al., 1995; Martin et al., 1997). Mutants obtained by this type of screen were not previously identified by scoring morphological characteristics, suggesting that mutations affecting reporter gene expression are specific to a particular signaling pathway. Similarly, map positions and *GUS* expression patterns show that *age1* and *age2* are not allelic to previously described auxin response mutants nor do they exhibit morphological changes that mimic other auxin response mutants. The complex pattern of *Aux/IAA* gene expression in the *age* backgrounds indicates that these mutations likely control late steps in specific signaling pathways that are re-

sponsible for proper control of various auxin-regulated genes. For instance, the fact that the *age2* plants contain increased levels of *GUS*, *GFP*, *IAA-1*, and *IAA-12* RNA but do not show changes in the amount of *IAA-4* RNA suggests that this mutation may be due to the loss of a specific negative regulator that normally suppresses the expression of a subset of auxin-responsive genes.

The complex phenotype exhibited by *age1* plants, including the increased sensitivity of *GUS* expression in specific cell types, the decrease in steady state levels of auxin-inducible RNAs, and the multiple phenotypic changes, indicate that this mutation is not due to changes in IAA biosynthesis or uptake. The increased sensitivity shown in specific cells suggests that the *age1* mutation may represent the loss of a negative regulator that permits expression of *BA*-driven *GUS* expression at lower auxin concentrations. In addition, if this negative regulator normally functions to suppress the expression or action of other genes that are involved in the regulation of early auxin genes, then mutations that alter its activity will lead to the aberrant expression of these genes. If one of these genes is involved in a negative feedback loop to control the steady state levels of *Aux/IAA* RNAs, then an increase in its expression can account for the decreased level of *Aux/IAA* gene expression, even though the sensitivity to auxin remains the same. The isolation and characterization of the *AGE1* and *AGE2* gene products will provide a better understanding of their function and their role in regulating auxin-mediated early gene expression.

METHODS

Plasmid Construction and Plant Transformation

All plasmids were constructed using standard recombinant DNA techniques; their authenticity was confirmed by DNA sequencing. The promoter-*GUS* fusions containing the *AuxRD A*, *AuxRD B*, *AuxRR* (positions -318 to +96), *AuxRD A* plus *B*, $4\times$ *AuxRD A*, $4\times$ *AuxRD B*, and the auxin nonresponsive *CORE* (-92 to +96) were constructed from previously described promoter-*CAT* constructs (Ballas et al., 1995). The corresponding *Sall*-*NcoI* or *XhoI*-*NcoI* fragments containing the auxin-responsive domains were joined in a three-way ligation with the *NcoI*-*SacI* fragment of *pUBQncoGUS* (Norris et al., 1993) and the *SacI*-*XhoI* fragment of *pBI101.1*. The *UBQ3-GUS* construct was generated by ligating the *HindIII*-*SacI* fragments from *pUBQ3ncoGUS* and *pBI101.1*.

The *BA-GFP* gene was constructed as follows. The *BA* region of the *PS-IAA4/5* promoter was isolated as an *XhoI*-*NcoI* fragment from *pBA-CAT*, and the C-terminal region of *GFP* with the *nos* terminator was isolated as an *NcoI*-*EcoRI* fragment from *pBIN35mGFP4* (Haseloff et al., 1997). The fragments were joined in a three-way ligation with *EcoRI*-*XhoI*-digested *pBluescript SK+* (Stratagene, La Jolla, CA) to yield *pBA-N-GFP*. The N-terminal portion of *GFP* was introduced into this construct as a 300-bp *NcoI* fragment generated by polymerase chain reaction (PCR), yielding *pBA-GFP* containing an *NcoI* site at the initiating methionine. The *BA-GFP-nos* fragment was introduced into the *XhoI*-*EcoRI*-digested binary vector *pPZP121* (Hajdukiewicz et al., 1994), which was used to transform by electroporation *Agrobacterium tumefaciens* LBA4404, for transforming Nossen (No-0) plants, and GV3101 (pMP90), for transforming Columbia (Col-0) plants. Transformation of *Arabidopsis thaliana* with the *Agrobacterium* strains was performed according to standard procedures (Valvekens et al., 1988; Bechtold et al., 1993).

Plant Growth Conditions and Reporter Gene Assay

Surface-sterilized seeds were plated on germination media (GM) (0.5 \times Murashige and Skoog salts [Gibco BRL, Gaithersburg, MD], 1% sucrose, 1 \times B5 vitamins, and 0.5 g/L Mes, pH 5.8) containing 0.8 or 1.6% Bacto agar (Difco, Detroit, MI). For synchronous seedling germination, the plates were kept in the dark for 2 days at 4°C and then transferred to 23°C with continuous light. Quantitative β -glucuronidase (*GUS*) assays were performed as follows. Ten-day-old kanamycin-resistant seedlings grown under continuous light were treated with or without 20 μ M indoleacetic acid (IAA) for 6 hr, as described previously (Theologis et al., 1985). The seedlings were washed three times with 50 mM sodium phosphate, pH 7.0, and *GUS* activity was determined using 4-methylumbelliferyl β -D-glucuronide as a substrate (Gallagher, 1992). For histochemical analysis of *GUS* expression, 5-day-old seedlings were grown vertically on GM containing 0.8% agar, treated with the concentration of chemicals listed in Table 1 for 2 hr, rinsed three times with staining buffer lacking 5-bromo-4-chloro-3-indolyl β -D-glucuronide (X-gluc) (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, and 0.1% Triton X-100), and then incubated for 18 hr in staining buffer containing 1 mM X-gluc. To remove chlorophyll from the green tissues, we incubated the stained plants in 70% ethanol. *GUS* staining patterns were recorded using a Nikon SMZ-2T or a Zeiss Axiophot microscope. Green fluorescence protein (GFP) imaging was

performed on a Zeiss Axiophot microscope (Thornwood, NY) using a standard fluorescein filter set (extension filter, BP450-490; dichroic mirror, FT 510; emission filter, LP 520).

DNA and RNA Gel Blot Analyses

Plant DNA was isolated using the cetyltrimethylammonium bromide extraction procedure described by Murray and Thompson (1980). RNA was isolated from 5-day-old seedlings treated with the IAA concentration indicated in Figure 7 by using the procedure of Theologis et al. (1985). We performed DNA and RNA hybridization analyses with Nytran membranes (Schleicher & Schuell) by using ^{32}P -labeled probes, as described previously (Abel et al., 1995).

Thermal Asymmetric Interlaced PCR

For mapping the integration site of the *BA-GUS* transgene in the WT/BA3 line, thermal asymmetric interlaced (TAIL)-PCR was performed following the procedure described by Liu et al. (1995), using the following primers: arbitrary primers TAIL-2 (5'-TC[A/T]TCIG[A/C/G/T]-ACIT[GC]CTC-3') and TAIL-3 (5'-CA[A/T]CTIC[A/C/G/T]AGIA[GC]-TCG-3'); and TL-DNA border-specific primers TL-1 (5'-CCCTAT-CTCGGGCTATTCTTTTGA-3'), TL-2 (5'-TATAAGGGATTTTGCCGATTTTCGG-3'), and TL-3 (5'-AACCACCATCAAACAGGATTTTC-3'). TL-1, TL-2, and TL-3 were used for primary, secondary, and tertiary TAIL-PCR reactions, respectively. The amplified fragments were subcloned into *pBluescript KS-* vector and sequenced. From the sequence obtained, two additional PCR primers, BA3F1 (5'-TAC-TCGCAACATAGCTTACATCA) and BA3F2 (5'-ATGCTTAGCTCT-GTCCTCTAACA), were synthesized and used to screen the *yUPYAC* library for yeast artificial chromosome (YAC) clones containing this region of genomic DNA. Thirty-five PCR cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min were performed, and the templates yielding a product were used to identify the chromosome location by using the map generated by Ecker et al. (<http://cbil.humgen.upenn.edu/~atgc/physical-mapping/physmaps.html>).

Mutant Screen and Genetic Analysis

Seeds of the WT/BA3 transgenic line were mutagenized with 0.3% ethyl methanesulfonate for 17 hr at room temperature, as described by Haughn and Sommerville (1986). These M_1 seeds were germinated on soil and allowed to self-pollinate. Approximately 200 surface-sterilized M_2 seeds were placed in wells 1 and 4 of a four-cell Falcon 1009 Petri plate (Becton Dickinson and Co., Lincoln Park, NJ) containing 1.6% agar in GM (see Figure 4). The seeds were placed in a parallel line 0.5 cm from the divider separating cells 1 and 4 from cells 2 and 3. To facilitate coordinated seedling emergence, we placed the plates in the dark for 2 days at 4°C and then maintained them vertically at 23°C under an 18-hr-light and 6-hr-dark cycle, with cells 2 and 3 being closest to the ground. After 10 to 14 days, the agar in cells 2 and 3 was carefully removed without damaging the seedling roots and replaced with incubation buffer containing 1 \times GM, 0.1% ethanol, and 10^{-8} or 5×10^{-9} M IAA. After a 6-hr incubation in the dark at room temperature, the auxin incubation medium was replaced by X-gluc staining solution (1 mM X-gluc, 0.25 \times Murashige and Skoog salts, 0.5% sucrose, and 50 mM sodium phosphate, pH 7.0), and the plates were incubated at 37°C for 15 hr. The candidate mutant plants were isolated by using forceps, washed extensively in

sterile water, and placed on GM containing 0.8% agar. After 7 to 10 days, the recovered seedlings were transferred to soil, and seeds were collected.

From the 45,000 seedlings screened using 10^{-8} M IAA, 100 plants were rescued, and 45 of these set seed. After a secondary screen using 10^{-8} M IAA, 28 lines showed GUS staining. The *age1* line was isolated from this pool of seedlings. From the 80,000 seedlings screened using 5×10^{-9} M IAA, 42 plants were rescued, and 20 of these set seed. After a secondary screen using 10^{-8} M IAA, 13 lines showed GUS staining. The *age2* line was isolated from this second pool of seedlings.

The mapping of mutations was performed using cleaved amplified polymorphic sequence (CAPS) and simple sequence length polymorphism (SSLP) markers following standard procedures (Bell and Ecker, 1994). The scoring of individuals in the mapping population was conducted for *age1* by looking for the morphological characteristics of *age1* and for *age2* by staining with X-gluc solution for 15 hr at 37°C without auxin treatment.

The *axr1-12*, *axr2-1*, *axr4-1*, and *aux1-7* lines were obtained from M. Estelle (Indiana University, Bloomington). The *axr3-1* line was provided by O. Leyser (University of York, UK), and the *hls3-12* line was from J. Ecker (University of Pennsylvania, Philadelphia). The *BA-GUS* gene was introduced into these backgrounds by crossing them with WT/BA3 plants.

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