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RESEARCH ARTICLE

Arabidopsis SHY2/IAA3 Inhibits Auxin-Regulated Gene Expression

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In Arabidopsis, *SHY2* encodes *IAA3*, a member of the auxin-induced *Aux/IAA* family. Gain-of-function mutations in *SHY2/IAA3* cause enlarged cotyledons, short hypocotyls, and altered auxin-regulated root development. Here we show that the gain-of-function mutation *shy2-2* decreases both the induction and repression of auxin-regulated genes, suggesting that *SHY2/IAA3* acts as a negative regulator in auxin signaling. *shy2-2* affects auxin induction of many previously characterized primary response genes, implying that it might repress primary auxin responses. In addition, *shy2-2* also affects expression of multiple auxin-nonresponsive genes. Light regulates expression of *SHY2/IAA3*, suggesting a possible link between light and auxin response pathways.

INTRODUCTION

Auxins regulate numerous cellular and developmental responses in plants, including cell division, expansion, and differentiation; patterning of embryos, vasculature, and other tissues; and distribution of growth between primary and lateral root and shoot meristems (Thimann, 1977; Sachs, 1991). This multiplicity of regulatory activities has spurred considerable interest in mechanisms of auxin signaling and response. Although auxin may regulate some cellular responses (such as expansion or polarity) through direct effects on membrane or cytoskeletal functions, auxin also regulates expression of numerous genes whose products probably perform most developmental responses (Guilfoyle, 1999).

Three major classes of early auxin response genes have been identified from various plant species: *Aux/IAA*, *SAUR* (small auxin upregulated), and *GH3* (Guilfoyle, 1999). Some *ACS* genes, encoding 1-aminocyclopropane-1-carboxylate synthase, which is required for ethylene biosynthesis, also can be induced by auxin (Abel et al., 1995a). Auxin induces many of these genes rapidly, specifically, and in the absence of de novo protein synthesis, and these genes are considered primary response genes. The protein synthesis inhibitor cycloheximide can induce some *Aux/IAA*, *SAUR*, *GH3*, and *ACS* genes, indicating that a short-lived protein normally represses these transcripts (Franco et al., 1990; Abel et al., 1995b; Roux and Perrot-Rechenmann, 1997; Hsieh et al., 2000).

Aux/IAA genes are the best characterized auxin-responsive genes, and there are at least 24 such genes in Arabidopsis (Liscum and Reed, 2001). Analysis of *IAA1* to *IAA14* transcripts revealed different spatial expression patterns and varied profiles of induction by auxin (Abel et al., 1995b). Some, such as *IAA3* and *IAA6*, were induced within minutes and returned to baseline levels after 2 hr. These genes, as well as genes whose induced expression persisted for several hours, behaved as primary auxin response genes. Others, such as *AXR2/IAA7* and *IAA8*, were induced much more slowly, and this induction depended on protein synthesis. Therefore, they may be secondary response genes.

Functional auxin response elements (AuxREs) containing the consensus core sequence 5′-TGTCTC-3′ have been identified in promoters of primary response genes (Guilfoyle, 1999; Hagen and Guilfoyle, 2001). ARF (auxin response factor) proteins can bind to these elements, and there are 23 ARF genes in Arabidopsis. ARF proteins have an N-terminal DNA binding domain, and most ARFs have domains III and IV, which are conserved among both ARFs and Aux/IAA proteins, at their C termini. Between the N-terminal domain and the C-terminal domain is a middle region that is quite divergent among different ARFs. Transient assays using carrot protoplasts showed that the ARF proteins can regulate expression of promoters having AuxREs (Ulmasov et al.,

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1997). Some of the ARF proteins (ARF5, ARF6, ARF7, and ARF8) with Q-rich middle domains will activate transcription, whereas others (ARF1) may repress transcription (Ulmasov et al., 1999).

Aux/IAA genes are targets of auxin regulation, and they encode proteins likely to regulate auxin-responsive gene expression. Semidominant mutations in several *Aux/IAA* genes, such as *SHY2/IAA3*, *AXR3/IAA17*, *AXR2/IAA7*, *MSG2/IAA19*, *IAR2/IAA28*, and *SLR/IAA14*, have been isolated, and each one causes pleiotropic auxin-related phenotypes, suggesting that *Aux/IAA* genes play a central role in auxin signaling (Reed, 2001). *Aux/IAA* genes encode short-lived nuclear proteins that contain four highly conserved motifs called domains I, II, III, and IV. Aux/IAA proteins that have been tested have half-lives as short as 6 to 8 min (Abel et al., 1994). All of these semidominant mutations cause amino acid changes in a very conserved amino acid sequence (VGWPPV) in domain II, and they probably increase protein function by stabilizing the corresponding proteins, indicating that domain II serves as a degradation signal (Worley et al., 2000; Ouellet et al., 2001; Ramos et al., 2001). Domain III is a dimerization domain that mediates homodimerization and heterodimerization among Aux/IAA and ARF proteins (Kim et al., 1997; Ulmasov et al., 1997; Morgan et al., 1999; Ouellet et al., 2001). Transient expression studies showed that Aux/IAA proteins can repress the auxin-dependent activation of AuxRE-mediated reporter genes (Ulmasov et al., 1997). Because there is no evidence that Aux/IAA proteins bind directly to DNA, it is likely that the interactions among Aux/IAA proteins and ARF proteins regulate gene expression. Some of the *aux/iaa* mutants show altered expression of auxin-inducible genes or reporter constructs, consistent with Aux/IAA proteins regulating auxin-induced transcription (Timpte et al., 1994; Abel et al., 1995b; Leyser et al., 1996; Rogg et al., 2001).

Semidominant *shy2* (short hypocotyl 2) mutations were identified in screens for suppressors of the long hypocotyl phenotype of phytochrome-deficient *hy2* or *phyB* mutants (Kim et al., 1996; Reed et al., 1998). *SHY2* encodes *IAA3*, one of the *Aux/IAA* genes (Soh et al., 1999; Tian and Reed, 1999). *shy2-1*, *shy2-2*, and *shy2-3* have missense mutations in conserved domain II, and *shy2-2* plants have higher steady state levels of SHY2/IAA3 protein than wild-type plants (Colón-Carmona et al., 2000). *shy2-2* seedlings have short hypocotyls and enlarged cotyledons both in the dark and in the light, have upcurled leaves in the light, and flower early (Kim et al., 1996, 1998; Reed et al., 1998; Tian and Reed, 1999). *shy2-2* seedlings also make leaves in the dark. Consistent with *SHY2/IAA3* having a role in auxin responses in roots, *shy2-2* seedlings also have fewer lateral roots, less wavy root growth on tilted hard agar plates, and slower root gravitropic response than wild-type seedlings (Tian and Reed, 1999). *shy2-1* mutants, which have the same mutation as *shy2-2*, have similar shoot phenotypes and express *CAB* and *PSBA* genes when grown in the dark (Kim et al., 1998). *shy2-3* plants have similar but less severe phenotypes; therefore, *shy2-3* is a weaker allele. The dark phenotypes of these mutants suggest that *SHY2/IAA3* may regulate light responses. We deduced that *shy2-2* is a gain-of-function mutation because it is semidominant, a *shy2-2* mutant transgene confers mutant phenotypes in wild-type plants, and it can be suppressed by intragenic loss-of-function mutations (Tian and Reed, 1999).

The *shy2-24* mutation is an intragenic suppressor of *shy2-2*, and it introduces a stop codon just upstream of the *shy2-2* mutation. Therefore, *shy2-24* is a putative null mutant. In contrast to *shy2-2*, *shy2-24* mutants have no drastic leaf or hypocotyl phenotype. However, they have root phenotypes, such as increased number of lateral roots, enhanced wavy root growth on tilted hard agar plates, and faster root gravitropic response.

The Aux/IAA protein biochemistry results discussed above suggested that *shy2* mutant phenotypes might arise from altered auxin-regulated gene expression. To determine whether this is true, and to identify potential regulatory targets of *SHY2/IAA3*, we used gene chips, RNA gel blot hybridizations, and promoter::reporter constructs to compare gene expression in the wild type, *shy2-2*, and *shy2-24* mutants with or without auxin treatment. We found that *shy2-2* affects the expression of many of the previously identified auxin-inducible genes, such as *Aux/IAA*, *SAUR*, *GH3*, and *ACS* genes, as well as numerous previously unknown auxin-responsive genes. *shy2-2* also negatively regulates itself. In addition, the *shy2-2* mutation affects many genes not regulated by auxin, which might be responsible for developmental phenotypes. Light also regulates the expression of *SHY2/IAA3*, suggesting that light and auxin signaling pathways interact.

RESULTS

Autoregulation of *SHY2/IAA3*

Six-day-old light-grown wild-type, *shy2-2*, and *shy2-24* seedlings were either mock treated or treated with 20 μM indoleacetic acid (IAA) for 2 hr, and the overall expression levels of *SHY2/IAA3* were examined by RNA gel blot hybridization. As shown in Figure 1, wild-type seedlings had a low steady state level of *SHY2/IAA3* expression, and upon auxin treatment, the transcript level was increased approximately threefold. In gain-of-function *shy2-2* seedlings, both the steady state level and the induction of *SHY2/IAA3* by auxin were reduced severely compared with wild-type seedlings, suggesting that shy2-2 negatively autoregulates its own gene. In loss-of-function *shy2-24* seedlings, the transcript level also was lower than in wild type at both steady state and induced levels. The *shy2-24* mutation causes a premature stop codon, and the decreased transcript level in this case might arise from transcript instability.

Previous RNA gel blot hybridization data indicated that

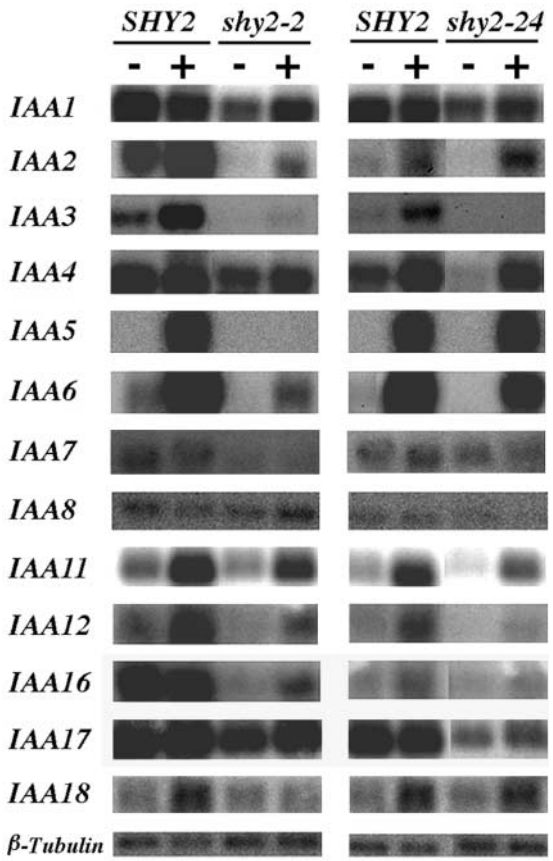

Figure 1. RNA Gel Blot Analysis of Expression of *Aux/IAA* Genes in *SHY2* (Wild Type) and *shy2-2* and *shy2-24* Mutants.

Poly(A)$^+$ RNA (5 μg) was isolated from 6-day-old *SHY2*, *shy2-2*, and *shy2-24* light-grown seedlings that had been either mock treated (−) or treated with 20 μM IAA for 2 hr (+). RNA was separated in 1% agarose, transferred to a nylon membrane, and hybridized with ^{32}P-labeled *Aux/IAA* probes or with a ^{32}P-labeled *β-tubulin* probe. *shy2-2* and *shy2-24* were assayed in different experiments, and the wild-type controls for each experiment are shown. Exposure times were 72 hr for *IAA1*, *IAA2*, *IAA4*, *IAA11*, *IAA12*, *IAA16*, and *IAA17* and 288 hr for *IAA3*, *IAA5*, *IAA6*, *IAA7*, *IAA8*, and *IAA18*.

SHY2/*IAA3* mRNA is most abundant in etiolated seedlings and in leaves, stems, and flowers of light-grown plants; and that it is present in roots as well (Abel et al., 1995b). To determine more precisely where *SHY2*/*IAA3* is expressed and how *shy2* mutations affect the expression, we transformed wild-type plants with a reporter gene consisting of 2.2 kb of DNA upstream of the *SHY2*/*IAA3* start codon fused to the *Escherichia coli* β-glucuronidase gene (*GUS*). This same upstream fragment, when fused to *shy2-2* and transformed into wild-type plants, conferred *shy2-2*–like phenotypes, suggesting that it is a functional promoter (Tian and Reed, 1999). Six independent transgenic lines each with an insertion at a single locus were generated and analyzed by histochemical staining for GUS activity. All six lines showed similar 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-gluc) staining patterns. We treated seedlings of one line with auxin and found that the expression of *GUS* mRNA was induced approximately twofold, as expected. This line then was crossed to gain-of-function and loss-of-function *shy2* mutants.

Figure 2A shows the X-gluc staining of 9-day-old wild-type, *shy2-2*, and *shy2-24* seedlings carrying the $P_{SHY2/IAA3}$::*GUS* transgene. Expression in light-grown wild-type seedlings was prominent in hypocotyls and cotyledons. Newly formed leaf primordia did not show any staining. This expression pattern varied over time. For example, after 14 days of growth, the first pair of leaves had detectable staining, as did hypocotyls and cotyledons, but newly formed leaves did not stain. After 21 days of growth, staining disappeared in hypocotyls and persisted in older leaves (data not shown). Dark-grown wild-type seedlings stained in the hypocotyls but not in the cotyledons, and this pattern persisted over time. Overall, X-gluc staining was stronger in dark-grown seedlings than in light-grown seedlings. There was no staining in the roots in either light- or dark-grown seedlings.

Consistent with our mRNA expression data (Figure 1), we observed less X-gluc staining in *shy2-2* $P_{SHY2/IAA3}$::*GUS* seedlings than in wild-type seedlings in both light and dark (Figure 2A). Light-grown *shy2-2* $P_{SHY2/IAA3}$::*GUS* seedlings had almost no staining, and dark-grown *shy2-2* $P_{SHY2/IAA3}$::*GUS* seedlings either had no staining or had staining only in cotyledons but not in the hypocotyl. In *shy2-24* $P_{SHY2/IAA3}$::*GUS* seedlings, X-gluc staining was identical to that in the wild type, which is consistent with our suggestion that the decreased *SHY2*/*IAA3* transcript level in *shy2-24* reflects transcript instability rather than decreased promoter activity. Quantitative GUS activity assays (Figure 2B) supported these results.

Light Regulates *SHY2*/*IAA3* Expression

The expression of $P_{SHY2/IAA3}$::*GUS* was greater in dark-grown than in light-grown seedlings, implying that light might repress *SHY2*/*IAA3* gene expression. To test this idea, dark-grown wild-type seedlings were either given a pulse of red light and then returned to darkness or shifted to continuous white light. After 4 hr, RNA from the light-treated seedlings was isolated, and the expression of *SHY2*/*IAA3* was compared with that in dark-grown seedlings by RNA gel blot hybridization. As shown in Figure 3, consistent with the X-gluc staining data, light inhibited *SHY2*/*IAA3* expression. In the same experiment, both light treatments induced the expression of *CAB*, as expected.

We found that this light regulation of *SHY2*/*IAA3* depended on growth conditions. The experiments showing light repression of *SHY2*/*IAA3* expression were all performed in medium containing sucrose. In contrast to these

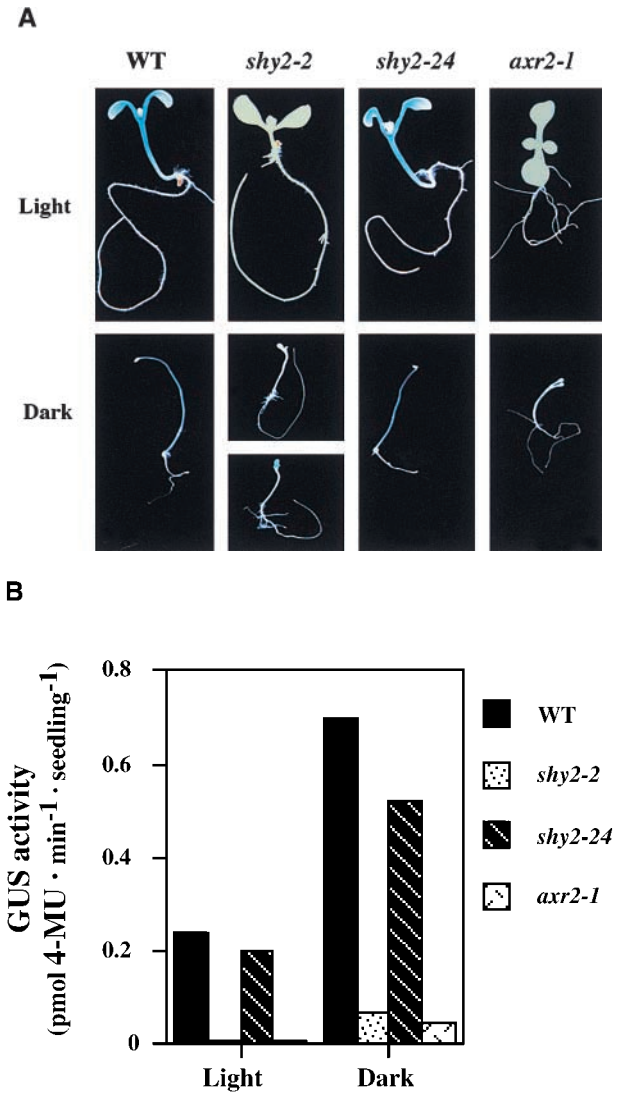

Figure 2. Histochemical Analysis of Expression of $P_{SHY2/IAA3}$::*GUS*.

(A) X-gluc staining of 9-day-old wild type (WT), *shy2-2*, *shy2-24*, and *axr2-1* $P_{SHY2/IAA3}$::*GUS* seedlings grown in the light or in the dark.
(B) 4-Methylumbelliferyl β-D-glucuronide (4-MU) assays of light-grown and dark-grown wild-type (WT), *shy2-2*, *shy2-24*, and *axr2-1* $P_{SHY2/IAA3}$::*GUS* seedlings.

results, in the absence of sucrose, light induced *SHY2/IAA3* expression. Seedlings were grown in the dark for 6 days in the absence of sucrose and were given a pulse of red light and returned to darkness for 2 or 4 hr. As shown in Figure 4, wild-type seedlings had less basal expression of *SHY2/IAA3* than did wild-type seedlings grown in the presence of sucrose (Figure 3), and red light induced *SHY2/IAA3* expression in both wild-type and *shy2-24* seedlings, despite the deduced instability of the *shy2-24* transcript. Red light did not induce expression in *shy2-2* seedlings, presumably because of the negative autoregulation by *shy2-2*. As expected, the expression of *CAB* was induced by red light in wild-type seedlings. Previous studies showed that *shy2-1* seedlings grown in the presence of sucrose had increased expression of *CAB* in the dark (Kim et al., 1998). As shown in Figure 4, in the absence of sucrose, *shy2-2* seedlings also had increased expression of *CAB* compared with that of the wild type, and the induction of *CAB* in *shy2-2* seedlings was abolished. We discuss a simple model to explain these contrasting effects of light under different growth conditions below.

shy2-2 Impedes Auxin-Regulated Expression of Multiple Genes

To determine how *SHY2/IAA3* affects expression of other genes, we performed a gene chip experiment. Wild-type, *shy2-2*, and *shy2-24* seedlings were grown in the light for 6 days, mock treated or treated with 20 μM IAA for 2 hr, and their RNA was isolated. Probes made from a mixture of RNA isolated from three independent experiments were hybridized to an Affymetrix gene chip having ~8000 Arabidopsis genes. Approximately two-thirds of the genes (5500 genes) had detectable signals in our experiment. As listed in Tables 1 and 2, by using a cutoff of ≥2.5-fold auxin induction or repression in at least two genotypes, we identified 74 auxin-induced genes and 26 auxin-repressed genes. Twenty of the 74 auxin-induced genes were previously identified genes of the *Aux/IAA*, *SAUR*, *GH3*, and *ACS* gene families.

We compared the expression of these genes among the wild-type and *shy2* mutants. Consistent with the subtle phenotypes of *shy2-24* plants, expression levels of auxin-responsive genes in *shy2-24* generally paralleled those in the wild type (except for *SHY2/IAA3* itself). *shy2-24* plants had reduced expression of just four genes, and none of these four genes was auxin responsive. Thus, the differences between wild-type and *shy2-24* plants are slight, and in practice, the *shy2-24* mutant data served as a quasi-replicate of wild-type data.

In contrast to *shy2-24*, many genes had altered expression in *shy2-2* seedlings. Forty-two of the 74 auxin-induced genes were less induced in *shy2-2* seedlings than in wild-type seedlings. Seventeen of these 42 genes were previously identified auxin-inducible *Aux/IAA*, *SAUR*, *GH3*, and *ACS* genes. Fourteen of the 26 auxin-repressed genes had decreased repression in *shy2-2* seedlings. In addition, the chip has 19 more genes of the *Aux/IAA*, *SAUR*, *GH3*, and *ACS* gene families that were either induced (16 genes) or repressed (three genes) by auxin <2.5-fold. On the basis of previous RNA gel blot hybridization data, we also consider these genes to be auxin-responsive genes (Abel et al., 1995b). Six of these genes also were less induced in *shy2-2* seedlings (Figure 5).

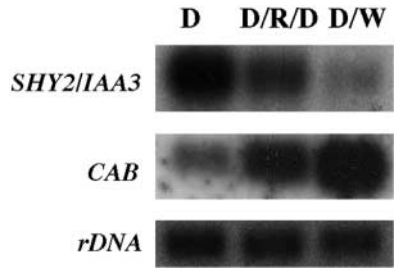

Figure 3. Inhibition of *SHY2/IAA3* Expression by Light in the Presence of Sucrose.

Six-day-old dark-grown wild-type seedlings were either given a pulse of red light (R) and then returned to darkness (D) or shifted to continuous white light (W). mRNA was isolated after 4 hr, and RNA gel blots were hybridized with *SHY2/IAA3* cDNA, *CAB*, or *rDNA* probes. Exposure times were 1 hr (*CAB* and *rDNA*) and 72 hr (*SHY2/IAA3*).

Aux/IAA Genes

In addition to the gene chip experiment, we also examined expression of selected *Aux/IAA* genes by RNA gel blot hybridization (Figure 1). In total, we assessed expression of 22 *Aux/IAA* genes, 12 of them by both methods. In most cases for which we obtained data from both experiments, these were qualitatively consistent. The collected data revealed that other than *SHY2/IAA3*, expression of 11 *Aux/IAA* genes was lower in *shy2-2* seedlings than in wild-type seedlings. As shown in Figures 1 and 5, for *IAA2*, *AXR2/IAA7*, *IAA11*, *AXR3/IAA17*, and *IAA18*, both steady state and auxin-induced levels were lower in *shy2-2* than in the wild type. For *IAA1*, *IAA5*, *IAA6*, *IAA13*, *MSG2/IAA19*, and *IAA20*, steady state levels were similar but auxin-induced levels were less in *shy2-2* seedlings than in the wild type. For all of these genes, expression levels in *shy2-24* generally paralleled those in the wild type. The data also revealed 10 *Aux/IAA* genes (*IAA4*, *IAA8*, *IAA9*, *IAA10*, *IAA12*, *SLR/IAA14*, *IAA16*, *IAA26*, *IAA27*, and *IAR2/IAA28*) whose expression was not affected by *shy2* mutations either at the steady state level or at the auxin-induced level (Figures 1 and 5 and data not shown). Although RNA gel blot hybridization showed that the auxin induction level of *IAA12* was reduced in *shy2-24* seedlings, quantitative data from the gene chip experiment indicated that the difference was less than twofold. Therefore, we considered the expression of *IAA12* in wild-type and *shy2-24* seedlings to be similar.

Among the *Aux/IAA* genes characterized previously, *AXR2/IAA7* and *IAA8* are suggested to be secondary response genes. Gene chip and RNA gel blot hybridization data showed that *shy2-2* seedlings had reduced levels of *AXR2/IAA7*. To provide additional insight into how *shy2* mutations affect *AXR2/IAA7* expression, we fused 2.3 kb of DNA upstream of *AXR2/IAA7* to the *GUS* gene and introduced this construct into wild-type plants. The same upstream fragment, when fused to *axr2-1* (which has a gain-of-function mutation in *AXR2/IAA7*) and transformed into wild-type plants, conferred *axr2-1*–like phenotypes, suggesting that it is a functional promoter (P. Nagpal and J.W. Reed, unpublished results). We generated seven independent lines, each with an insertion at a single locus, and analyzed them for histochemical staining of GUS activity. As shown in Figure 6, $P_{AXR2/IAA7}$::*GUS* expression was detected in shoot and root meristems in light-grown wild-type seedlings (Figures 6A and 6D) and only in shoot meristems, but not primary root meristems, in dark-grown seedlings (Figure 6G and data not shown). In addition, we observed X-gluc staining in the vasculature of hypocotyl/root junctions and occasionally in lateral root tips in dark-grown wild-type seedlings (Figure 6L and data not shown).

We crossed this construct into *shy2* mutant seedlings. Compared with the wild type, both light- and dark-grown *shy2-2* seedlings had ectopic $P_{AXR2/IAA7}$::*GUS* expression in cotyledons, leaf primordia, and vasculature of hypocotyls in addition to expression in shoot meristems (Figures 6B, 6H, and 6K). They did not have any $P_{AXR2/IAA7}$::*GUS* expression in root meristems or in other parts of the roots (Figure 6E and data not shown). These data suggest that although the total *AXR2/IAA7* expression level was reduced in *shy2-2* seedlings, *shy2-2* had contrasting effects in different tissues. *SHY2/IAA3* may regulate *AXR2/IAA7* expression positively in aerial parts of the seedlings but negatively in the roots. The X-gluc staining in light-grown *shy2-24* $P_{AXR2/IAA7}$::*GUS* mutant seedlings was similar to that of wild-type seedlings (Figures 6C and 6F). In dark-grown *shy2-24* seedlings, X-gluc staining also was detected in the shoot meristems (Figure 6I) and the vasculature of hypocotyl/root junctions, as in wild-type seedlings. However, compared with wild-type seedlings, dark-grown *shy2-24* seedlings had stronger X-gluc staining in lateral root tips, and they also had X-gluc staining in primary root tips (data not shown).

axr2-1 is a gain-of-function mutation in *AXR2/IAA7*, and *axr2-1* seedlings have similar shoot phenotypes to *shy2-2*

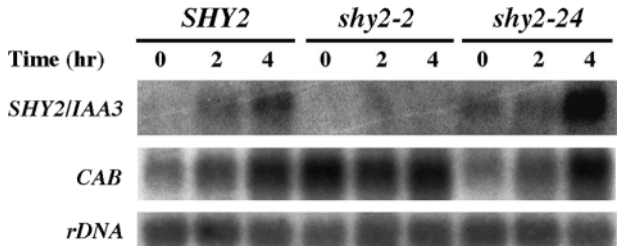

Figure 4. Induction of *SHY2/IAA3* Expression by Light in the Absence of Sucrose.

Six-day-old dark-grown seedlings were given a pulse of red light and returned to darkness for 0, 2, or 4 hr. RNA was isolated as described in Methods, and RNA gel blots were hybridized with *SHY2/IAA3*, *CAB*, and *rDNA* probes. Exposure times were 1 hr (*CAB* and *rDNA*) and 360 hr (*SHY2/IAA3*).

Table 1. Auxin-Induced Genes

Category	Coded Protein/Other Features[a]	GenBank Number	Affymetrix Number	Number of AuxREs[b]	Auxin Induction (fold)[c]		
					SHY2	*shy2-2*	*shy2-24*
Previously identified auxin-regulated genes							
	IAA1	**AAA16569**	**13289**	**7**	**~12.4**	**~4.6**	**~9.7**
	IAA2	**AAB97164**	**13297**	**3**	**3.8**	**3.5[d]**	**7.5**
	IAA5	**AAC49046**	**13660**	**3**	**~77.2**	**~4.4**	**~69.0**
	IAA6	**AAC49047**	**13661**	**4**	**~4.0**	**~1.2**	**~5.5**
	IAA13	**AAB80649**	**13293**	**2**	**4.3**	**3.2[d]**	**4.3**
	IAA19	**AAB84356**	**13296**	**7**	**17.7**	**5**	**13.6**
	IAA20	**AAB84357**	**13298**	**4**	**~4.5**	**~−1.2**	**~4.7**
	SAUR-1[e]	**CAB45438**	**20488**	**1**	**2.7**	**−1.9**	**3.1**
	SAUR-9	CAA18505	12947	ND[f]	3	1.8	2.5
	SAUR-10	**AAD20125**	**13781**	**3**	**5.5**	**−1.3**	**4.6**
	SAUR-13	**CAB38618**	**19695**	**3**	**2.6**	**−1**	**2.4**
	SAUR-15	**AAB30527**	**12608**	**2**	**~15.5**	**~1.1**	**~17.9**
	SAUR-25	**CAB36843**	**13395**	**3**	**~4.3**	**~1.6**	**~5.4**
	GH3-1[e]	**AAC61292**	**12553**	**2**	**14.4**	**6.9**	**25.7**
	GH3-2	CAB38206	13565	ND	17.9	12.1	16.8
	GH3-3	**AAB87114**	**16995**	**6**	**74.5**	**~137.4[d]**	**131.2**
	GH3-5	**CAA19720**	**16989**	**4**	**8.4**	**5.4**	**11.1**
	ACS4	**AAA85039**	**17107**	**3**	**3.3**	**−1.2**	**4.1**
	ACS6	**AAC63850**	**12891**	**5**	**3.2**	**2.5[d]**	**3.1**
	ACS8	**CAB38925**	**16387**	**6**	**~20.6**	**~4.0**	**~20.7**
Transcription related							
	HAT2	**AAA56901**	**18950**	**4**	**9.3**	**4.5**	**8.9**
	Zinc finger protein	**AAA87298**	**16581**	**7**	**~14.4**	**~4.5**	**~11.9**
	ARF19 (IAA22)[e]	AAB91321	13300	ND	2.9	2.1	2.8
	Putative heat shock transcription factor	AAB84350	18765	ND	3	~2.8	~5.3
	Contains similarity to GATA-type zinc fingers	AAB61058	17180	ND	~8.0	3.4[d]	~6.7
Signal transduction							
	Similar to protein kinases	**AAD30630**	**16878**	**5**	**5.6**	**2.7**	**5.5**
	Putative protein kinase	**AAC31848**	**15005**	**2**	**~7.4**	**~2.6**	**~5.6**
	Receptor protein kinase–like protein	**CAA18216**	**19418**	**1**	**2.8**	**1.9[d]**	**3.3**
	Putative protein kinase	AAC34357	19025	ND	4.2	1.9[d]	4.1
	Putative receptor-like protein kinase	AAC02766	14112	ND	3.6	2.5	4.4
	Putative protein kinase	AAC79621	12388	ND	2.6	~4.9	1.9
	Putative protein kinase	AAC14522	12357	ND	2.5	2.1	2.7
Transport							
	Similar to nitrate and oligopeptide transporters	**AAD39317**	**19835**	**3**	**3.6**	**1.4**	**3.8**
	AUX1-like amino acid permease	AAD29811	20612	ND	2.7	2	2.7
Cell wall establishment							
	Putative expansin	**AAB87577**	**19660**	**3**	**2.5**	**1.6**	**3**
	Similar to xyloglucan endotransglycosylase–related protein XTR4	**AAD39577**	**19490**	**4**	**2.6**	**−1.1**	**3.2**
	Contains similarity to expansins	AAC72858	19346	2	3.4	1.7[d]	5.4
	Similar to putative glucan synthase	AAF24822	18515	ND	3.6	~2.7	−1.2
	Putative glucosyl transferase	AAD20156	15496	ND	3.4	3.2	2.7

Continued

Table 1. (continued).

Category	Coded Protein/Other Features[a]	GenBank Number	Affymetrix Number	Number of AuxREs[b]	Auxin Induction (fold)[c]		
					SHY2	*shy2-2*	*shy2-24*
Metabolic enzymes							
	Putative cytochrome P450	**AAC34228**	**19045**	**6**	**8.8**	**2.7**	**2.3**
	Cytochrome P450–like protein	**CAB38204**	**14032**	**4**	**3.9**	**4.8[d]**	**3.7**
	Similar to mitochondrial protein and AAA-type ATPase	**CAB46004**	**15424**	**1**	**∼6.2**	**∼2.0[d]**	**∼6.9**
	Peroxidase ATP24a	**CAA72484**	**18946**	**3**	**2.9**	**1.5**	**4.2**
	Lupeol synthase	**AAD05032**	**15653**	**4**	**∼3.0**	**∼−1.5[d]**	**∼5.5**
	Similar to mandelonitrile lyase	**AAD39305**	**18869**	**8**	**2.8**	**∼2.1**	**3**
	Spermine synthase	**AAF01311**	**17999**	**6**	**5.2**	**2.1[d]**	**5.9**
	Glutamate decarboxylase	**AAA33709**	**18508**	**6**	**3.3**	**1.9[d]**	**2.7**
	Similar to glutaredoxin	**AAB07873**	**14766**	**3**	**5.9**	**2.9**	**∼6.4**
	Short-chain alcohol dehydrogenase–like protein	CAB41928	20685	ND	3.2	3.1	4.1
	12-oxophytodienoate reductase OPR1	AAC78440	18253	ND	2.6	3.4	2.6
	Putative glutathione *S*-transferase	AAC95196	18966	4	2.6	4.7	3.6
	Putative alanine acetyl transferase	AAD15402	14918	ND	∼4.8	∼3.2	∼3.3
	Putative laccase (diphenol oxidase)	AAD20177	17287	ND	∼4.5	∼2.5	2.3
	Monooxygenase	CAA07574	17877	5	3.5	4.1	3.5
	Putative peroxidase	AAD22357	20328	ND	∼4.6	∼2.8	∼5.0
	Similar to indole-3-acetate β-glucosyltransferase	AAD30627	20297	ND	3.3	2.5	3.3
	Putative pectinesterase	AAC62855	17338	ND	2.5	2.1	2.9
Others							
	Similar to TOLB (colicin tolerance) protein precursor	AAC80599	20519	ND	4.8	3.1	3.5
	GASA5	AAA98520	19565	ND	3.6	2.6	3.4
	Dehydrin	AAB00374	19186	ND	3.3	3.4	5.5
	ATAF2	CAA52772	18591	ND	2.5	2.1	3.1
	Similar to hookless1 (HLS1)	AAC17822	20122	2	∼13.7	∼10.0	∼10.7
	Translation factor EF-1 α–like protein	CAA16563	13439	ND	4	2.1	2.5
Unknown							
	Hypothetical protein	**CAB10516**	**13016**	**2**	**13.7**	**5.8**	**15.8**
	Hypothetical protein	**AAD15394**	**12095**	**3**	**7**	**2.9**	**3.5**
	Putative protein	**CAB39613**	**14951**	**3**	**3.9**	**2.2**	**4.1**
	Unknown protein	**AAD21443**	**18885**	**2**	**3.4**	**1.8**	**3.5**
	Hypothetical protein	**AAD23726**	**16180**	**6**	**∼5.7**	**∼2.5[d]**	**∼4.2**
	Unknown protein	**AAC27828**	**12090**	**4**	**∼21.6**	**3.7**	**14.5**
	Hypothetical protein	**AAD23725**	**15288**	**1**	**∼14.6**	**2.8**	**6.2**
	Predicted protein of unknown function	AAD22649	13656	3	4.7	4.1	4.5
	Unknown protein	AAB82622	16247	ND	4.3	3.2	4.1
	Unknown protein	AAC63640	20550	ND	3.2	1.9	3.3
	Hypothetical protein	AAC24177	17154	ND	∼3.8	2[d]	∼4.0

[a] Boldface indicates that *shy2-2* causes less auxin induction compared with the wild type. Roman indicates that the auxin induction is not affected by *shy2-2*.

[b] Number of TGTC_C and G_GACA sequences in the 2-kb fragments upstream of the start codon.

[c] Fold difference of expression levels after auxin treatment relative to without auxin treatment. ∼ indicates that one of the expression levels for comparison is below the detection limit; therefore, the auxin induction fold is approximate.

[d] *shy2-2* had both reduced basal and reduced auxin-induced expression levels.

[e] Gene numbers for ARF, SAUR, and GH3 genes are from Hagen and Guilfoyle (2001).

[f] ND, not determined.

Table 2. Auxin-Repressed Genes

Category	Coded Protein/Other Features[a]	GenBank Number	Affymetrix Number	Number of AuxREs[b]	Auxin Induction (fold)[c]		
					SHY2	*shy2-2*	*shy2-24*
Transcription related							
	Zinc finger protein	**AAA87299**	**17071**	**4**	**∼−3.9**	**1**	**∼−2.9**
	RING-H2 finger protein RHA4a	**AAC69852**	**20040**	**ND[d]**	**−4**	**−1.7**	**−3.5**
	TINY	**AAC29139**	**19722**	**5**	**−2.6**	**−1.5**	**−3**
	ATK1	**CAA57121**	**12928**	**2**	**∼−3.4**	**1.5**	**∼−2.9**
	Putative transcription factor	AAD53097	17610	ND	−2.7	−1.2	∼−2.9
	Putative transcription factor	AAC83598	18790	ND	−3.3	−2	∼−3.0
	Putative WRKY DNA-binding protein	AAD17441	14679	ND	∼−3.9	∼−5.1	∼7.1
	R2R3-MYB transcription factor	CAB09190	18747	ND	∼−2.8	∼−2.3	∼−2.6
Signal transduction							
	Putative histidine kinase	AAD03576	13362	ND	−2.6	−1.7	−2.5
	Receptor protein kinase–like protein	AAC14033	16356	ND	−4.3	−4.2	∼−4.2
Transport							
	Nodulin-26–like protein	**CAA16760**	**19847**	**4**	**−10.7**	**−2.4**	**∼−9.9**
	Similar to Arabidopsis Fe(II) transport protein	AAB71447	19718	ND	∼−3.6	∼−3.7	−3
Metabolic enzymes							
	Putative cytochrome P450 protein	**AAD10659**	**14366**	**2**	**−5.8**	**−3.4**	**−6.5**
	Peroxidase	**CAA66963**	**16971**	**4**	**∼−18.0**	**−1.7**	**∼−13.5**
	Putative peroxidase	**AAC79614**	**18150**	**3**	**−3.1**	**−1.1**	**−2.9**
	Putative endochitinase	**AAB64046**	**12542**	**4**	**∼−4.1**	**−1.1**	**∼−3.0**
	UDP-glucose 4-epimerase–like protein	**CAB45812**	**17748**	**4**	**∼−4.1**	**1.6**	**−3.5**
	CER1-like protein	**AAC23640**	**19237**	**3**	**−3.1**	**−1.5**	**−3.6**
	Putative cytochrome P450	AAD22364	19549	ND	−3.8	−3.6	−2.2
	Putative pectinesterase	AAC17097	12352	ND	−3.6	−1.8	−3
Others							
	HSR201–like protein	**CAB10318**	**16045**	**7**	**−4.1**	**−1.5**	**−5.5**
	Putative RNA binding protein	**AAC23648**	**18817**	**4**	**∼−3.5**	**−1.6**	**∼2.9**
	pEARLI 1–like protein	CAB41722	18983	ND	−4.3	−3.3	−4.5
	Similar to NPH-3 (nonphototropic hypocotyl-3)	CAA16538	16213	ND	∼−3.8	∼−3.5	−1.6
Unknown							
	Putative protein	**CAA22575**	**16510**	**5**	**−4.7**	**1**	**−3.7**
	Chromosome I bacterial artificial chromosome F3F20 genomic sequence	AAD30618	16832	ND	−2.7	−2.8	−2

[a] Boldface indicates that *shy2-2* causes less auxin repression compared with the wild type. Roman indicates that the auxin repression is not affected by *shy2-2*.

[b] Number of TGTC_C and G_GACA sequences in the 2-kb fragments upstream of the start codon.

[c] Fold difference of expression levels after auxin treatment relative to without auxin treatment. ∼ indicates that one of the expression levels for comparison is below the detection limit; therefore, the auxin repression fold is approximate.

[d] ND, not determined.

seedlings, such as short hypocotyls and upcurled leaves (Wilson et al., 1990; Timpte et al., 1992, 1994; Nagpal et al., 2000). They also have agravitropic shoot and root growth. However, *axr2-1* seedlings have more lateral roots, whereas *shy2-2* seedlings have fewer lateral roots than does the wild type. We introduced $P_{SHY2/IAA3}$::*GUS* and $P_{AXR2/IAA7}$::*GUS* into *axr2-1* mutants to examine how *axr2-1* affected the expression of these two genes. Consistent with previous mRNA data (Abel et al., 1995b; Nagpal et al., 2000), *axr2-1* $P_{AXR2/IAA7}$::*GUS* seedlings had almost no staining when grown either in light or in darkness, indicating that *AXR2*/*IAA7* also negatively regulates itself (data not shown). Similarly, *axr2-1* $P_{SHY2/IAA3}$::*GUS* seedlings had almost no staining when grown either in light or in darkness, suggesting that *AXR2*/*IAA7* has a negative effect on *SHY2*/*IAA3* expression (Figure 2A). Because *AXR2*/*IAA7* is considered a secondary response gene, these results indicate that products of both primary (*SHY2*/*IAA3*) and secondary (*AXR2*/*IAA7*) auxin-responsive genes can feed back to repress *SHY2*/*IAA3* expression.

To determine how *shy2* mutations affect *IAA2* expression, we transformed the previously described P_{IAA2}::*GUS* construct (Luschnig et al., 1998) into wild-type plants and

crossed a transgenic line with an insertion at a single locus into *shy2* mutants. Light-grown wild-type P_{IAA2}::*GUS* seedlings had prominent X-gluc staining in petioles and root tips. They also had staining in cotyledons, leaves, and mature tissues of roots. Compared with wild type, *shy2-2* P_{IAA2}::*GUS* seedlings had much less staining in petioles, consistent with the decreased *IAA2* expression in *shy2-2* seedlings in the chip experiment, but they had similar staining in other tissues. *shy2-24* P_{IAA2}::*GUS* seedlings had similar X-gluc staining to wild type (data not shown).

SAUR Genes

There are 16 *SAUR* genes on the chip, and 11 of them had detectable signals. Among these, auxin induced 10 *SAUR* genes and repressed one, *SAUR6*. As shown in Figure 7A, auxin inductions of *SAUR1*, *SAUR10*, *SAUR13*, *SAUR15*, *SAUR16*, and *SAUR25* were abolished almost completely in *shy2-2* seedlings. Expression of *SAUR9*, *SAUR12*, *SAUR32*, and *SAUR36* were not changed by *shy2* mutations (data not shown). Compared with the wild type, *shy2-2* seedlings had similar repression of the *SAUR6* gene by auxin. However, both the baseline and the auxin-repressed levels of *SAUR6* were higher in *shy2-2* seedlings than in wild-type seedlings.

We examined the effects of *shy2* mutations on *SAUR15* (previously named *SAUR-AC1*) spatial expression using the $P_{SAUR\text{-}AC1}$::*GUS* fusion (Gil and Green, 1997). Figure 8A shows X-gluc staining of 9-day-old wild-type, *shy2-2*, and *shy2-23* (which has the same mutation as *shy2-24*) seedlings. In the wild type, $P_{SAUR\text{-}AC1}$::*GUS* was expressed in the elongating region of the hypocotyls of light- and dark-grown seedlings and in the cotyledons and primary leaves of light-grown seedlings. Occasionally, we observed moderate expression in the roots. Compared with the wild type, *shy2-2* seedlings had much less GUS activity, and X-gluc staining was most abundant in cotyledons and shoot meristems rather than in the hypocotyls, in both light- and dark-grown seedlings. The difference of $P_{SAUR\text{-}AC1}$::*GUS* expression between *shy2-2* and wild-type seedlings was more obvious in dark-grown seedlings. Staining in *shy2-23* seedlings was similar to that in the wild type. Quantitative GUS activity assays supported these results (Figure 8B).

GH3 Genes

The *shy2-2* mutation had less profound effects on *GH3* genes. There are seven *GH3* genes on the chip, and five of them had detectable signals. As shown in Figure 7B, among these *GH3* genes, expression of *GH3-2* and *GH3-11* was almost normal in *shy2-2*. The auxin induction, but not the baseline levels, of *GH3-3* and *GH3-5* was reduced by 20 to 50% in *shy2-2* mutants, and the auxin induction of *GH3-1* was reduced only slightly in *shy2-2*.

ACS Genes

There are three *ACS* genes on the chip (*ACS4*, *ACS6*, and *ACS8*). As shown in Figure 7C, *shy2-2* reduced either auxin induction or baseline expression levels of all of these genes.

Other Auxin-Induced Genes

In addition to the 25 previously identified auxin-responsive genes described above, there are 38 more auxin-regulated genes that were affected by the *shy2-2* mutation. Twenty-four of these genes were auxin-inducible genes, and *shy2-2* seedlings had reduced baseline expression levels or induction levels (Table 1), suggesting that, as for the *Aux/IAA*, *SAUR*, *GH3*, and *ACS* genes, *SHY2/IAA3* also is a repressor of transcription of these genes. Fourteen of these 38 genes were auxin-repressed genes (Table 2). However, the repression of these genes by auxin was reduced in *shy2-2* seedlings, and some of them were induced by auxin in *shy2-2*, indicating that *shy2-2* might inhibit transcription factors that normally repress their transcription.

Auxin-Nonresponsive Genes

We searched the chip data for all of the auxin-nonresponsive genes expressed differently between *shy2* mutants and the wild type either with or without auxin treatment. As listed in Table 3, there were 81 such genes in several categories affected by the *shy2-2* mutation. These genes may be responsible for establishing cellular consequences visible as *shy2-2* mutant phenotypes. We discuss the possible physiological roles of some of these genes below.

DISCUSSION

SHY2/IAA3 Affects Gene Expression

shy2-2 seedlings have short hypocotyls, slightly auxin-resistant hypocotyl and root growth, reduced gravitropic root response, and decreased lateral root number (Soh et al., 1999; Tian and Reed, 1999). We have interpreted these phenotypes as indicating that *shy2-2* seedlings have reduced auxin responses. The experiments described in this article confirm at a molecular level that *shy2-2* seedlings have decreased auxin responses. Of the 74 genes that were induced >2.5-fold by auxin in wild-type seedlings, 42 genes were not induced or were induced much less in *shy2-2* seedlings. Similarly, of the 26 genes that were repressed >2.5-fold by auxin in wild-type seedlings, 14 genes were less repressed in *shy2-2* seedlings. These results indicate that SHY2/IAA3 can decrease both induction and repression of a substantial fraction of auxin-regulated genes.

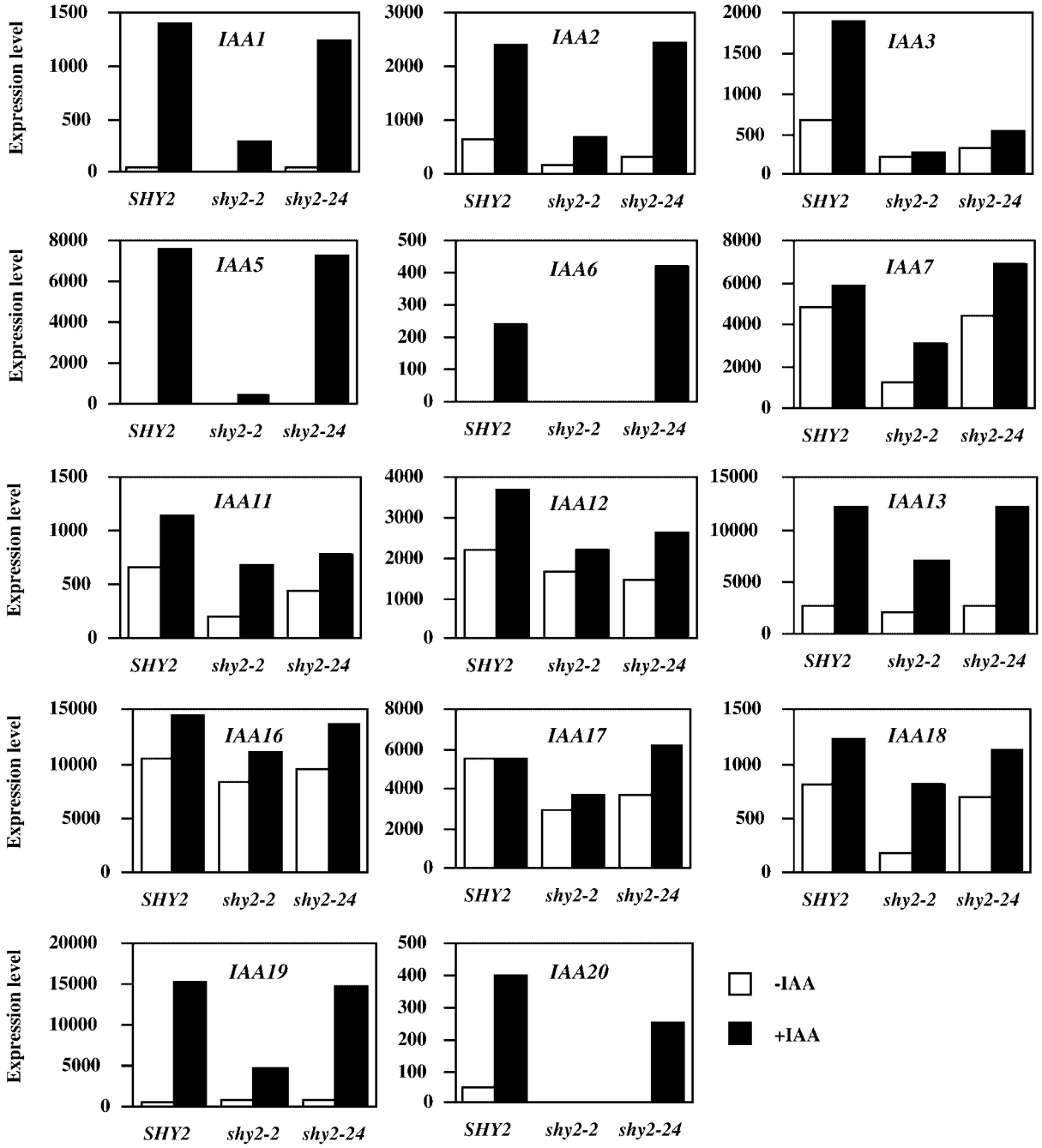

Figure 5. Analysis of *Aux/IAA* Gene Expression in *SHY2* (Wild-Type), *shy2-2*, and *shy2-24* Seedlings.

Six-day-old light-grown seedlings were either mock-treated (open bars) or treated with 20 μM IAA (closed bars) for 2 hr. Total RNA was isolated, and the gene chip experiment was performed as described in Methods. The expression levels (*y* axis) were computed using Affymetrix software and represent differential hybridizations between perfectly matched and mismatched oligodeoxyribonucleotides.

Genetic studies have shown that *shy2-2* causes a gain of function, and *shy2-2* seedlings have higher SHY2/IAA3 protein levels than do wild-type seedlings (Tian and Reed, 1999; Colón-Carmona et al., 2000). Therefore, increased SHY2/IAA3 protein activity is responsible for *shy2-2* phenotypes and for the changes in gene expression profiles. The protein synthesis inhibitor cycloheximide can superinduce several primary auxin response genes (Franco et al., 1990; Abel et al., 1995b), implying that a short-lived protein normally represses expression of auxin-induced genes. That stabilization of SHY2/IAA3 by the *shy2-2* mutation decreases auxin regulation of gene expression suggests that SHY2/IAA3 may be such a repressor. Although there is no evidence that the SHY2/IAA3 protein can bind to DNA, it might dimerize with ARF proteins that have either activator or repressor middle domains, thereby impeding either activation or repression of gene expression by ARFs. Recent evidence indicates that auxin can regulate stability of other Aux/IAA proteins, suggesting that regulated turnover of SHY2/IAA3 may mediate auxin gene expression responses (Gray et al., 2001; Zenser et al., 2001).

Auxin rapidly induces expression of *SHY2/IAA3*, and the *shy2-2* mutation almost completely abolished both the basal expression level and the auxin induction of *SHY2/IAA3*. Auxin induction of *SHY2/IAA3*, therefore, potentiates a negative feedback on expression of *SHY2/IAA3* and other auxin-induced genes. Such negative feedback might allow precise quantitative control of auxin responses.

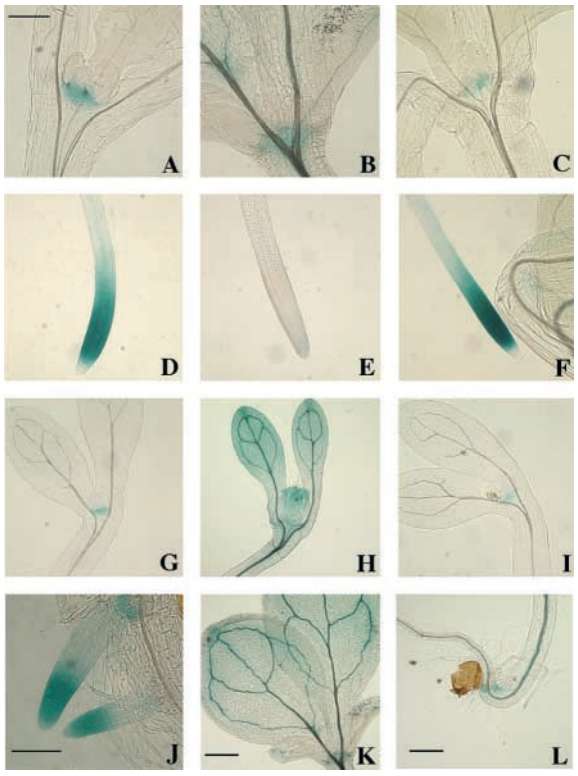

Figure 6. Histochemical Analysis of $P_{AXR2/IAA7}$::*GUS* Expression.

X-gluc staining of 9-day-old light-grown (**[A]** to **[F]**, **[J]**, and **[K]**) and dark-grown (**[G]** to **[I]** and **[L]**) wild-type (**[A]**, **[D]**, **[G]**, **[J]**, and **[L]**), *shy2-2* (**[B]**, **[E]**, **[H]**, and **[K]**), and *shy2-24* (**[C]**, **[F]**, and **[I]**) $P_{AXR2/IAA7}$::*GUS* seedlings.
(A) to **(C)** and **(G)** to **(I)** Shoot meristems.
(D) to **(F)** Primary root tips.
(J) Lateral root tips.
(K) Cotyledons and leaves.
(L) Hypocotyl/root junction.
Bar in **(A)** = 0.25 mm for **(A)** to **(I)**; bar in **(J)** = 0.15 mm; and bars in **(K)** and **(L)** = 0.5 mm.

Genes Regulated by shy2-2

Most *Aux/IAA*, *SAUR*, *GH3*, and *ACS* genes affected by *shy2-2* are primary auxin response genes, indicating that *shy2-2* inhibits primary auxin responses. *shy2-2* also affected expression of one previously characterized secondary response gene, *AXR2/IAA7*. *shy2-2* might regulate *AXR2/IAA7* and other secondary or late response genes directly or indirectly through primary response genes. *shy2-2* does not affect expression of some other known auxin-regulated genes, indicating that SHY2/IAA3 has some specificity in its regulatory targets. This specificity might arise from functional specialization of the protein or from tissue-specific *SHY2/IAA3* expression.

Some of the semidominant mutants in other *Aux/IAA* genes also have altered expression of primary auxin response genes. For example, *axr2-1* caused reduced expression and auxin induction of a number of *Aux/IAA* genes, especially *SHY2/IAA3*, *IAA5*, and itself, and it also caused decreased induction of *SAUR-AC1* expression (Timpte et al., 1994; Abel et al., 1995b; Nagpal et al., 2000). *iaa28-1* caused reduced expression and auxin induction of the synthetic *BA*::*GUS* auxin-responsive promoter::reporter fusion (Rogg et al., 2001). In contrast, *axr3-1* caused ectopic *SAUR-AC1* expression in the root vasculature (Leyser et al., 1996). These results suggest that to mediate auxin responses precisely, these *Aux/IAA* genes might form a tightly regulated network in which some Aux/IAA proteins (such as IAA28, AXR2/IAA7, and SHY2/IAA3) act as repressors and some Aux/IAA proteins (such as AXR3/IAA17) act as activators. They may have overlapping and/or distinct downstream targets, again depending on the specificity of the proteins and their spatial expression patterns.

Other than the *Aux/IAA*, *SAUR*, *GH3*, and *ACS* genes, *shy2-2* affects expression of a number of auxin-responsive and auxin-nonresponsive genes. Some of the auxin-responsive genes have multiple AuxREs in their promoters (Table 1) and may be primary or secondary auxin response genes. *shy2-2* may regulate expression of the auxin-nonresponsive genes indirectly.

The genes that are misregulated in *shy2-2* presumably

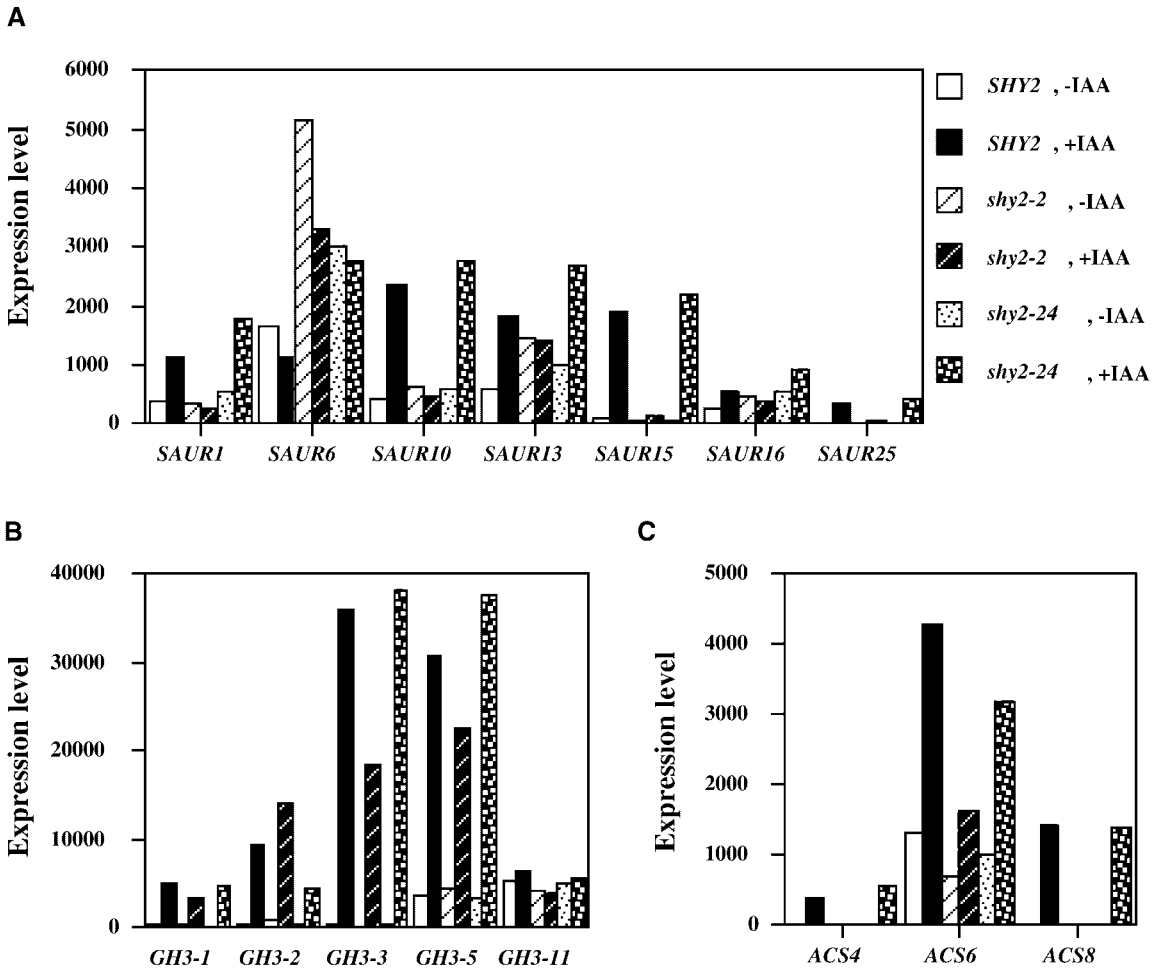

Figure 7. Analysis of *SAUR* **(A)**, *GH3* **(B)**, and *ACS* **(C)** Gene Expression in *SHY2* (Wild-Type), *shy2-2*, and *shy2-24* Seedlings.

Six-day-old light-grown seedlings were either mock treated (−IAA) or treated with 20 μM IAA (+IAA) for 2 hr. Total RNA was isolated, and the gene chip experiment was performed as described in Methods. The expression levels (*y* axis) were computed using Affymetrix software and represent differential hybridizations between perfectly matched and mismatched oligodeoxyribonuleotides.

cause the mutant phenotypes; therefore, they may provide clues about cellular or signaling functions that are altered in the mutant. For example, several genes encoding protein kinases were less induced in *shy2-2* than in wild-type seedlings, and these might be components of signal transduction pathways that regulate *SHY2*/*IAA3*-mediated inhibition of auxin-regulated gene expression. Several metabolic enzymes that are misregulated, such as cytochrome P450s and a putative tyrosine aminotransferase homologous to the ROOTY protein, might affect biosynthesis or breakdown of auxin or other signaling molecules (Barlier et al., 2000; Hull et al., 2000; Bak et al., 2001). Genes encoding enzymes that might synthesize or modify the cell wall may affect cell enlargement, leading to the short hypocotyls and upcurled leaves of *shy2-2* mutants. Further molecular and genetic studies will help reveal how much each of these genes contributes to *shy2* mutant phenotypes.

Novel Auxin-Regulated Genes

A number of other genes were induced or repressed by auxin but unaffected by the *shy2-2* mutation, and these may reveal aspects of auxin response that are independent of SHY2/IAA3 action. Expression of an AUX1-like permease is induced by auxin, possibly to allow auxin influx into cells to activate auxin responses. Expression of a gene encoding an IAA β-glucosyltransferase, which is involved in IAA conjugation (Normanly and Bartel, 1999), is induced by auxin, indicating that negative feedback controls on auxin responses

might act to increase auxin conjugation and affect auxin signaling. *HOOKLESS1* encodes a putative acetyltransferase that affects differential cell growth during apical hook formation (Lehman et al., 1996), and expression of an *HLS1* homolog is also induced significantly (>10-fold) by auxin, consistent with the idea that these acetyltransferases function in auxin responses.

The genes listed in Tables 1 and 2 are those that are either induced or repressed by >2.5-fold after 2 hr of auxin treatment. Previous studies showed that some primary response genes had transient responses to auxin and some were induced <2.5-fold (Abel et al., 1995b). Moreover, we assayed only approximately one-fourth of the genes in Arabidopsis. Therefore, our results include only a subset of auxin-responsive genes in the genome. Other experiments exploring the kinetics of global gene regulation response to auxin might reveal additional aspects of auxin responses.

SHY2/IAA3 Acts in Hypocotyls and Cotyledons

The $P_{SHY2/IAA3}$::*GUS* fusion was expressed in the hypocotyls of dark-grown and young light-grown wild-type seedlings, in cotyledons of light-grown seedlings, and in leaves of older seedlings. *shy2-2* mutants have short hypocotyls, enlarged cotyledons, and upcurled leaves, and $P_{SHY2/IAA3}$::*GUS* expression in these organs suggests that *SHY2/IAA3* regulates hypocotyl, cotyledon, and leaf growth cell autonomously. Consistent with this idea, several auxin-regulated promoter::GUS fusions were misexpressed in hypocotyls, cotyledons, and leaves of *shy2-2* seedlings. For example, compared with wild-type seedlings, light-grown *shy2-2* seedlings had less expression of $P_{SAUR\text{-}AC1}$::*GUS* and $P_{SHY2/IAA3}$::*GUS* in hypocotyls and less expression of P_{IAA2}::*GUS* in the petioles of the cotyledons. They also had more expression of $P_{AXR2/IAA7}$::*GUS* in the cotyledons. Dark-grown *shy2-2* seedlings also had almost no $P_{SHY2/IAA3}$::*GUS* expression in hypocotyls but more expression in the cotyledons than wild-type seedlings. Similarly, dark-grown *shy2-2* seedlings had more expression of $P_{AXR2/IAA7}$::*GUS* and $P_{SAUR\text{-}AC1}$::*GUS* in cotyledons than wild-type seedlings. Because *shy2-2* mutants have enlarged cotyledons in the dark, these effects on gene expression in cotyledons might be an indirect effect of cotyledon growth in *shy2-2* seedlings.

Both *shy2-2* and *shy2-24* mutants have altered root gravitropism and lateral root numbers. However, we observed no X-gluc staining in roots of plants carrying the $P_{SHY2/IAA3}$::*GUS* transgene. A previous report showed that *SHY2/IAA3* was expressed at a very low level in roots (Abel et al., 1995b), and our reverse transcriptase–polymerase chain reaction from root RNA also showed expression in the root (data not shown). These results suggest that this transgene does not reflect normal expression precisely, that the expression in the roots was too low to be detected by X-gluc staining, or that the mRNA experiments suffered from nonspecific cross-hybridization. If it is true that *SHY2/IAA3* is not expressed in the roots, then it is possible that it regulates root growth through some indirect mechanism, for example, by affecting polar auxin transport from shoot to root. We did find that *shy2-2* caused reduced $P_{AXR2/IAA7}$::*GUS* expression in roots. *shy2-3* also caused ectopic $P_{AXR3/IAA17}$::*GUS* expression in root tips (O. Leyser, personal communication). All of these results suggest a role of *SHY2/IAA3* in roots.

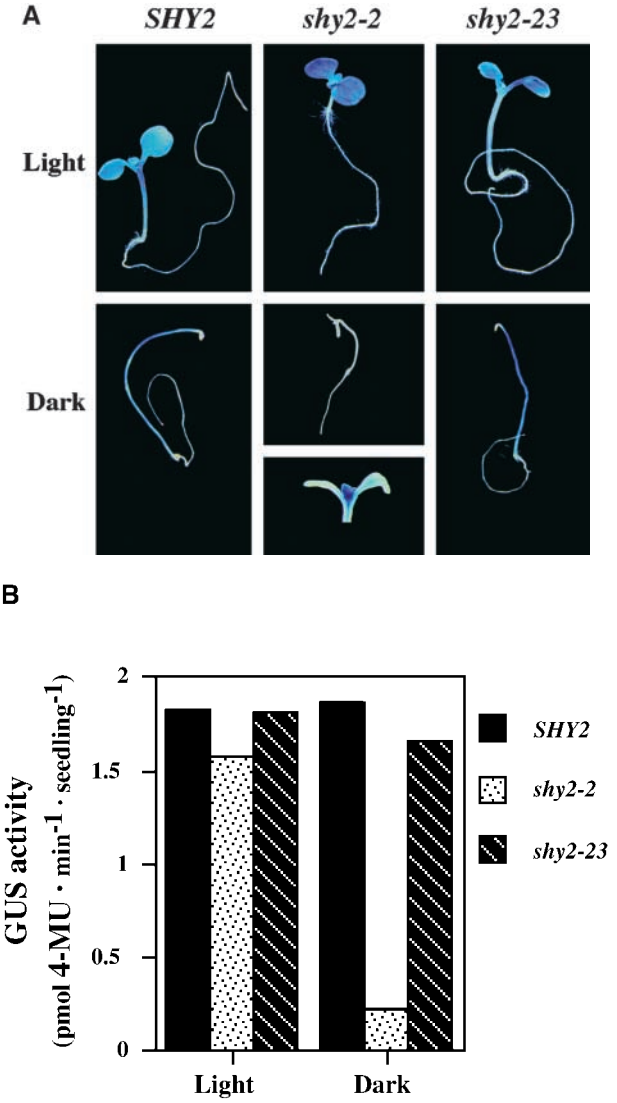

Figure 8. Histochemical Analysis of $P_{SAUR\text{-}AC1}$::*GUS* Expression.

(A) X-gluc staining of 9-day-old light- and dark-grown *SHY2* (wild-type), *shy2-2*, and *shy2-23* $P_{SAUR\text{-}AC1}$::*GUS* seedlings.

(B) 4-Methylumbelliferyl β-D-glucuronide (4-MU) assay of 9-day-old dark-grown *SHY2* (wild-type), *shy2-2*, and *shy2-23* $P_{SAUR\text{-}AC1}$::*GUS* seedlings.

Table 3. Auxin Nonresponsive Genes Affected by *shy2-2*

Category	Coded Protein/Other Features[a]	GenBank Number	Affymetrix Number	*shy2-2/SHY2*, −IAA[b] (fold difference)	*shy2-2/SHY2*, +IAA[c] (fold difference)
Transcription related					
	Ethylene-responsive element binding factor 1	**BAA32418**	**16063**	**∼−23.5**	**−7.1**
	NAM (no apical meristem)–like protein	**AAB81668**	**13806**	**∼−5.1**	**−1.3**
	Similar to gb|X92204 NAM gene product from *Petunia hybrida*	**AAD39614**	**12643**	**∼−3.1**	**−2.9**
	Putative c2h2 zinc finger transcription factor	**AAB80922**	**15665**	**∼−4.9**	**−1.6**
	RING-H2 finger protein RHA1b	**AAC68670**	**16553**	**−3.3**	**−2.5**
Signal transduction					
	Receptor-like serine/threonine protein kinase ARK3	**CAA20203**	**16360**	**∼−6.2**	**−3.8**
	Putative receptor-like protein kinase	**AAD32284**	**12497**	**−3**	**−2.7**
	Serine/threonine kinase–like protein	**CAA18465**	**13550**	**−3.5**	**−3.2**
	Wall-associated kinase 1	**CAA08794**	**15616**	**−3.4**	**−2.6**
	Putative serine/threonine protein kinase	**CAB38914**	**18459**	**−3.4**	**−5.5**
	Calmodulin-like protein	**CAB42906**	**13217**	**−9.5**	**−6.8**
	Putative calmodulin binding protein	**CAA18193**	**19857**	**−6.8**	**−2.9**
Light related					
	Chlorophyll *a/b* binding protein	CAB41095	16004	3	2.8
	Lhcb2 protein	AAD28772	15153	5.3	4.6
	Putative photosystem I reaction center subunit II	AAD15351	18086	2.7	3
	PII protein	AAC78333	17505	3.7	4.7
Transport					
	Putative ligand-gated ion channel protein	**AAC33239**	**18844**	**∼−7.9**	**−3.3**
	Contains similarity to sugar transporters	**AAC26243**	**14116**	**−3.2**	**−2.9**
	Contains similarity to *Medicago sativa* corC (magnesium and cobalt efflux protein)	AAC19312	20468	4.8	4.1
	Sulfate transporter protein	AAC78252	17042	4.3	4.5
Cell wall establishment					
	Similar to the family of glycosyl hydrolases	**AAC13634**	**12336[d]**	**−7.4**	**−1**
	Xyloglucan endotransglycosylase related protein	**AAC05572**	**16620**	**−2.8**	**−2.6**
	Putative polygalacturonase	**CAA23048**	**13628**	**−3.2**	**−2.8**
	Putative β-glucosidase	**CAB43970**	**18917**	**∼−7.6**	**−3.8**
	Putative xyloglucan endo-1,4-β-D-glucanase	**CAB39603**	**16630**	**−4.4**	**−3.7**
	Endo-xyloglucan transferase	**AAC39467**	**16927**	**−3.5**	**−2.4**
	β-1,3-Glucanase 2	**CAB68132**	**13212**	**−9.7**	**−8.1**
	β-1,3-Glucanase	**AAA32756**	**12364**	**−17.9**	**−12.1**
	Xyloglucan endotransglycosylase–like protein	**CAA22967**	**19294**	**−3.6**	**−5.9**
	Contains similarity to *Nicotiana alata* pistil extensin-like protein	**AAC28181**	**15049**	**−3.8**	**−3.9**
	Strong similarity to extensin-like protein gb|Z34465 from *Zea mays*	**AAC17609**	**19826**	**∼−10.8**	**∼−7.5**
	Contains similarity to *Zea mays* embryogenesis transmembrane protein	**AAC33958**	**17185**	**∼−13.4**	**−14.1**
	Xyloglucan endotransglycosylase–related protein	AAB18367	17533	6.3	16
	Thioglucosidase	CAA55787	12740	∼6.0	∼5.5
Metabolic enzymes					
	Cytochrome P450–like protein	**CAB10312**	**18951**	**∼−8.5**	**∼−6.5**
	Putative cytochrome P450	**AAC26690**	**12989**	**−8.5**	**−3.5**
	Peroxidase ATP19a	**CAB51413**	**13610**	**−2.7**	**−2.7**
	Peroxidase	**CAA67092**	**12400**	**−2.8**	**−2.7**
	Strong similarity to Arabidopsis peroxidase ATP11A	**AAB71454**	**17932**	**−4.6**	**−6.8**
	Peroxidase	**CAA66967**	**20296**	**−5.2**	**−4.4**
	Putative peroxidase	**CAB39666**	**15562**	**∼−14.3**	**−7.2**
	Putative oxidoreductase	**AAC79100**	**14338**	**∼−38.4**	**−8.5**
	Caffeoyl-CoA *O*-methyltransferase–like protein	**CAB38951**	**19348[d]**	**−4**	**−1.2**
	Hydroxymethylglutaryl CoA reductase	**AAA32814**	**12920[d]**	**−1.2**	**∼−208.3**

Continued

Table 3. (continued).

Category	Coded Protein/Other Features[a]	GenBank Number	Affymetrix Number	*shy2-2/SHY2*, −IAA[b] (fold difference)	*shy2-2/SHY2*, +IAA[c] (fold difference)
	Putative tropinone reductase	**AAC95203**	**20491**	**−3.1**	**−6.1**
	Putative anthocyanin 5-aromatic acyltransferase	**AAB95283**	**18876**	**−4.3**	**−4.8**
	Putative tyrosine aminotransferase	**AAD23027**	**17008**	**−7.7**	**−5.7**
	Chitinase-like protein	**CAA19692**	**12514**	**∼−10.9**	**∼−10.0**
	Putative trehalose-6-phosphate synthase	**AAD08939**	**13706**	**∼−4.4**	**−2.3**
	Putative glutathione *S*-transferase	**AAC32912**	**12764**	**−2.5**	**−2.4**
	Squalene epoxidase homolog	**CAA06769**	**19704**	**∼−4.8**	**∼−5.5**
	Similar to PHZF, catalyzing the hydroxylation of phenazine-1-carboxylic acid to 2-hydroxy-phenazine-1-carboxylic acid	AAD15344	14497	6	∼5.1
	Cytochrome P450 monooxygenase-like protein	CAB38208	14117	3.7	4.9
	Pyrophosphate-dependent phosphofructo-1-kinase	CAB38956	19741[d]	−1.6	10.9
	Drought-inducible cysteine proteinase RD21A precursor-like protein	CAB51416	12746	6.5	8
	Carbonic anhydrase	AAA50156	15144	3.3	3.1
Disease responses					
	Putative disease resistance protein	**AAC12833**	**12251**	**∼−5.2**	**−2.7**
	Pathogenesis-related protein 1	**CAA65420**	**12933[d]**	**−3.3**	**−1.3**
	Downy mildew resistance protein RPP5	**AAF08790**	**20208**	**∼−5.0**	**∼−5.5**
	Putative disease resistance protein	**CAA18120**	**19443**	**∼−3.8**	**−5.1**
	Putative disease resistance response protein	CAB43056	14013	∼11.3	∼8.6
	Resistence protein-like	CAA16927	17294	∼5.3	∼5.0
	Contains similarity to pathogenesis-related protein 1 precursors and SCP-like extracellular proteins	AAD17355	20395	∼4.9	∼4.4
Others					
	Contains similarity to WB domains, G-β repeats	**AAC35546**	**19589[d]**	**−1.1**	**∼−30.4**
	Putative surface protein	**AAD25652**	**14076[d]**	**−1**	**−3.8**
	Thioredoxin *h*	**AAC49356**	**13187**	**−3**	**−3**
	Thioredoxin *h*	**AAC49356**	**13189**	**−4.6**	**−4.1**
	Peroxiredoxin TPx2	**AAD28243**	**15116**	**−3.8**	**−3.9**
	Pre-hevein–like protein	**AAA20642**	**15162**	**−3.4**	**−2.6**
	ORF1	**CAA50905**	**16439**	**−3.3**	**−3.7**
	Putative protein	**CAA19766**	**13937**	**∼−6.4**	**∼−7.0**
	Unknown protein	**AAD15461**	**15846**	**−7.7**	**−7.1**
	Unknown protein	**AAD15461**	**14704**	**−26.3**	**−13.4**
	Selenium binding protein–like	CAB46000	18215	4.1	5.3
	Ubiquitin fusion degradation protein 1 homolog	CAB10321	19879	∼5.6	2.9
	T15B16.1 gene product	AAC72861	13935	13.2	20
	Hypothetical protein	AAC78273	18761	∼16.7	∼24.0
	Putative protein	CAB45069	15531	∼13.4	∼23.5
	Putative protein	CAB43702	12660	5.9	8
	Putative protein	CAB41004	18648	∼4.3	∼6.8
	Putative protein	CAA19878	12169	4.6	∼23.7

[a] Boldface indicates that gene expression is decreased in *shy2-2*. Roman indicates that gene expression is increased in *shy2-2* compared with the wild type.

[b] Fold difference of expression levels in *shy2-2* relative to the wild type without auxin treatment. ∼indicates that one of the expression levels for comparison is below the detection limit; therefore, the fold difference is approximate.

[c] Fold difference of expression levels in *shy2-2* relative to the wild type after auxin treatment. ∼indicates that one of the expression levels for comparison is below the detection limit; therefore, the fold difference is approximate.

[d] *shy2-2* causes reduced basal expression level or auxin-induced level but not both.

In contrast to the dramatic phenotypes of *shy2-2* gain-of-function seedlings, *shy2-24* mutants do not have strong hypocotyl, cotyledon, or leaf phenotypes, most likely because of genetic redundancy. *IAA4* is phylogenetically related most closely to *SHY2/IAA3*, and previous work showed that *IAA4* is expressed throughout the seedlings, including the cotyledons, hypocotyls, and roots (Abel et al., 1995b). Therefore, IAA4, or other Aux/IAA proteins with similar expression patterns, could act redundantly with SHY2/IAA3 to regulate hypocotyl or cotyledon growth.

Role of *SHY2/IAA3* in Light Responses

The *shy2-2* mutation can bypass the function of phyB, and *shy2-2* seedlings have short hypocotyls and expanded cotyledons and make leaves in the dark. Therefore, *shy2-2* might cause activation of light responses and photomorphogenesis. Consistent with this idea, in both dark and light, several light-regulated genes, such as *CAB*, and some photosynthesis factors are upregulated in *shy2-2* (Kim et al., 1998) (Table 3). Recently, auxin was shown to downregulate expression of some light-induced genes (Gil et al., 2001). Therefore, the overexpression of light-induced genes in *shy2-2* might arise from the reduced auxin responses.

Our data that light regulated *SHY2/IAA3* expression suggest further links between light and SHY2/IAA3 activity. Overall, $P_{SHY2/IAA3}$::*GUS* expression was lower in light than in darkness, and RNA gel blot hybridizations also showed that light regulates *SHY2/IAA3* expression in dark-grown wild-type seedlings. In the presence of sucrose, a pulse of red light or a shift to continuous white light inhibited *SHY2/IAA3* expression, whereas in the absence of sucrose, a red light pulse induced *SHY2/IAA3* expression. These results suggest that both light and sucrose signaling pathways modulate *SHY2/IAA3* gene expression and/or protein activity.

Considering that SHY2/IAA3 can inhibit expression of its own gene, our data could be explained by light regulation of SHY2/IAA3 protein activity. In the dark in the absence of sucrose, there may be little SHY2/IAA3 protein present, and light can induce the gene by an unknown mechanism. Sucrose increases *SHY2/IAA3* expression in the dark, similarly to its effect on other light-regulated genes such as *CAB* (Brusslan and Tobin, 1992), so there may be more SHY2/IAA3 protein present. In this situation, light might activate the autoinhibitory activity of SHY2/IAA3, and this apparently overcomes the light activation of gene expression seen in the absence of sucrose. Light did not activate *SHY2/IAA3* expression in the *shy2-2* mutant, suggesting that light normally regulates SHY2/IAA3 protein activity. Consistent with the possibility that photoreceptors regulate SHY2/IAA3 protein activity, oat phyA can phosphorylate SHY2/IAA3 in vitro (Colón-Carmona et al., 2000) and SHY2/IAA3 also interacts with Arabidopsis phyB (Q. Tian, L. Krall, and J.W. Reed, unpublished results).

METHODS

Growth Conditions and Tissue Treatment

Arabidopsis thaliana seedlings were surface-sterilized, plated on Murashige and Skoog (1962) (MS)/agar plates (1 × MS salts [Gibco BRL], 0.8% [w/v] phytagar [Gibco BRL], and 1 × Gamborg's B5 vitamin mix [Sigma]) with or without 2% (w/v) sucrose, and stored at 4°C for 40 hr before being moved to the appropriate light conditions. For dark growth, seed were treated with constant white light for 6 hr to induce germination and then wrapped in three layers of aluminum foil to create a dark condition. For the red light induction experiments, we used light-emitting diode red light sources, which emit light with a peak at 670 nm and a half-bandwidth of 25 nm (Quantum Devices, Inc., Barneveld, WI). Seedlings were first grown in the dark for 6 days, and then they were given a pulse of red light with an intensity of 460 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ for 5 min and returned to darkness for the indicated period of time. For the white light induction experiment, seedlings were treated with constant white light with an intensity of 45 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ for 4 hr after 6 days of growth in the dark. For all experiments, the growth temperature was 21°C. For the auxin induction experiments, seedlings were grown in MS/sucrose liquid medium for 6 days with moderate shaking at 100 rpm. The growth temperature was 23°C, and the white light intensity was 6 to 10 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. After 6 days of growth, seedlings were either mock treated with 0.1% ethanol or treated with 20 μM indoleacetic acid (IAA) dissolved in ethanol for 2 hr.

RNA Gel Blot Analysis

Seedlings were taken carefully from the plates, or drained with 3MM Whatman paper if they were grown in liquid medium, and frozen with liquid nitrogen. The frozen tissues were homogenized with mortars and pestles in liquid nitrogen. For the light induction experiments, total RNA was isolated using the acid guanidinium thiocyanate–phenol extraction method as described previously (Chomczynski and Sacchi, 1987). For other experiments, total RNA was extracted using Trizol reagent (Gibco BRL). Poly(A)$^+$ RNA was extracted using oligo(dT)$_{25}$ Dynabeads according to the manufacturer's instructions (Dynal, Lake Success, NY). mRNA isolated from 50 μg of total RNA was loaded on formaldehyde gels and transferred onto nylon membranes (Hybond N$^+$; Amersham, Piscataway, NJ) by capillary blotting.

Blots were washed for 5 sec with 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), baked at 80°C for 1 hr, and hybridized as described (Church and Gilbert, 1984). All *Aux/IAA* cDNA probes were kindly provided by A. Theologis (Plant Gene Expression Center, Albany, CA). We either amplified the cDNA fragments by polymerase chain reaction (PCR) using $M_{13}F_1$ and $M_{13}R_3$ vector primers or degenerate primers recognizing *Aux/IAA* genes, or we cut the fragments from the cDNA clones using restriction endonucleases and used those fragments as probes. The ^{32}P probes were prepared by labeling the cDNA fragments (*Aux/IAA*) or the whole plasmids (*CAB*) using a random priming kit (Boehringer Mannheim, Indianapolis, IN). The blots were hybridized with the probes in Church buffer (250 mM $NaPO_4$, pH 7.4, 7% SDS, 1 mM EDTA, and 1% BSA) at 65°C overnight and then washed in 2 × SSC once for 15 min followed by 0.2 × SSC and 0.1% SDS twice for 15 min each time at 65°C. The blots then were exposed to x-ray films (Eastman Kodak, Rochester, NY).

Microarray Method

Total RNA was extracted using Trizol reagent (Gibco BRL). Seven micrograms of total RNA was used to synthesize cDNA. A custom cDNA kit (Gibco BRL) was used with a T7-$(dT)_{24}$ primer for this reaction. Biotinylated cRNA then was generated from the cDNA reaction using the Enzo BioArray High Yield RNA Transcript Kit (Affymetrix, Sunnyvale, CA). The cRNA then was fragmented in fragmentation buffer (1 × fragmentation buffer is 40 mM Tris acetate, pH 8.1, 100 mM KOAc, and 30 mM MgOAc) at 94°C for 35 min before chip hybridization. Fifteen micrograms of fragmented cRNA then was added to a hybridization cocktail [0.05 μg/μL fragmented cRNA, 50 pM control oligonucleotide B2, *BioB*, *BioC*, *BioD*, and *cre* hybridization controls, 0.1 mg/mL herring sperm DNA, 0.5 mg/mL acetylated BSA, 100 mM 2-(*N*-morpholino)-ethanesulfonic acid, pH 6.7, 1 M NaCl, 20 mM EDTA, and 0.01% Tween 20]. Arrays were hybridized for 16 hr in the GeneChip Fluidics Station 400 and were washed and scanned with the Hewlett-Packard GeneArray Scanner (Boise, ID). Affymetrix GeneChip Microarray Suite 4.0 software was used for washing, scanning, and basic analysis. Sample quality was assessed by examination of 3′ to 5′ intensity ratios of certain genes.

Construction of Promoter::Reporter Fusions

The 2.2-kb *SHY2/IAA3* promoter was amplified by PCR using primers 5′-AACTGCAGTGCTATAATCAACCAGCG-3′ and 5′-CGGGATCCTGAGGTTAACAAACTCATCC-3′ (underlined are PstI and BamHI sites, respectively). The 2.3-kb *AXR2/IAA7* promoter was amplified by PCR using primers 5′-AACTGCAGCAATATGTGTATGTGCACG-3′ and 5′-CGGGATCCGTTGGCCGATCATGTTACTTG-3′ (underlined are PstI and BamHI sites, respectively). These PCR products were cloned into pPZP211 (Hajdukiewicz et al., 1994) with PstI–BamHI sites. The 2.0-kb β-glucuronidase gene (*GUS*) was cut from pBI101.1 (Clontech, Palo Alto, CA) and placed at the 3′ ends of the *SHY2/IAA3* and *AXR2/IAA7* promoters with BamHI–EcoRI sites.

$P_{SHY2/IAA3}$::*GUS* was transformed into Landsberg *erecta* (L*er*) plants by vacuum infiltration (Bechtold et al., 1993). We obtained 35 independent T1 plants and screened T2 progeny of these plants for 3:1 segregation for kanamycin resistance, indicating a single locus insertion. Six individual lines segregated 3:1, and each of them showed a similar 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) staining pattern. We crossed one of these lines into *shy2* and *axr2-1* mutants. For each cross, we screened for homozygous lines either by phenotype (*shy2-2* and *axr2-1*) or by cleaved amplified polymorphic sequence polymorphism (*shy2-24*) (Tian and Reed, 1999). $P_{AXR2/IAA7}$::*GUS* was transformed into Wassilewskija (Ws) and L*er*. We obtained 13 T1 lines in Ws and three T1 lines in L*er*, and among them were seven individual lines in Ws and one line in L*er* with an insertion at a single locus. Each of the lines showed a similar staining pattern, and we used one line in Ws for the crossing. A previously described P_{IAA2}::*GUS* construct (Luschnig et al., 1998) was transformed into L*er*. We obtained 22 T1 lines, and seven of them had insertions at a single locus. Each of the seven lines showed similar X-gluc staining, and we crossed one of them into *shy2* mutants. Previously described $P_{SAUR\text{-}AC1}$::*GUS* (in Columbia background) (Gil and Green, 1997) was crossed directly into *shy2* mutants.

GUS Staining

Seedlings were taken carefully from the MS/sucrose plates and placed directly in 50 mM $NaHPO_4$ buffer, pH 7.0, containing 2 mM X-gluc. The 0.2 M X-gluc stock in *N*,*N*-dimethylformaldehyde was prepared fresh every time before use and diluted into 50 mM $NaHPO_4$ buffer. The seedlings then were vacuum infiltrated for 5 min and subsequently incubated at 37°C for 16 hr or until sufficient staining developed. After clearing in 70% ethanol overnight, the seedlings were photographed through a dissecting microscope or a compound microscope with differential interference contrast optics.

GUS Activity Assay

Seedlings were grown on MS/sucrose plates as described above. After 6 days, 20 seedlings were taken carefully from the plates and homogenized with blue pestles in 100 μL of GUS extraction buffer (50 mM $NaHPO_4$, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% *N*-lauroyl sarcosine, and 10 mM 2-mercaptoethanol) until no obvious debris was visible. The insoluble debris was spun out in a microcentrifuge. The supernatants were used directly for the assay or, if necessary, kept at −80°C until processing. For the GUS assay, 50 μL of the extracts was mixed with 150 μL of 1 mM 4-methylumbelliferyl β-D-glucuronide in GUS extraction buffer prewarmed to 37°C. The mixture was incubated at 37°C for 5, 30, 60, 120, and 240 min. At each time point, 40 μL of the mixture was removed and mixed with 360 μL of stop buffer (0.2 M Na_2CO_3). The resulting fluorescence was measured in a luminescence spectrometer (LS-5B; Perkin-Elmer), which was adjusted to read 900 units for 9 μM 4-methylumbelliferone. We read the fluorescence of each sample three times, plotted the average, and determined the GUS activity from the slope of this line.

Accession Numbers

Accession numbers for the genes described in this article are listed in the tables.

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