Functional Genomic Analysis of the *AUXIN/INDOLE-3-ACETIC ACID* Gene Family Members in *Arabidopsis thaliana* [W]

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Auxin regulates various aspects of plant growth and development. The *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) genes encode short-lived transcriptional repressors that are targeted by the TRANSPORT INHIBITOR RESPONSE1/AUXIN RECEPTOR F-BOX proteins. The Aux/IAA proteins regulate auxin-mediated gene expression by interacting with members of the AUXIN RESPONSE FACTOR protein family. Aux/IAA function is poorly understood; herein, we report the identification and characterization of insertion mutants in 12 of the 29 *Aux/IAA* family members. The mutants show no visible developmental defects compared with the wild type. Double or triple mutants of closely related *Aux/IAA* genes, such as *iaa8-1 iaa9-1* or *iaa5-1 iaa6-1 iaa19-1*, also exhibit wild-type phenotypes. Global gene expression analysis reveals that the molecular phenotypes of auxin-treated and untreated light-grown seedlings are unaffected in the *iaa17-6* and *iaa5-1 iaa6-1 iaa19-1* mutants. By contrast, similar analysis with the gain-of-function *axr3-1/iaa17-1* mutant seedlings reveals dramatic changes in basal and auxin-induced gene expression compared with the wild type. Expression of several type-A *ARABIDOPSIS RESPONSE REGULATOR* genes and a number of genes involved in cell wall biosynthesis and degradation is repressed in *axr3-1/iaa17-1*. The data suggest extensive functional redundancy among *Aux/IAA* gene family members and that enhanced stability of the AXR3/IAA17 protein severely alters the molecular phenotype, resulting in developmental defects.

INTRODUCTION

The plant hormone auxin, typified by indole-3-acetic acid (IAA), regulates a variety of physiological processes, including apical dominance, tropic responses, lateral root formation, vascular differentiation, embryo patterning, and shoot elongation (Davies, 1995). Although auxin was the first plant hormone identified, insights into the molecular mechanism of auxin action is only recently beginning to emerge (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005). Auxin enhances the transcription of several classes of early genes, such as the *Aux/IAA*, *Gretchen Hagen3* (*GH3*), and *SMALL AUXIN UP RNA* (*SAUR*) gene family members (Abel and Theologis, 1996). The central role of the *Aux/IAA* gene family members in auxin signaling has been suggested

by molecular genetic and biochemical studies (Leyser, 2002; Woodward and Bartel, 2005).

The *Aux/IAA* genes were originally identified based on their rapid induction by auxin in etiolated soybean (*Glycine max*) and pea (*Pisum sativum*) tissues (Walker and Key, 1982; Theologis et al., 1985). Similar genes were subsequently isolated in a variety of plant species, including *Arabidopsis thaliana*, tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*), and maize (*Zea mays*) (Reed, 2001). The *Arabidopsis* genome has 29 genes that encode putative Aux/IAA proteins (Arabidopsis Genome Initiative, 2000; Liscum and Reed, 2002; Remington et al., 2004). Auxin induces the expression of many, but not all, *Aux/IAA* gene family members. The *Arabidopsis IAA1* through *IAA14* and *IAA19* genes are auxin inducible with varying induction kinetics, and their mRNA accumulation varies in different parts of the plant (Abel et al., 1995; Tatematsu et al., 2004). However, several *Aux/IAA* genes, including *IAA17* and *IAA28*, show little or no response to exogenous auxin (Rogg et al., 2001; Tian et al., 2002; this study). The diversity in auxin responsiveness and tissue-specific expression among the various gene family members suggests that each member may have a distinct or overlapping function(s) during normal auxin responses required for plant development.

The *Aux/IAA* mRNAs are also rapidly induced by treatment with the protein synthesis inhibitor cycloheximide (Ballas et al., 1993; Abel et al., 1995; Koshiba et al., 1995). This observation led to the suggestion that auxin-mediated transcriptional activation might involve the removal of a short-lived transcriptional repressor (Theologis et al., 1985). Recent biochemical and

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genetic studies indicate that the Aux/IAA proteins are the postulated repressors of auxin-regulated transcriptional activation (Ulmasov et al., 1997b; Tiwari et al., 2001, 2003, 2004; Woodward and Bartel, 2005). The *Aux/IAA* genes encode short-lived nuclear proteins, and most of them contain four highly conserved domains (domain I, II, III, and IV) separated by variable regions (Abel et al., 1994, 1995; Reed, 2001). Each domain contributes to the functional properties of the protein. Domain I is responsible for the repressing activity of the proteins (Tiwari et al., 2004), whereas domain II confers instability to the proteins (Worley et al., 2000; Ouellet et al., 2001). Domains III and IV serve for homodimerization and heterodimerization with other *Aux/IAA* gene family members (Kim et al., 1997; Ouellet et al., 2001) as well as for heterodimerization with the AUXIN RESPONSE FACTORS (ARFs) (Ulmasov et al., 1997b; Ouellet et al., 2001).

The Aux/IAA proteins, while they do not bind to the auxin-responsive elements (*AuxREs*) directly, regulate auxin-mediated gene expression by controlling the activity of ARFs by protein–protein interactions (Ulmasov et al., 1997b; Tiwari et al., 2003). A typical ARF protein contains a B3-like DNA binding domain in its N terminus region and domains III and IV similar to those present in the Aux/IAAs in their C terminus region (Kim et al., 1997; Ulmasov et al., 1997a; Guilfoyle and Hagen, 2001). The ARF proteins bind to *AuxREs* in the promoter region of auxin-responsive genes, including *Aux/IAAs*, through their DNA binding domain (Ulmasov et al., 1997a, 1999b). The amino acid composition of their middle region determines whether an ARF protein is a transcriptional activator or repressor (Ulmasov et al., 1999a; Tiwari et al., 2003). These molecular observations suggest that the vast and diverse combinations of dimers among the *Aux/IAA* and *ARF* gene family members may regulate auxin-mediated gene expression in a cell- and tissue-specific manner (Abel et al., 1994; Kim et al., 1997; Kepinski and Leyser, 2002). The prospect arises that auxin signals are converted into specific responses by matching pairs of co-expressed ARF and Aux/IAA proteins (Weijers and Jurgens, 2004; Weijers et al., 2005).

The molecular genetic and biochemical data indicate that rapid turnover of Aux/IAA proteins is required for normal auxin response. Significant advances toward linking Aux/IAA protein degradation and auxin signaling have been made. Auxin treatment increases the instability of the pea IAA6 (PSIAA6):luciferase (LUC) and *Arabidopsis* IAA1:LUC fusion proteins (Zenser et al., 2001, 2003), suggesting that auxin directly modulates the degradation rate of Aux/IAA proteins. Biochemical and genetic studies have shown that IAA7/AXR2 and IAA17/AXR3 proteins are targeted for ubiquitin-mediated proteolysis by the SCF[TIR1] ubiquitin ligase complex. The interactions of IAA7/AXR2 and IAA17/AXR3 proteins with the SCF[TIR1] complex involve domain II and occur in an auxin-dependent manner (Gray et al., 2001; Dharmasiri et al., 2003; Dharmasiri and Estelle, 2004; Kepinski and Leyser, 2004). Auxin directly binds to the TRANSPORT INHIBITOR RESPONSE1/AUXIN RECEPTOR F-BOX (TIR1/AFB) proteins, resulting in the degradation of the Aux/IAA proteins (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005). Therefore, stability of the Aux/IAA proteins is the central regulator of auxin signaling.

Gain-of-function mutations in several *Aux/IAA* genes, including *shy2/iaa3* (Tian and Reed, 1999), *shy1/iaa6* (Kim et al., 1996; Reed, 2001), *axr2/iaa7* (Timpte et al., 1994; Nagpal et al., 2000), *bdl/iaa12*

(Hamann et al., 1999, 2002), *solitary root* (*slr*)/*iaa14* (Fukaki et al., 2002), *axr3/iaa17* (Leyser et al., 1996; Rouse et al., 1998), *iaa18* (Reed, 2001), *msg2/iaa19* (Tatematsu et al., 2004), and *iaa28* (Rogg et al., 2001), have pleiotropic affects on plant growth. These mutants have been identified from a variety of developmental and auxin-specific genetic screens, and the changes in growth can be associated with decreased or increased auxin responses. For example, *shy2-2/iaa3*, *axr2-1/iaa7*, and *iaa28-1* mutants show decreased apical dominance (Tian and Reed, 1999; Nagpal et al., 2000; Rogg et al., 2001), which is consistent with a reduced auxin response. By contrast, *axr3-1/iaa17-1* mutant plants show increased apical dominance and increased adventitious rooting, suggesting an enhanced auxin response (Leyser et al., 1996). Each of the *Aux/IAA* gain-of-function mutants is caused by a single amino acid change in domain II of the corresponding Aux/IAA proteins. Mutations in domain II stabilize the Aux/IAA proteins. Mutant axr3-1/iaa17 protein has a sevenfold increased half-life compared with the wild-type version of the protein (Ouellet et al., 2001). Similarly, the shy2-2/iaa3 protein accumulates in the *shy2-2/iaa3* mutant plants (Colon-Carmona et al., 2000). The increased protein level is thought to inhibit the activity of the ARF proteins by preventing them from binding to *AuxREs* (Tiwari et al., 2001, 2003, 2004; Kepinski and Leyser, 2002; Woodward and Bartel, 2005). The *Aux/IAA* gain-of-function mutations alter gene expression in untreated and auxin-treated plants. For example, the *SAUR-AC1* transgene is ectopically expressed, and the auxin-mediated induction of several *Aux/IAA* genes is severely impaired in the *axr3-1/iaa17-1* and *axr2-1/iaa7* mutants (Abel et al., 1995; Leyser et al., 1996; Ouellet et al., 2001). Microarray analysis has shown that the auxin-mediated gene expression profile is globally altered in the *shy2-2/iaa3* plants (Tian et al., 2002).

Here, we report the isolation and characterization of insertion mutants in the *Aux/IAA* genes. All the single mutants do not show obvious visible phenotypes. Construction of double (*iaa8-1 iaa9-1*) and triple (*iaa5-1 iaa6-1 iaa19-1*) mutants also failed to reveal visible phenotypes, suggesting a high degree of functional redundancy among the various gene family members. Consistent with these findings is the observation that the gene expression profiles of auxin-treated and untreated *iaa17-6* and *iaa5-1 iaa6-1 iaa19-1* mutant seedlings are similar to the treated or untreated wild-type plants. On the other hand, the gene expression profiles of the gain-of-function mutant *axr3-1/iaa17-1* are altered by the mutation. These data suggest that the stabilized axr3/iaa17 protein functions as a repressor in auxin signaling even though some of the phenotypes of *axr3-1/iaa17-1* are consistent with enhanced auxin responses (Leyser et al., 1996; Tiwari et al., 2001, 2003). Furthermore, the mutation also has global effects on non-auxin-regulated gene expression.

RESULTS

The *Arabidopsis Aux/IAA* Gene Family

The *Arabidopsis* genome contains 29 *Aux/IAA* genes scattered among the five chromosomes (Arabidopsis Genome Initiative, 2000, annotation version 5.0; Liscum and Reed, 2002; Figure 1A). The locations of the 10 previously described gain-of-function
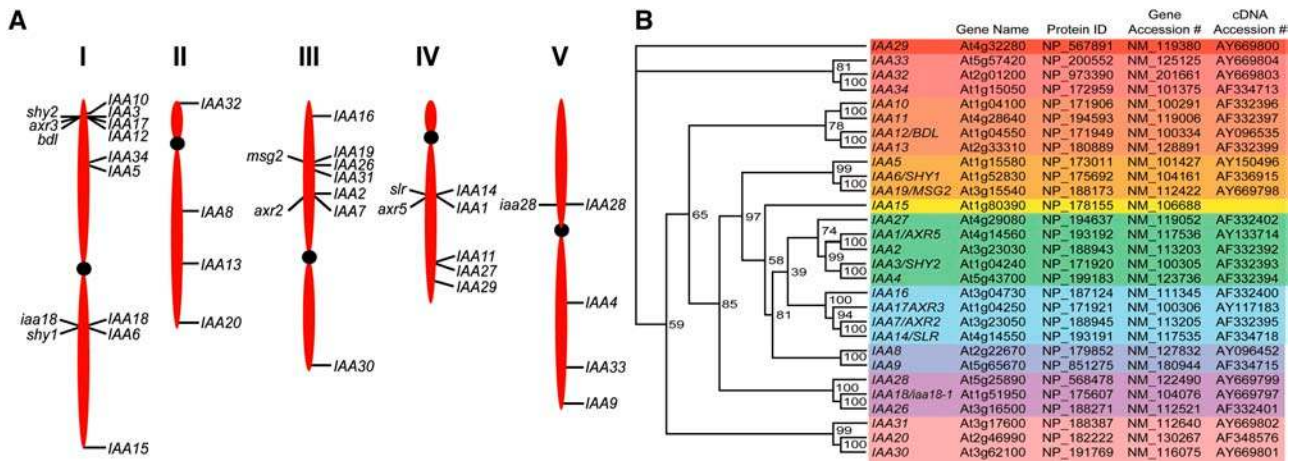
Figure 1. The *Aux/IAA* Gene Family of *Arabidopsis.*

(A) Chromosomal location of *Aux/IAA* genes. The locations of 29 putative *Aux/IAA* genes on the *Arabidopsis* chromosomes (I to V) are shown according to version 5.0 of the *Arabidopsis* genome annotation submitted to GenBank. Mutants that have been isolated in the *Aux/IAA* gene are shown on the left side of the chromosomes.

(B) Phylogenetic analysis. The gene names, accession numbers, protein IDs, and the accession numbers of the full-length ORFs used for this analysis are also shown. The full-length ORFs of *IAA18, IAA19, IAA28, IAA29, IAA30, IAA31, IAA32,* and *IAA33* were constructed during this study.

mutations, *shy2/iaa3* (Tian and Reed, 1999), *axr3/iaa17* (Leyser et al., 1996; Rouse et al., 1998), *bdl/iaa12* (Hamann et al., 1999, 2002), *iaa18* (Reed, 2001), *shy1/iaa6* (Kim et al., 1996; Reed, 2001), *msg2/iaa19* (Tatematsu et al., 2004), *axr2/iaa7* (Nagpal et al., 2000), *slr/iaa14* (Fukaki et al., 2002), *axr5/iaa1* (Yang et al., 2004), and *iaa28* (Rogg et al., 2001), are highlighted in Figure 1A. The *IAA3/IAA17* and *IAA1/IAA14* gene pairs are clustered on chromosomes I and IV, respectively. They have most likely risen during genome duplication and gene reshuffling (Blanc et al., 2000). The isolation of full-length open reading frames (ORFs) for all the gene family members except *IAA15* (Figure 1B) demonstrates that all *Aux/IAA* genes are transcriptionally active (Figure 1B). *IAA15* was previously thought to be a pseudogene (Abel et al., 1995); however, the amino acid alignment shown in Figure 2 shows that the predicted IAA15 protein (annotation version 5.0 of the *Arabidopsis* genome) contains all four conserved domains associated with the Aux/IAA proteins. Our efforts to isolate a full-length *IAA15* ORF have been unsuccessful. This may be due to its low abundance mRNA or due to its expression in developmental stages other than those tested. The updated amino acid alignment shown in Figure 2 has been derived from full-length ORF cDNAs and reveals that not all the Aux/IAA proteins contain the four conserved domains I, II, III, and IV, a hallmark of these proteins (Abel et al., 1994; Reed, 2001). For example IAA10, IAA11, IAA20, and IAA29-34 do not contain the conserved amino acid residues in domain I shared by other members of the gene family. In addition domain II is quite different in IAA20, IAA30, IAA32, and IAA34. Domains I and II are divided into two subdomains in the IAA8/IAA9 and IAA10/IAA11 pairs, respectively, due to insertions of additional amino acid residues (Figure 2). Phylogenetic analysis shows that the *Aux/IAA* genes fall into 10 branches marked with different colors in Figure 1B. The Aux/IAA proteins vary widely in size, ranging from ∼18 (IAA31) to ∼36 kD (IAA9) (see Supplemental Table 1 online). Their isoelectric points

also vary widely from 4.51 (IAA30) to 9.74 (IAA12) (see Supplemental Table 1 online). The amino acid identity among the various gene family members ranges from 83% (IAA20/IAA30 pair) to 10% (e.g., IAA8/IAA33 pair) as shown in Supplemental Table 2 online. The overall identity among the various proteins is low, even between members of the same phylogenetic branch (see Supplemental Table 2 online).

Isolation of *Aux/IAA* T-DNA Insertion Mutants

We initiated this project using a PCR-based screening approach to identify T-DNA insertion mutants for a large number of *Aux/IAA* genes. A total of 80,000 T-DNA insertion lines in the Columbia (Col) ecotype were initially screened, and 11 lines were identified (Alonso et al., 2003). Subsequently, the laboratory participated in generating the Garlic lines in collaboration with the former Torrey Mesa Research Institute, and one additional line was isolated (Sessions et al., 2002). A dSpm insertion line for *IAA5* was also identified (Tissier et al., 1999). Taken together during the last 6 years, we identified 13 T-DNA insertion lines located between the start and stop codons of 12 *Aux/IAA* genes. Figure 3 and Supplemental Table 3 online provide a summary of all the mutants isolated and characterized during the course of this study. A recent search at the Salk T-DNA express line collection (http://signal.salk.edu/cgi-bin/tdnaexpress) reveals that there are additional T-DNA insertion lines in the coding regions of *IAA5, IAA7, IAA11, IAA15, IAA17, IAA18, IAA28, IAA29, IAA30, IAA33,* and *IAA34,* but the analysis and characterization of these lines has not been completed during this study. All the lines have been backcrossed at least once and partially characterized phenotypically. We plan to deposit all the lines in the Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/∼plantbio/Facilities/abrc/abrchome.htm) for further molecular and phenotypic characterization by the community.
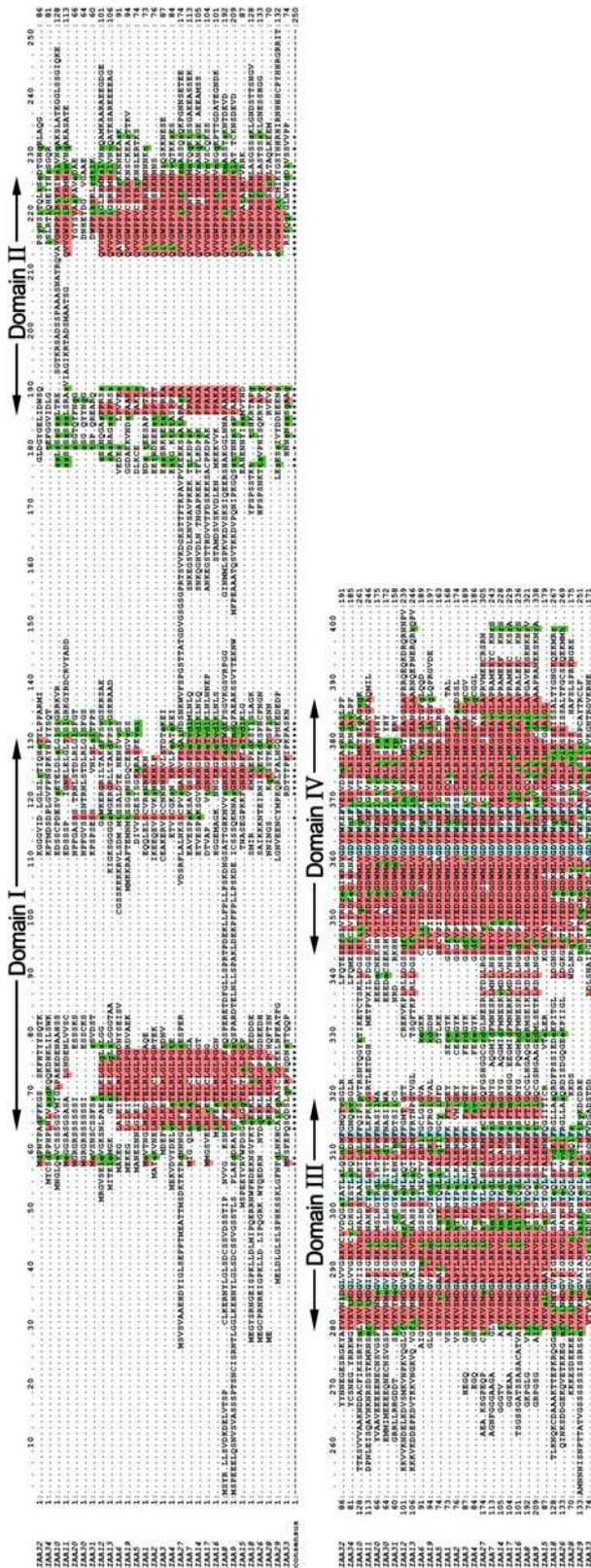
Figure 2. Alignment of the Aux/IAA Proteins Reveals Several Highly Conserved Domains.

Phenotypes of Insertion Mutants

Figure 4 compares 6-week-old mature wild-type plants (Col) with each of the insertion lines. All the mutants fail to show an obvious visible phenotype during growth and development (Figure 4). These observations suggest that highly similar proteins encoded by one or more members of the *Aux/IAA* gene family are capable of performing overlapping functions during plant growth and development. The most evolutionarily linked genes would encode the most similar proteins and might also contain similar *cis*-regulatory elements. Therefore, the most closely related *Aux/IAA* genes would be good candidates to encode proteins in the appropriate cells for compensating the loss of an individual gene family member. It is also possible that a more distantly related member of the gene family whose expression pattern overlaps with that of the disrupted gene may encode a protein that performs the functions of the missing protein.

To explore the extent of functional redundancy among the various gene family members, we constructed double and triple mutant plants of closely related *Aux/IAA* gene family members, for example, *IAA8/IAA9* or *IAA5/IAA6/IAA19* subfamilies (see Figure 1B). If closely related proteins are responsible for compensating for the loss of a single Aux/IAA protein, lines containing double and triple gene disruptions should reveal the overlapping functions during growth. Surprisingly, the *iaa8-1 iaa9-1*, *iaa5-1 iaa6-1*, *iaa5-1 iaa19-1*, and *iaa6-1 iaa19-1* double mutants and the *iaa5-1 iaa6-1 iaa19-1* triple mutant grow and develop like the wild-type plants under greenhouse conditions (Figures 5A to 5C). Furthermore, the roots of the *iaa5-1 iaa6-1 iaa19-1* triple mutant show wild-type sensitivity to increasing concentrations of exogenous IAA, indicating that the auxin response of the root tissue is not affected by the removal of these three genes (Figure 5D). The findings are surprising because among the three *Aux/IAA* genes used to construct the triple mutant, at least *IAA5* and *IAA6* share similar expression patterns in terms of tissue specificity, kinetics of auxin-mediated induction, and auxin dose–response curves (Abel et al., 1995). In addition, the phenotypes of gain-of-function mutants of *SHY1/IAA6* and *MSG2/IAA19* suggest that they may participate in a light signaling pathway and differential growth response that is mediated by auxin, respectively (Kim et al., 1996; Tatematsu et al., 2004).

Global Gene Expression Analysis

Although the single, double, and triple *Aux/IAA* mutants do not show any detectable phenotypic differences, it is possible that the loss of *Aux/IAA* gene function affects global gene expression. This prompted us to perform microarray analysis with one single loss-of-function mutant (*iaa17-6*), one single gain-of-function mutant (*axr3-1/iaa17-1*), and the triple mutant (*iaa5-1 iaa6-1*

The deduced amino acid sequences of the Aux/IAA proteins were aligned using ClustalW (Thompson et al., 1994). The conserved domains I, II, III, and IV present in the Aux/IAA proteins are indicated at the top of the alignment. The sequences used in this analysis are the same as those used for constructing the phylogenetic tree shown in Figure 1B.
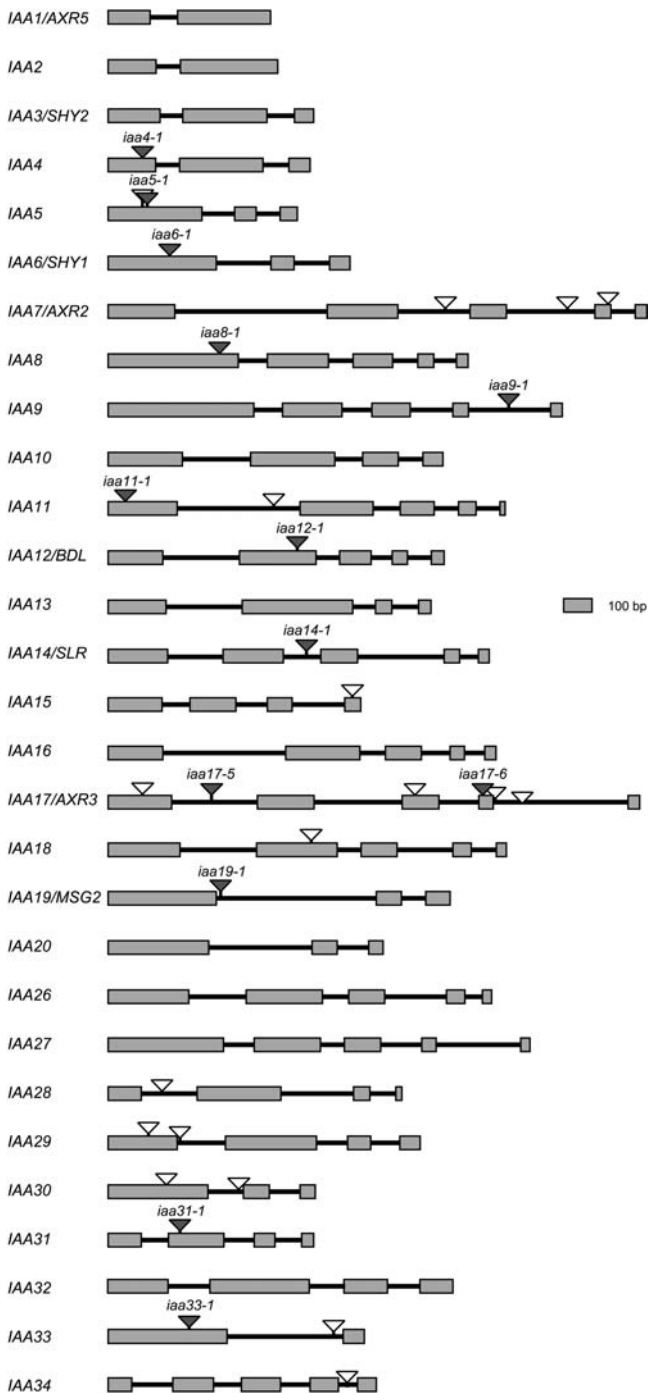
Figure 3. Location of T-DNA or dSpm Insertions in the *Aux/IAA* Gene Family Members.

Boxes represent exons. Insertions with gray triangles denote lines whose characterization has been completed. Insertions with white triangles denote lines not characterized.

iaa19-1; note the three genes of the mutant form a distinct clade on the phylogenetic tree; Figure 1B). We chose *iaa17-6* as the representative of the single mutants because the *AXR3/IAA17* gene and its mutant alleles have been well characterized (Leyser et al., 1996; Rouse et al., 1998; Gray et al., 2001; Ouellet et al., 2001). We identified and characterized two independent T-DNA insertion alleles for *AXR3/IAA17*, *iaa17-5*, and *iaa17-6* (Figure 3); both of them failed to show an obvious growth phenotype (Figure 4). The T-DNA of the *iaa17-5* allele is located in the first intron (Figure 3). RT-PCR analysis revealed that a properly processed *AXR3/IAA17* transcript was still made in the plants homozygous for the *iaa17-5* insertion, suggesting that the T-DNA insertion may be removed by splicing (data not shown). By contrast, RT-PCR analysis with RNA from *iaa17-6* plants showed no detectable transcript for this gene (data not shown).

Effect of Mutations on Global Gene Expression in Auxin-Treated Seedlings

Light-grown seedlings of wild-type, *iaa17-6*, *axr3-1/iaa17-1*, and *iaa5-1 iaa6-1 iaa19-1* were treated for 2 h with the carrier solvent ethanol (control sample) or 5 μM IAA (auxin-treated sample). Each experiment was performed in triplicate, and total RNA was independently isolated to generate biotin-labeled complementary RNA (cRNA) for hybridization (see Methods). The data were then subjected to a set of statistical analyses that consider signal strength and variance within and among the biological replicates (see Methods). Figure 6 shows the scatterplots (*M-A* plots; Dudiot et al., 2002) representing the auxin-regulated transcriptional profiles of wild-type, *iaa17-6*, *iaa5-1 iaa6-1 iaa19-1*, and *axr3-1/iaa17-1* mutants. A cursory examination of the scatterplots indicates that the wild-type gene expression profile is globally altered by exogenous auxin treatment. However, the loss of *IAA17* and *IAA5/IAA6/IAA19* genes do not cause changes in auxin-induced gene expression, suggesting that the gene regulation response to exogenous auxin is intact in these two mutants. However, there is a reduced level of scattering in the expression profile of the *axr3-1/iaa17-1* mutant seedlings (Figure 6), indicating that auxin-mediated gene expression is altered by this gain-of-function mutation. This is consistent with the reduced auxin sensitivity and the altered growth habit seen in *axr3-1/iaa17-1* plants (Leyser et al., 1996).

We identified a preliminary set of genes whose expression was affected at least twofold by auxin treatment using the log$_2$ expression values from the robust multiarray analysis (RMA) output file (Irizarry et al., 2003). From this initial set of genes, we identified those genes whose expression pattern of expression was statistically significant (see Methods). As shown in Table 1, of the 22,800 genes analyzed, in wild-type seedlings only 168 genes were identified as auxin induced, and only 57 genes were identified as auxin-repressed genes. A complete list of all the auxin-regulated genes according to their functional classification can be found in Supplemental Tables 4 and 5 online. The lists include various classes of known auxin-regulated genes, such as *Aux/IAA*, *GH3*, *SAUR*, and *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE*, consistent with similar studies reported previously (Tian et al., 2002; Pufky et al., 2003; Redman et al., 2004; Okushima et al., 2005).
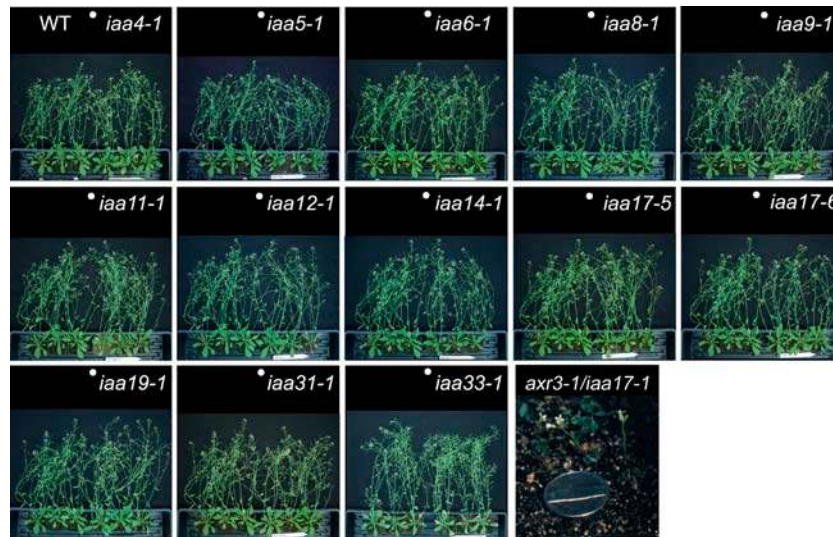
Figure 4. Growth Phenotype of Insertion Mutants of Various *Aux/IAA* Gene Family Members.

Three wild-type (left) and three mutant plants (right) were grown simultaneously and are shown for each line. White dots indicate the boundaries between the wild-type and the mutant plants.

The question immediately arises as to whether the three mutants have a defective gene expression profile in the presence of exogenous auxin. We extracted the gene sets that were induced or repressed by auxin in the wild type and compared them with those extracted from auxin-treated mutants seedlings (*iaa17-6*, *axr3-1/iaa17-1*, and *iaa5-1 iaa6-1 iaa19-1*). We used fold change ratio of induction or repression levels between mutants and the wild type as criteria, with a cutoff fold change ratio value of ≥2. Almost all auxin-regulated genes respond normally to exogenous auxin treatment in the *iaa17-6* and *iaa5-1 iaa6-1 iaa19-1* mutants (see Table 1). This is consistent with the gene distribution observed by the scatterplot analyses (Figure 6). In fact, in the *iaa17-6* seedlings, only one auxin-repressed gene (At5g47450), which encodes TIP2;3, shows a reduced level of auxin-regulated repression (Table 1). A small number (four genes) show reduced level of induction by auxin treatment in the *iaa5-1 iaa6-1 iaa19-1* mutant (Table 1). Three of the four genes, *IAA5*, *IAA6*, and *IAA19*, are the inactivated genes in the mutant (Table 1; see Supplemental Figure 1 online). The fourth gene (At5g47450) encodes a protein of unknown function (Table 1). By contrast, in the *axr3-1/iaa17-1* mutant, 77 (46%) of the 168 auxin-induced genes and 37 (65%) out of 57 auxin-repressed genes show impaired auxin-mediated regulation (Table 1; see Supplemental Tables 6 and 7 online). The mutation affects a group of auxin-regulated genes that encode proteins with diverse functions (Table 1; see Supplemental Tables 6 and 7 online). Among the 28 *Aux/IAA* genes present on the ATH1 GeneChip, only *IAA1*, *IAA2*, *IAA4*, *IAA5*, *IAA6*, *IAA11*, *IAA13*, *IAA19*, and *IAA29* are auxin induced in wild-type seedlings (see Supplemental Figure 1 online). The *axr3-1/iaa17-1* mutation impairs the auxin inducibility of three *Aux/IAA* genes, *IAA5*, *IAA6*, and *IAA11* (see Supplemental Figure 1 online). The expression of *IAA2* is not affected by the mutation (see Supplemental Figure 1 online). The remaining five auxin-inducible *Aux/IAA* genes (*IAA1*, *IAA4*, *IAA13*, *IAA19*, and *IAA29*) remain auxin inducible, but the amplitude of the response is dampened (see Supplemental Figure 1 online). Furthermore, the basal expression level of *AXR2/IAA7* and *AXR3/IAA17* is reduced in untreated *axr3-1/iaa17-1* seedlings compared with the wild type (see Supplemental Figure 1 online).

Effect of the Mutations on Global Gene Expression in Non-Auxin-Treated Seedlings (Basal Expression)

To identify the genes affected by the mutants in the absence of exogenous auxin, we compared the gene expression profiles of each mutant with the wild-type profile in the absence of auxin treatment. Among the 22,810 genes, only 2/5 genes were induced and 4/1 genes were repressed in *iaa17-6* and *iaa5-1 iaa6-1 iaa19-1*, respectively (Table 2). Given the small number of genes whose expression is altered in the untreated mutant seedlings, we conclude that the single and triple loss-of-function mutations do not affect their molecular phenotype. Numerous genes, however, are differentially expressed in untreated *axr3-1/iaa17-1* seedlings compared with the wild type. Among the 22,810 genes, 108 and 78 genes are identified as repressed or induced genes in mock-treated *axr3-1/iaa17-1* (Table 2). The mutation affects the expression of diverse classes of genes (Table 2). The genes are listed in Supplemental Tables 8 and 9 online. Among the 108 repressed genes in the *axr3-1/iaa17-1* mutant (Table 2), 28 genes encode cell wall structural proteins and proteins involving cell wall degradation enzymes, including xyloglucan endotransglycosylases (XTHs), pectinmethylesterases (PMEs), expansins, extensins, Pro-rich proteins (PRPs), and arabinogalactan proteins (AGPs) (Table 3; see Supplemental Table 8 online). Their expression characteristics in the presence and absence of auxin are shown in Figure 7. In addition to the genes that encode cell wall–related proteins, the expression of
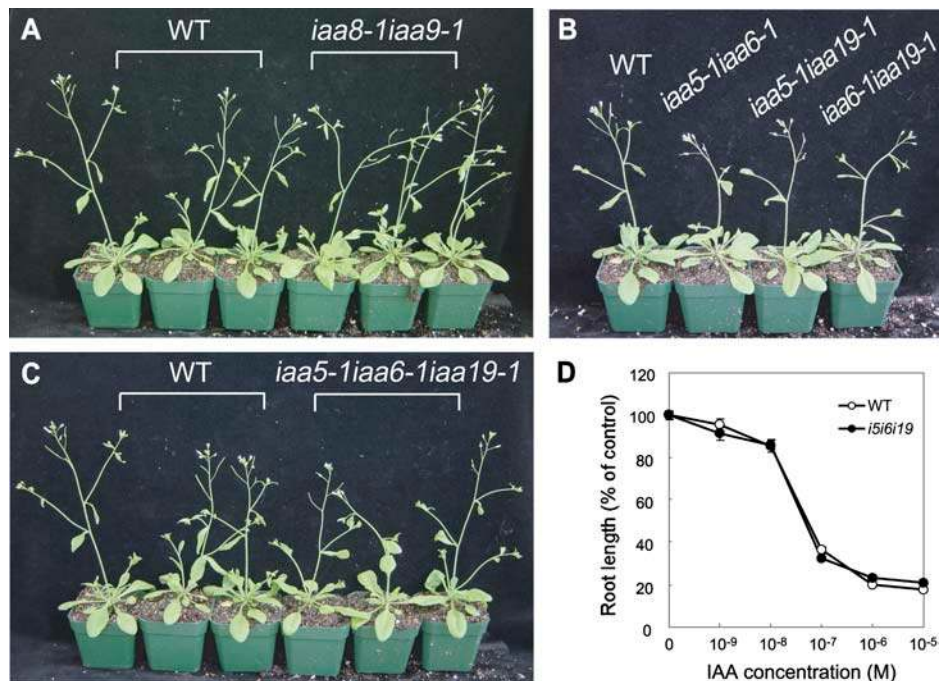
Figure 5. Phenotypes of *iaa8 iaa9* Double and *iaa5 iaa6 iaa19* Triple Mutants during Developmental Stages.

(A) Four-week-old soil-grown plants of the wild type and the *iaa8-1 iaa9-1* double mutant.
(B) Four-week-old soil-grown plants of the wild type or the *iaa5-1 iaa6-1*, *iaa5-1 iaa19-1*, and *iaa6-1 iaa19-1* double mutants.
(C) Four-week-old soil-grown plants of the wild type and the *iaa5-1 iaa6-1 iaa19-1* triple mutant.
(D) Root growth inhibition assay of wild-type and *iaa5-1 iaa6-1 iaa19-1* triple mutant seedlings. Four-day-old seedlings were transferred to medium containing the indicated concentrations of IAA, and the increase in root length was measured after 5 d. Root length is shown as a percentage of the root length in the absence of auxin. Each point represents the average of at least 10 seedlings. Bars represent SE.
The double and triple mutants have been deposited with the ABRC (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm) and the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info/).

several peroxidase genes is enhanced or repressed in untreated *axr3-1/iaa17-1* seedlings (see Supplemental Figure 2 and Supplemental Tables 8 and 9 online). The class III plant peroxidases (POXs; EC 1.11.1.7) are encoded by 73 genes in *Arabidopsis*, and they have been named P1 to P73 (Tognolli et al., 2002). Among the 73 POX genes, eight of them (P2, P24, P35, P39, P44, P59, P60, and P73) show reduced expression in untreated *axr3-1/iaa17-1* mutant seedlings compared with untreated wild-type seedlings, while two of them, P14 and P34, are upregulated (see Supplemental Table 9 online).

Untreated *axr3-1/iaa17-1* seedlings also show altered expression of genes that encode components of the cytokinin signal transduction pathway. Eleven type A *ARABIDOPSIS RESPONSE REGULATORS* (*ARRs*) have been identified in *Arabidopsis* (Hwang et al., 2002). Four of them, namely, *ARR3* (At1g59940), *ARR5* (At3g48100), *ARR6* (At5g62920), and *ARR7* (At1g19050), are repressed by the *axr3-1/iaa17-1* mutation (Figure 8; see Supplemental Table 8 online). Although *ARR4* (At1g10470) appears to be repressed in *axr3-1/iaa17-1*, this observation was not supported by our statistical analysis (Figure 8; see Supplemental Table 8 online). The expression of *ARR8* (At2g41310) and *ARR9* (At3g57040) is similar in the *axr3-1/iaa17-1* and wild-type seedlings (Figure 8). The expression of these two genes is unaffected by cytokinin treatment (D'Agostino et al., 2000). The expression

intensities of the remaining four *ARR* genes (*ARR15*, *ARR16*, *ARR17*, and *ARR22*) are similar to background level, indicating that these four genes are not expressed under our experimental conditions (data not shown).

Does the *axr3-1/iaa17-1* Mutation Induce Auxin-Regulated Genes?

It has been suggested that the phenotype of the *axr3-1/iaa17-1* plants is consistent with enhanced auxin responses (Leyser et al., 1996). The question then arises whether untreated *axr3-1/iaa17-1* seedlings express auxin-regulated genes. In order to examine whether the expression profile of the *axr3-1/iaa17-1* seedlings resembles wild-type seedlings treated with auxin, we compared the list of auxin-induced or repressed genes in the wild type with the list of induced or repressed genes in *axr3-1/iaa17-1* in the absence of auxin treatment. We expect that if the *axr3-1/iaa17-1* mutation causes enhanced auxin responses, auxin-induced genes should be enhanced in *axr3-1/iaa17-1*; likewise, auxin-repressed genes should be repressed in *axr3-1/iaa17-1*. Figure 9 shows the results of such a comparison. A very small number of genes (seven) are overlapping between auxin-induced genes in the wild type and enhanced genes in the *axr3-1/iaa17-1* mutant (Figure 9A). Likewise, only six genes are overlapping among
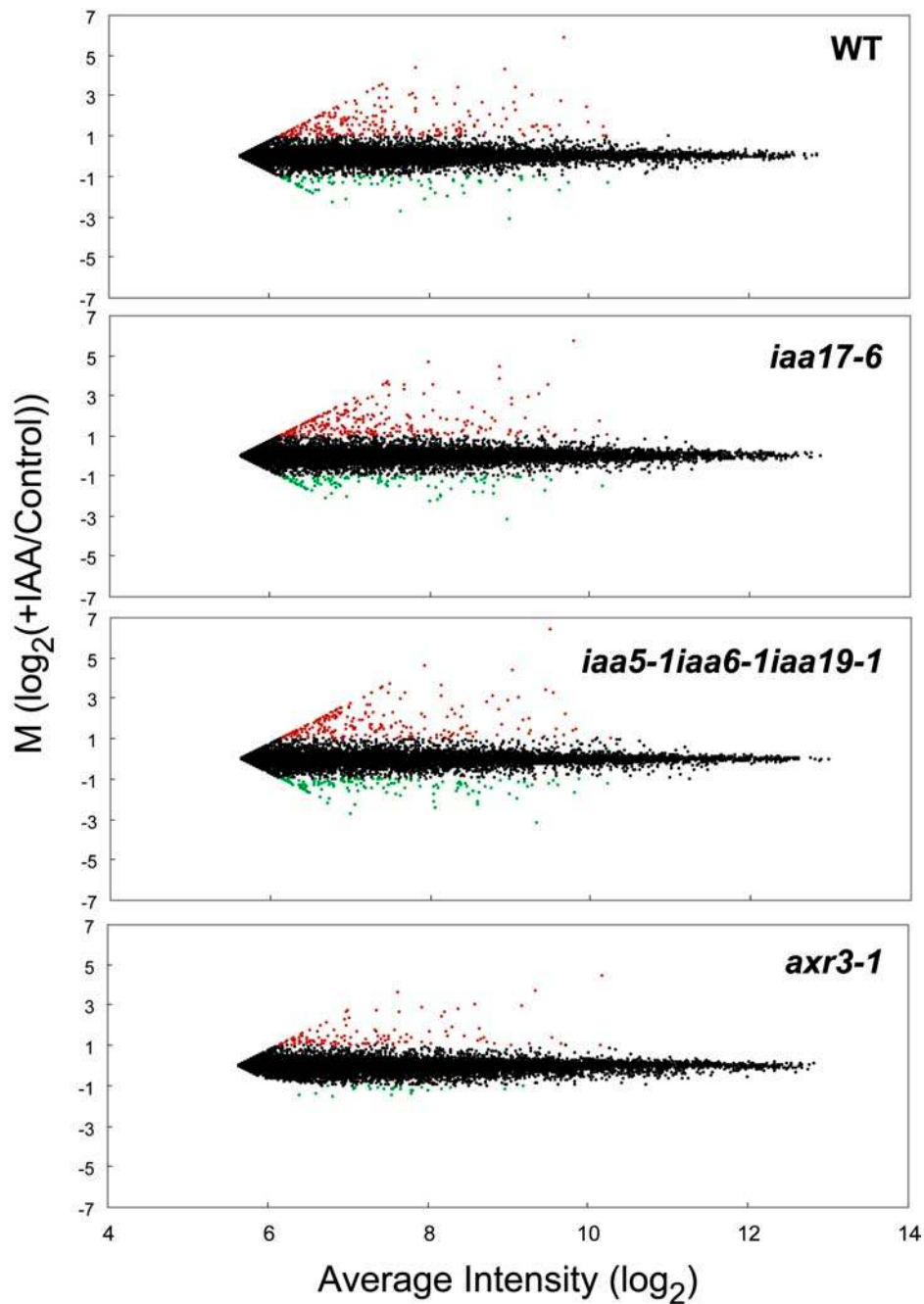
Figure 6. Global Gene Expression Profiling.

M-A plots (Dudiot et al., 2002) showing changes of auxin-mediated gene expression in the wild type, *iaa17-6*, *axr3-1/iaa17-1*, and *iaa5-1 iaa6-1 iaa19-1*. Each plot represents the $\log_2$ ratio of the average of the auxin-treated samples (I) to the control samples (C) [$M = \log_2 (I/C)$] versus overall average intensity [$A = \log_2 \sqrt{(I{*}C)}$] (Dudiot et al., 2002). The genes induced by auxin treatment ($M > 1$) are shown in red, and the genes repressed by auxin treatment ($M < -1$) are shown in green. The data were further analyzed for variance to extract the statistically valid auxin-regulated genes discussed in Tables 1 and 2 and Figure 8.

Table 1. Summary of Genes with Impaired Auxin-Mediated Regulation in the Mutants

Functional Classification	Auxin-Induced Wild Type	Genes with Reduced Induction in Mutants			Auxin-Repressed Wild Type	Genes with Reduced Repression in Mutants		
		iaa17-6	iaa5 iaa6 iaa19	axr3-1		iaa17-6	iaa5 iaa6 iaa19	axr3-1
Auxin	30	0	3*	13	4	0	0	3
Ethylene	9	0	0	6	0	0	0	0
Other phytohormone	5	0	0	0	0	0	0	0
Cell wall	9	0	0	6	5	0	0	5
Metabolism	21	0	0	11	14	0	0	11
Development	3	0	0	3	1	0	0	1
Stress/defense	8	0	0	3	2	0	0	1
Signaling	32	0	1**	10	8	0	0	3
Transporter/channel	5	0	0	3	5	1***	0	4
Others	14	0	0	8	11	0	0	8
Unknown	32	0	0	14	7	0	0	1
Total	168	0	4	77	57	1	0	37

*, At1g15580 (IAA5), At1g52830 (IAA6), and At3g15540 (IAA19); **,At5g44260 (unknown protein); ***, At5g47450 (aquaporin, TIP2;3).

auxin-repressed genes in the wild type and repressed genes in *axr3-1/iaa17-1* (Figure 9B). This relatively small overlap suggests that >90% (212 of 225 genes) of the auxin-regulated genes (induced and repressed; see Table 1) do not show increased or repressed expression in the *axr3-1/iaa17-1* untreated seedlings. For example, previous reports indicated that the *SAUR-AC1/AtSAUR-15* promoter driving β-glucuronidase is ectopically expressed in roots of the *axr3-1/iaa17-1* seedlings (Leyser et al., 1996). In untreated wild-type and *axr3-1/iaa17-1*plants, the

SAUR-AC1/AtSAUR-15 gene is similarly expressed, but its auxin inducibility is impaired in *axr3-1/iaa17-1* seedlings (Figure 9E). Figures 9C and 9D show the expression characteristics of the overlapping set of genes. Their expression is auxin regulated in the wild type as well as in the two loss-of-function mutants *iaa7-6* and *iaa5-1 iaa6-1 iaa19-1*. However, their expression in untreated *axr3-1/iaa17-1* is similar to the auxin-treated wild-type seedlings. Among the 13 overlapping genes, the biological role of the myb-transcription factor, *ATR1* (At5g60890), has been

Table 2. Number of Genes with Repressed or Enhanced Expression in Mutants in the Absence of Exogenous Auxin

Functional Classification	Suppressed Genes in Mutants			Enhanced Genes in Mutants		
	iaa17-6	iaa5 iaa6 iaa19	axr3-1[a]	iaa17-6	iaa5 iaa6 iaa19	axr3-1[b]
Auxin	1[c]	0	3	0	0	1
Ethylene	0	0	0	0	0	0
Other phytohormone	0	0	4	0	0	0
Cell wall	0	1[d]	28	0	1[e]	8
Metabolism	0	0	22	1[f]	1[g]	22
Development	0	0	1	0	0	3
Stress/defense	0	0	6	0	0	2
Signaling	0	0	5	0	0	11
Transporter	1[h]	0	1	0	0	7
Others	1[i]	0	24	1[j]	0	5
Unknown	1[k]	0	14	0	3[l]	19
Total	4	1	108	2	5	78

[a] See Supplemental Table 8 online for complete gene list.
[b] See Supplemental Table 9 online for complete gene list.
[c] At1g04250 (IAA17/AXR3).
[d] At5g65730 (At-XTH6/XTR10).
[e] At4g22470 (extensin-like).
[f] At5g36220 (cytochrome P450 CYP81D1).
[g] At5g03860 (putative malate synthase).
[h] At5g47450 (aquaporin, TIP2;3).
[i] At4g16870 (retrotransposon-like).
[j] At2g04460 (putative retroelement pol polyprotein).
[k] At3g05410 (unknown).
[l] At3g27220 (unknown), At4g33560 (unknown), and At4g24110 (unknown).

Table 3. Induced and Repressed Cell Wall–Related Genes in the *axr3-l/iaal7-1* Mutant

Putative Gene Product	Repressed	Induced
XTHs	7	0
PMEs	2	1
Expansins	2	1
Extensins	3	2
PRPs	7	3
AGPs	5	0
Gly-rich proteins	1	0
Other cell wall–related enzymes	1*	1**
Total	28	8

*, Endo-β-1,4-glucanase; **, exostosin-like glycosyltransferase.

previously described. The expression of *ATR1* is repressed by auxin, and its encoded protein functions as a positive regulator of *ASA1* (At3g54640) and *CYP79B2* (At4g39950) expression (Bender and Fink, 1998; Smolen and Bender, 2002). *ASA1* encodes an enzyme that catalyzes the first step in Trp biosynthesis (Niyogi and Fink, 1992). *CYP79B2* and a closely related gene, *CYP79B3* (At2g22330), encode cytochrome P450 enzymes that are involved in converting Trp to indole-3-acetaldoxime, the precursor of IAA (Zhao et al., 2002). Auxin-mediated repression of *ATR1*, therefore, appears to be a feedback mechanism, and the reduced level of ATR1 expression in the untreated *axr3-1/iaa17-1* seedlings is consistent with the enhanced auxin response phenotypes seen in these plants.

DISCUSSION

Functional Redundancy among the *Aux/IAA* Gene Family Members

The Aux/IAA proteins constitute a large family of plant-specific transcription factors that play a central role in auxin-regulated transcriptional activation/repression. According to the current view of auxin action, the Aux/IAAs are repressors of ARF activity. They are targeted by the TIR1/AFB receptors for proteolytic degradation, resulting in ARF-dependant transcriptional activation/repression of auxin-regulated gene expression, thereby modulating auxin-dependent growth and development (Woodward and Bartel, 2005). Thus far, gain-of-function mutations in 10 of 29 *Arabidopsis* Aux/IAA members have been described using forward genetics (i.e., *iaa1/axr5*, *iaa3/shy2*, *iaa6/shy1*, *iaa7/axr2*, *iaa12/bdl*, *iaa14/slr*, *iaa17/axr3*, *iaa18*, *iaa19/msg2*, and *iaa28*). Phenotypic analysis of the mutants reveals distinct and overlapping functions among the various members. For example, expression of the stabilized mutant form of *iaa3/shy2* or *iaa17/axr3* proteins from a heat shock promoter causes different effects on root hair development (Knox et al., 2003). The phenotype of the gain-of-function mutants can be mimicked by the expression of corresponding Aux/IAA proteins with the domain II mutation under the control of their own promoters (Fukaki et al., 2002, 2005; Weijers et al., 2005). Promoter-swapping experiments among IAA12/BDL,

IAA13, and IAA3/SHY2 using stabilized forms of these proteins reveal distinct functions of these proteins during plant development, embryonic root formation, and seedling growth (Weijers et al., 2005). The functional difference of each Aux/IAA member may be due to its specific preference for a particular ARF partner.

Analysis of transgenic plants expressing stabilized mutant forms of Aux/IAA proteins can contribute to obtaining new insights into the function of the uncharacterized Aux/IAAs. However, since some of the effects of the gain-of-function mutations appear to be created by the ectopic accumulation of the mutant protein, the phenotypes are unlikely to accurately reflect the biological function of the corresponding Aux/IAA protein. It is possible that some of them may not be normally involved in auxin sensing at all, having auxin effects only in gain-of-function mutant states. Moreover, whether these proteins are positive or negative regulators of auxin responses cannot be inferred even if they are components of the auxin signaling apparatus. Thus, it is crucial to analyze the loss-of-function mutant phenotypes of these genes to elucidate their role in auxin signal transduction.

We therefore attempted to identify and characterize loss-of-function mutants of Aux/IAA family members using a reverse genetics approach. Currently, the T-DNA or dSpm transposon insertion mutants for 18 out of 29 *Aux/IAA* genes are available (SIGnAL; http://signal.salk.edu/cgi-bin/tdnaexpress; Alonso et al., 2003). An initial characterization has been conducted for 12 Aux/IAA insertion alleles in this study, but all of them, including previously identified mutants, failed to show dramatic defects on plant development. In addition, the *iaa8-1 iaa9-1* and *iaa5-1 iaa6-1 iaa19-1* double and triple mutants, whose protein products are more closely related to each of these gene sets and produce distinct clades in the phylogenetic tree, also grow normally under greenhouse conditions. Global gene expression profiles of *iaa17-6* single and *iaa5-1 iaa6-1 iaa19-1* triple mutant seedlings are also almost the same as those of the wild-type seedlings treated with or without 5 μM IAA for 2 h. Although considering a small number of possible combinations, these observations suggest that the inactivation of single or triple *Aux/IAA* gene function(s) does not affect the auxin-mediated gene expression, suggesting a broad functional redundancy among the *Aux/IAA* gene family members. There is still a possibility that each single *Aux/IAA* insertional mutant and the double and triple mutants that we have described do have specific phenotypes but would warrant additional scrutiny to detect them. It has been previously reported that the loss-of-function mutants of *iaa3/shy2* and *iaa7/axr2* have subtle but significant phenotypes (Tian and Reed, 1999; Nagpal et al., 2000). Therefore, all our *Aux/IAA* insertional mutants may reveal specific phenotypes by meticulous and careful observations or by determining their sensitivity/resistance to chemical genetic screens and various environmental stresses.

The Aux/IAA family members with a high degree of similarity most likely compensate for the loss of one or more gene functions. However, given that we did not see obvious phenotypes with the *iaa8-1 iaa9-1* double and *iaa5-1 iaa6-1 iaa19-1* triple mutants, the compensatory function might be provided by family members that share a lower degree of similarity but whose
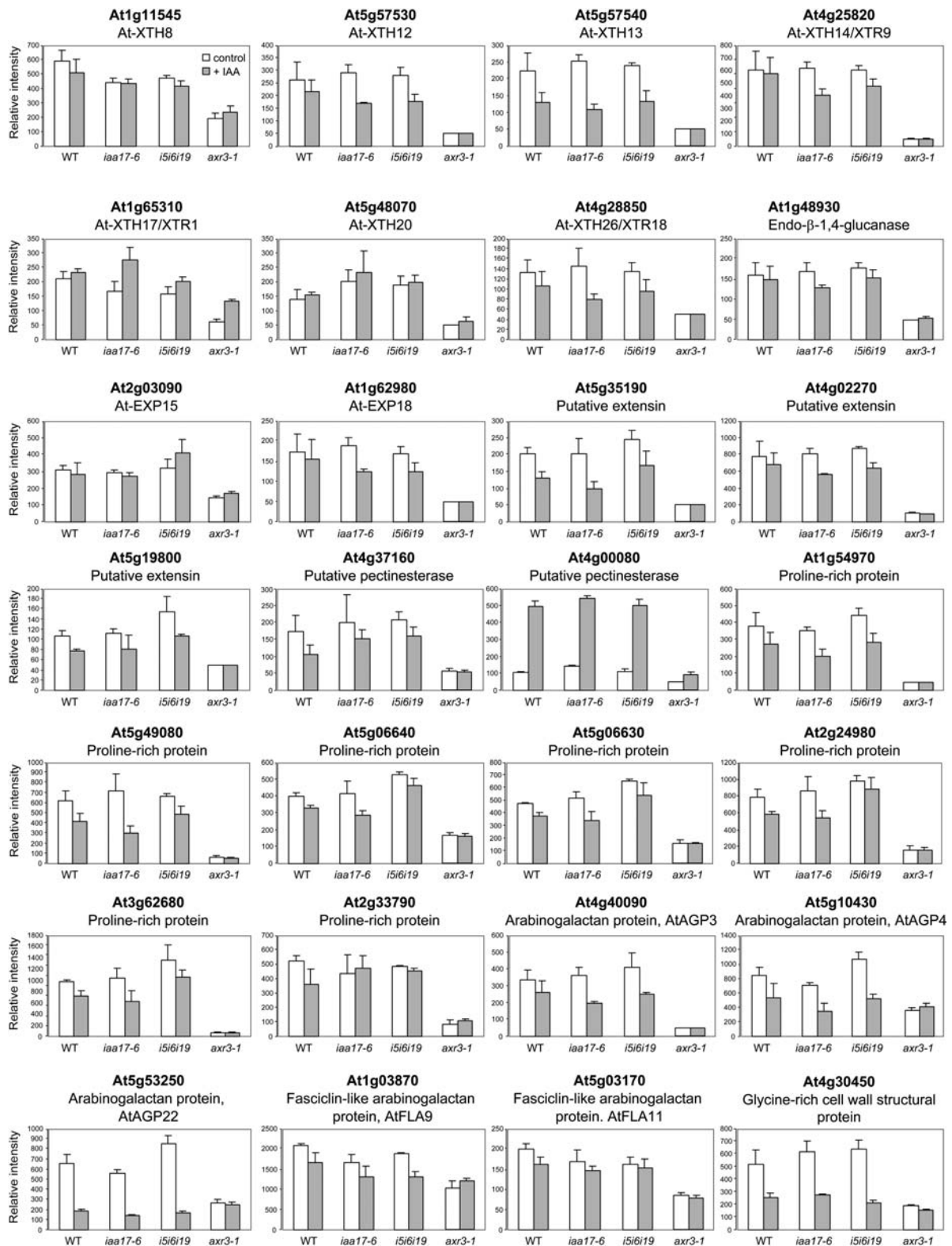
Figure 7. Expression Characteristics of Cell Wall–Related Genes in Wild-Type, *iaa17-6*, *axr3-1/iaa17-1*, and *iaa5-1 iaa6-1 iaa19-1* Seedlings.

Data represent the average relative expression intensity of control (open bars) or auxin-treated (gray bars) samples from triplicate experiments. See Methods for further details. Bars represent SD.
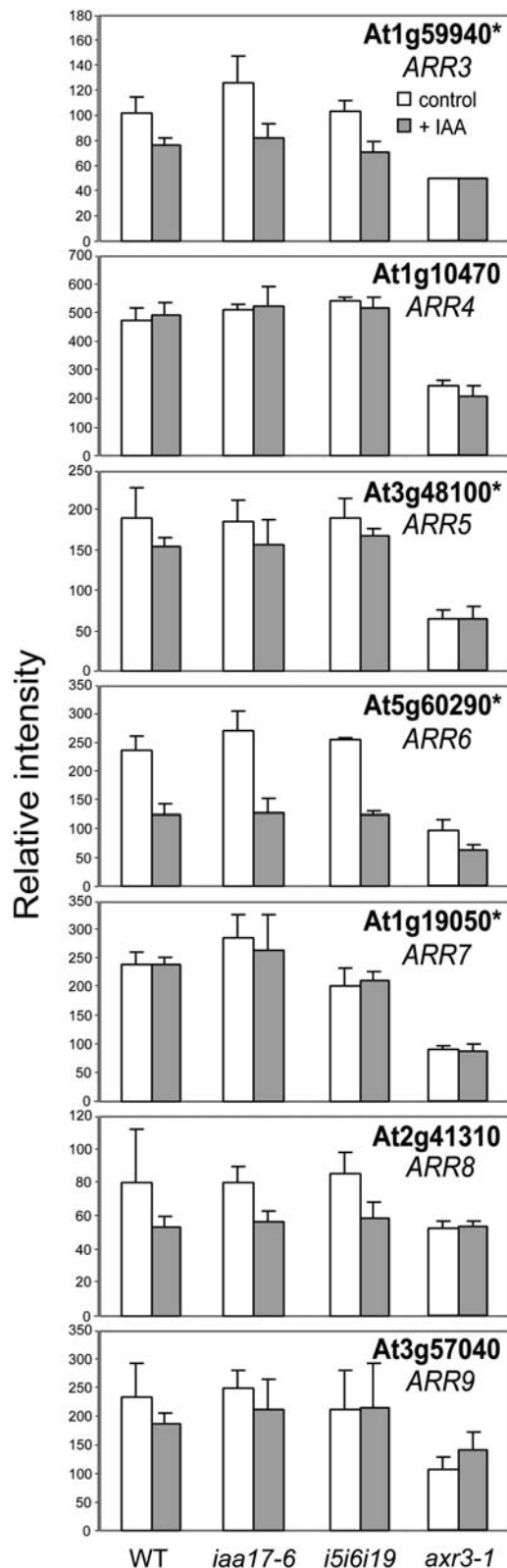
Figure 8. The Expression of Several Type A *ARR* Genes Is Suppressed in *axr3-1/iaa17-1* Seedlings.

Histograms show expression patterns of seven type A *ARR* genes in the wild type, *iaa17-6*, *axr3-1/iaa17-1*, and *iaa5-1 iaa6-1 iaa19-1*. Data represent the average relative expression intensity of control (open bars) or auxin-treated (gray bars) samples from triplicate experiments. The asterisks indicate genes whose expression is statistically different in wild-type (Col) and *axr3-1/iaa17-1* seedlings. Bars represent SD.

expression profiles more closely correlate to those of the inactivated genes. An analysis of available microarray databases using the GENEVESTIGATOR program (http://www.genevestigator. ethz.ch; Zimmermann et al., 2004) shows that (1) each *Aux/IAA* gene is widely expressed during plant development, (2) each tissue expresses different sets of multiple *Aux/IAA* genes, and (3) each member has distinct expression profiles in terms of expression levels and specificity. Furthermore, cell type–specific microarray analysis reveals that each *Aux/IAA* member has different expression profiles in various types of root cells (Birnbaum et al., 2003; Weijers and Jurgens, 2004). For example, although *IAA5*, *IAA6*, and *IAA19* share sequence similarity, their expression patterns show distinct tissue specificity. An alternative analysis using another database (http://www.atted.bio. titech.ac.jp/) also indicates that none of the three IAA genes (*IAA5*, *IAA6*, and *IAA19*) exhibit significant correlative expression profiles with each other, suggesting that these genes are not involved in the same biological pathways. These findings are consistent with the distinct type of defects caused by the gain-of-function mutations *iaa6/shy1* and *iaa19/msg2* during plant development (Reed, 2001; Liscum and Reed, 2002; Tatematsu et al., 2004). On the other hand, although IAA1 and IAA19 are relatively divergent members among the Aux/IAA family members, the phenotype of *iaa19/msg2* is partially overlapping with that of *iaa1/axr5* (Yang et al., 2004). Interestingly, the tissue-specific expression of *IAA19* is partially but significantly correlated with that of *IAA1* (http://www.atted.bio.titech.ac.jp/). It appears that the level and pattern of expression as well as the sequence similarity affect the phenotypes caused by the gain-of-function mutation of each *Aux/IAA* gene. The generation of double or higher-order loss-of-function mutants with family members whose expression pattern is similar may yield loss-of-function phenotypes.

While the results presented may be viewed as disappointing, they reveal important information about the function of these important proteins. Overlapping functions among gene family members have been recently unmasked in yeast from studies of the oxysterol binding protein (OSH) gene family (Beh et al., 2001). The *Saccharomyces cerevisiae* genome encodes seven *OSH* genes; a combination of genetics, genomics, and sterol lipid analysis was used to characterize *OSH* deletion mutants. All 127 combinations and permutations of OSH deletion alleles were constructed, and the results show that the loss of individual *OSH* genes does not alter growth (Beh et al., 2001). However, elimination of the entire *OSH* gene family is lethal (Beh et al., 2001). The findings suggest that each *OSH* gene performs a multitude of nonessential functions and contributes to a common essential function. The possibility exists that each member of the *Aux/IAA* gene family performs a plethora of nonessential functions, while contributing to one essential function by analogy with
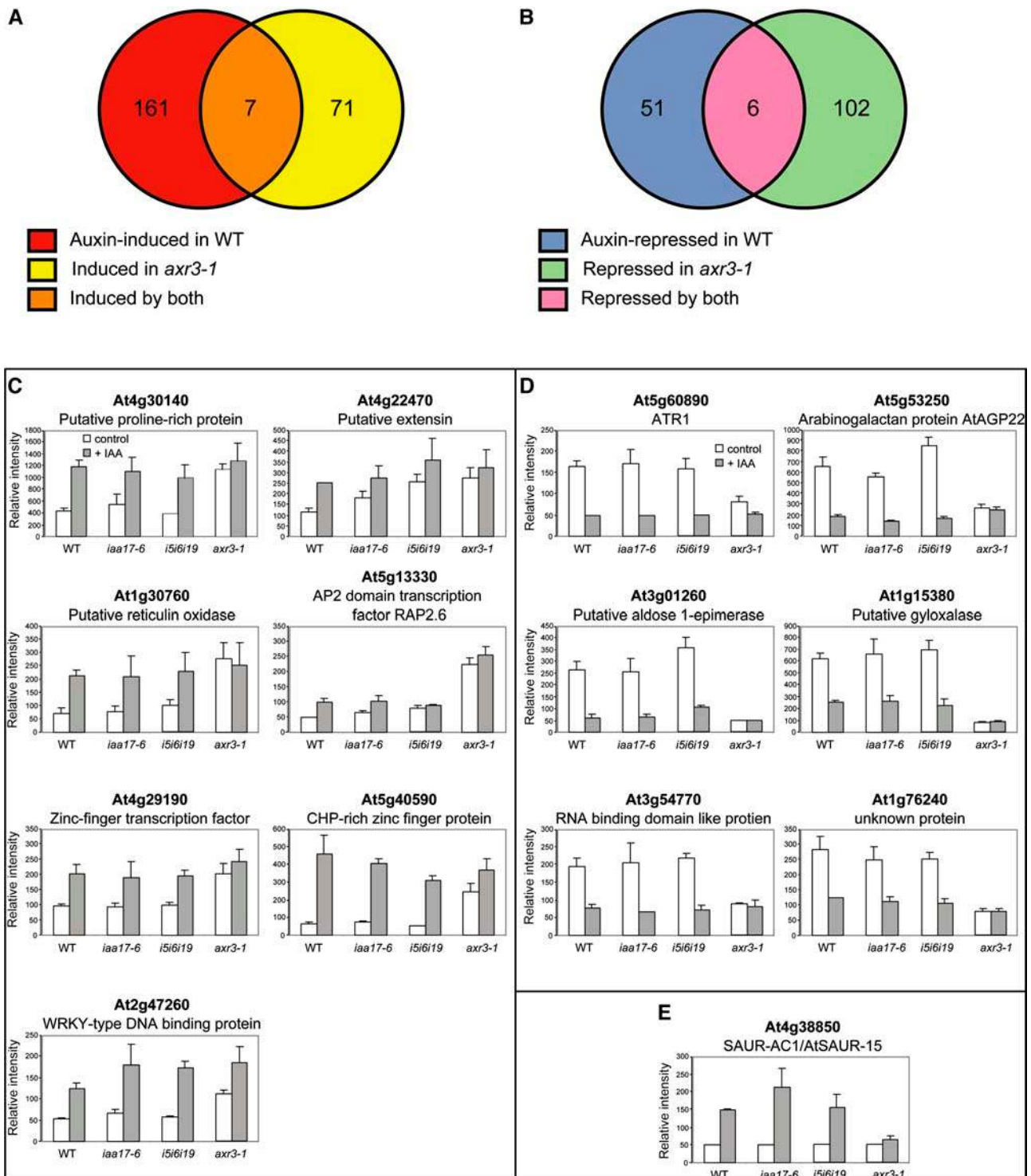
Figure 9. Comparision of the Auxin-Regulated Genes in the Wild Type with the Genes Whose Expression Is Altered in *axr3-1/iaa17-1* Seedlings.

(A) Venn diagram showing the number of overlapping and unique genes that are auxin induced in wild-type seedlings and whose expression is enhanced in control-treated *axr3-1/iaa17-1* seedlings.
(B) Venn diagram showing the number of overlapping and unique genes that are downregulated by auxin treatment of wild-type seedlings and downregulated in control-treated *axr3-1/iaa17-1* seedlings.

the yeast *OSH* gene family members. Since the OSH experiment cannot be duplicated for the *Arabidopsis Aux/IAA* family for obvious reasons, the question arises as to how to determine the function of each *Aux/IAA* gene. In the short term, the use of RNA interference technology to inactivate a large set of genes that share a common DNA sequence has the potential to provide useful information. In the long term, the development of gene transplacement in a cell- and tissue-specific manner in *Arabidopsis* has the potential to provide the answers to this difficult biological problem (Scherer and Davis, 1979).

Similar functional analysis with the *ARF* gene family members has also revealed functional redundancy among the *ARF* gene family members but not as severe as with that observed with the *Aux/IAA* family (Okushima et al., 2005). A number of single loss-of-function mutants have been isolated during the past 10 years, such as *arf2/hss*, *arf3/ettin*, *arf5/mp*, and *arf7/nonphototropic hypocotyl4/msg1*, which result in distinct developmental defects (Sessions et al., 1997; Hardtke and Berleth, 1998; Harper et al., 2000; Okushima et al., 2005). In addition, ARF double mutants have strong and visible phenotypes (Hardtke et al., 2004; Ellis et al., 2005; Okushima et al., 2005; Wang et al., 2005; Weijers et al., 2005; Wilmoth et al., 2005; H. Quach and A. Theologis, unpublished data). The higher degree of biological functional specificity among the ARF proteins may be due to their DNA binding specificity and tissue-specific expression. The Aux/IAAs, on the other hand, act as repressors of ARF function (Tiwari et al., 2001, 2003) and may be more promiscuous in order to tightly regulate ARF funtion.

The *axr3/iaa17-3* Mutation and Its Effects on Global Gene Expression

The *axr3-1/iaa17-1* mutation is a gain-of-function mutation that has pleiotropic effects on *Arabidopsis* growth and development (Leyser et al., 1996). Biochemical evidence indicates that the mutation increases the stability of the encoded protein (Ouellet et al., 2001). Based on the current model of auxin action, the stabilized protein acts as repressor of ARF activity. Protoplast transfection assays have shown that the overexpression of AXR3/IAA17 attenuates the auxin-dependent expression from a promoter that contains *AuxREs* because the protein acts as a repressor of ARF function (Tiwari et al., 2001, 2003). The axr3-1/iaa17-1 protein completely eliminates auxin-induced reporter gene expression (Tiwari et al., 2001, 2003). Consistent with this model, we found that the expression of 114 auxin-regulated genes is impaired in *axr3-1/iaa17-1* (Table 1). Similarly, *shy2-2/iaa3* affects auxin-regulated gene expression (Tian et al., 2002).

Since different ARF proteins can activate or repress transcription, repressing their activity will result in altered auxin-regulated gene expression, thus leading to dramatic changes in growth and development in the *axr3-1/iaa17-1* plants.

The microarray data also reveal that although the auxin-mediated regulation of many auxin-induced and repressed genes is impaired in *axr3-1/iaa17-1*, the basal expression level of most of these auxin-regulated genes is similar in both the wild type and the *axr3-1/iaa17-1* mutant (Figure 9). These findings are similar to the seemingly paradoxical observations made by Leyser et al. (1996) when this mutant was first characterized, as having reduced sensitivity to exogenous auxin as well as enhanced auxin-related phenotypes (such as strong apical dominance). Similarly, other gain-of-function mutants in *Aux/IAA* genes, such as *slr1-1/iaa14*, show phenotypes that reflect a reduced auxin response (e.g., no lateral roots) as well as enhanced auxin response (e.g., strong apical dominance; Fukaki et al., 2002). It appears that stabilized Aux/IAA proteins have the capacity to exert opposite effects on developmental events that are mediated by auxin.

The *axr3-1/iaa17-1* mutation also affects the expression of a large number of non-auxin-regulated genes with diverse functions (Table 2). Among this class of genes, a large percentage encodes proteins involved in cell wall biosynthesis and degradation. Plant cell walls contain several classes of structural proteins, including extensins, PRPs, Gly-rich proteins, and AGPs (Showalter, 1993). These proteins form the proteoglycan backbone of the extracellular matrix. In addition, the plant cell wall contains many proteins that are involved in mediating cell elongation, including XTHs, PMEs, and expansins (Showalter, 1993). These cell wall structural proteins and enzymes are thought to be essential for cell elongation. For example, XTHs are involved in cell elongation by hydrolyzing the cell wall component, xyloglucan, and subsequent loosening of the cell wall (Rose et al., 2002). Among the seven *XTH* genes that were suppressed in the *axr3-1/iaa17-1* mutant, *At-XTH17* is expressed in the elongation and differentiating regions of the roots, and *At-XTH20* is specifically expressed in the basal mature region of the roots (Vissenberg et al., 2005). Repression of these two *XTH* genes may be responsible for root growth inhibition in the *axr3-1/iaa17-1* mutant. Alterations in cell wall expansion may account for the pleiotropic morphological phenotypes that include small and curled leaves, short inflorescence, and reduced root length observed in the mutant (Leyser et al., 1996). Furthermore, several POX genes are differentially expressed in the *axr3-1/iaa17-1* mutant. Plant POXs appear to be involved in the event associated with cell wall construction, such as lignification and cross-linking of cell wall proteins, as well as auxin catabolism and

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Figure 9. (continued).

(C) Expression profiles of the seven overlapping genes whose expression is auxin induced in wild-type seedlings and enhanced in control *axr3-1/iaa17-1* seedlings shown in **(A)**. Data represent the average relative expression intensity of control (open bars) or auxin-treated (gray bars) seedlings from triplicate experiments. Bars represent SD.

(D) Expression profiles of the six overlapping genes whose expression is downregulated in wild-type seedlings and downregulated in control-treated *axr3-1/iaa17-1* seedlings shown in **(B)**.

(E) Expression characteristics of the *SAUR-AC1* gene.

stress response (Hiraga et al., 2001). Altered expression levels of several *POX* genes might also be associated with growth inhibition in the *axr3-1/iaa17-1* mutant.

In addition to the altered expression of cell wall–associated proteins, the *axr3-1/iaa17-1* seedlings show altered expression of genes that encode components of the cytokinin signal transduction pathway. Type A *ARRs* are rapidly and specifically induced by cytokinin treatment and are known as primary response genes for cytokinins (Brandstatter and Kieber, 1998; Imamura et al., 1998; D'Agostino et al., 2000). Since the expression levels of several type A *ARRs*, namely, *ARR3*, *ARR5*, *ARR6*, and *ARR7*, are suppressed in the *axr3-1/iaa17-1* seedlings, it is likely that the cytokinin response or level of endogenous active cytokinin content is suppressed in the *axr3-1/iaa17-1* mutant. The phenotypes of *axr3-1/iaa17-1* can be partially rescued by cytokinin treatment (Leyser et al., 1996), and roots of *axr3-1/iaa17-1* seedlings exhibit an altered response to exogenous cytokinin application as well as severe auxin resistance (Leyser et al., 1996). Although we have not determined the endogenous cytokinin content, the lower expression levels of type A *ARR* genes suggest that at least a downstream cytokinin signaling pathway(s) is impaired in the mutant. Because the *axr3-1/iaa17-1* mutant has similar levels of free and conjugated IAA as the wild type (Leyser et al., 1996), it is likely that the normal ratio of auxin to cytokinin is impaired in this mutant, resulting in the severe morphological phenotypes associated with it (Leyser et al., 1996). It is of a great interest that inactivation of the cytokinin receptors enhances cytokinin content in *Arabidopsis* (T. Schmülling, personal communication). This suggests that the cytokinin sensing pathway communicates with the biosynthetic pathway. We also examined the expression profiles of the genes that encode enzymes involved in cytokinin biosynthesis and catabolism (e.g., isopentenyl transferases and cytokinin oxidases) in the *axr3-1/iaa17-1* mutant, but they were not significantly different from those of the wild type (data not shown). AXR3/IAA17 may be involved in the crosstalk between cytokinin and auxin signaling (Swarup et al., 2002), and disrupting this communication results in the morphological and molecular phenotypes of the *axr3-1/iaa17-1* mutant.

The genes discussed above in the gain-of-function mutant seemingly have been classified as non-auxin-regulated genes because their expression is not regulated by auxin in the wild-type control 7-d-old light-grown seedlings. Their expression may be unmasked, however, in the mutant seedlings because of the ectopic expression of the axr3-1/iaa17-1 protein, resulting in activation of expression of genes that are not regulated by auxin in the wild type at the particular developmental stage investigated in this study. The possibility exists that these genes are indeed auxin regulated in developmental stages other than the stage investigated in this study, or the axr3-1/iaa17-1 protein may have effects on other pathways and systems not mediated via the TIR1/AFB-Aux/IAA-ARF system (e.g., associating with other proteins affecting their function). The new equilibrium of gene expression established in the *axr3-1/iaa17-1* background indicates that the Aux/IAA proteins may, in addition to serving as targets for TIR1/AFB receptors, mediate additional levels of gene expression, suggesting a broader role for these proteins in auxin signaling.

METHODS

Materials

All chemicals used for this study were ACS reagent grade or molecular biology grade. Oligonucleotides were purchased from Operon Technologies or synthesized in house with a Polyplex Oligonucleotide Synthesizer (GeneMachines).

Molecular Biology

Standard protocols were followed for DNA manipulations described by Sambrook et al. (1989). Standard protocols for DNA sequencing were used to confirm the accuracy of the DNA constructs.

Plant Growth Conditions

Arabidopsis thaliana ecotype Col was used throughout this study. Seeds were surface sterilized for 8 min in 5% sodium hypochlorite + 0.15% Tween-20, excessively rinsed in distilled water, and plated on 0.8% agar plates containing $0.5\times$ Murashige and Skoog salts (Murashige and Skoog, 1962; Life Technologies) + 0.5 mM MES, pH 5.7, + 1% sucrose + $1\times$ vitamin B5. The plates were incubated in the dark at 4°C for 2 d and were subsequently transferred to a 16-h-light/8-h-dark cycle at 22°C for light-grown seedlings or in the dark for etiolated seedlings. Mature plants were also grown under the light conditions mentioned above. The root auxin sensitivity assay was performed as follows: 4-d-old light-grown seedlings were transferred to vertically oriented agar plates containing appropriate concentrations of IAA. The root length was determined after an additional 5 d of growth. The root length was determined using the NIH Image 1.63 program (http://rsb.info.nih.gov/nih-image/download.html).

Phylogenetic Analysis

An unrooted dendogram was generated using ClustalW (Thompson et al., 1994). TreeView was used to generate the graphical output (Page, 1996). The numbers at the branching points (see Figure 1) indicate the percentage of times that each branch topology was found during bootstrap analysis ($n = 1000$).

Identification and Characterization of T-DNA Insertion Alleles

Screening for T-DNA Insertions

The identification of insertional mutants utilizes a PCR-based screen. For each gene, a forward primer annealing to 100 to 150 bp 5′ of the ATG and a reverse primer annealing to 100 to 150 bp 3′ of the translation stop codon were designed (see Supplemental Table 10 online). PCR amplification (35 cycles: 94°C for 15 s, 56°C for 15 s, and 72°C for 3 min) was performed, and the resulting PCR products were subjected to DNA gel blot analysis using radiolabeled *Aux/IAA* cDNA fragments as probes. The size of the genomic products ranged from 6 to 3.2 kb. Eight sets of DNA template derived from 10,000 plants each (80,000 lines total) were screened. Each set of template contained 40 tubes of DNA (10 each of DNA combined from column, row, plate, and individual superpools). Identification of an individual requires a PCR product in each of the four superpools. Using all combinations of forward and reverse primers with primers annealing to the left border and right border of the T-DNA, PCRs were run ($4 \times 40 \times 8 = 1280$ reactions per gene). All operations were adapted to a 384-well format, and handling of samples was performed with a BioMek robot. The products were analyzed by DNA gel blotting to allow increased sensitivity of detection and to assess the specificity of screening. Subsequent to this screen, two large databases containing sequence of DNA flanking T-DNA inserts in 100,000 and 20,000 independent lines have been screened in silico. Data for the 100,000 lines were

generated in a collaboration of the University of California at Berkeley with the Torrey Mesa Research Institute, and the 20,000 lines have been obtained by SIGNAL (http://signal.salk.edu/cgi-bin/tdnaexpress).

Confirmation of T-DNA Lines

The nature and location of the T-DNA insertion was confirmed by sequencing PCR products. Once the location of the T-DNA insertion was confirmed, we designed gene-specific PCR primers that flank the T-DNA for use in a codominant genotyping analysis. By performing two sets of PCR, one using the gene-specific primer pair and the other using a gene-specific primer and the T-DNA border primer, we could determine whether the individual is homozygous for no T-DNA insertion, heterozygous for the T-DNA insertion, or homozygous for the T-DNA insertion.

Molecular Characterization of the T-DNA Lines

To determine the number of T-DNA inserts present in the lines, we compared the DNA gel blot hybridization patterns arising from sibling plants that were either homozygous for the T-DNA insertion or homozygous for no T-DNA. To remove additional T-DNA loci from the lines of interest, backcrosses to wild-type Col were performed and plants homozygous for the T-DNA insertion were again identified.

RNA Preparation and Microarray Hybridization

Surface-sterile seeds (1.8 mg) were germinated in 40 mL of liquid Murashige and Skoog medium containing 1.5% sucrose and cultured in a 16-h-light/8-h-dark cycle with gentle shaking (100 rpm). After a 7-d culture period, the seedlings were treated with 5 μM IAA (IAA treated) or ethanol (control) for 2 h. Total RNA was prepared using the RNAqueous RNA isolation kit with plant RNA isolation aid (Ambion). After LiCl precipitation, RNA was purified using RNeasy columns (Qiagen) and reprecipitated with LiCl. RNA pellets were washed with 70% ethanol (three times) and resuspended in diethyl pyrocarbonate–treated water. Five micrograms of total RNA was used for biotin-labeled cRNA probe synthesis. cRNA probe synthesis, hybridization, and washing and scanning and detection of array images were performed according to the manufacturer's protocols (Affymetrix). Twenty-four independent hybridization experiments (three independent biological replicates for eight different samples [two treatments × four genotypes]) were performed in this study.

Microarray Data Analysis

Affymetrix GeneChip Microarray Suite version 5.0 software was used to obtain signal values for individual genes. The cell intensity files (CEL files) were used for background correction and normalization using the $\log_2$ scale RMA procedure (Irizarry et al., 2003). The R environment (Ihaka and Gentleman, 1996) was used for running the RMA program. Data analysis and statistical extraction were performed using $\log_2$-converted expression intensity data within Microsoft Excel 98. Based on preliminary analysis, a hybridization signal <5.64 (= $\log_2$ 50) was considered as background; all signals <5.64 were converted to 5.64 prior to further analysis. The entire data set is provided in the supplemental data online and has been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE629, GSM9595 to GSM9603, and GSM9605 to GSM9619.

We used an *M-A* plot (Dudiot et al., 2002) to display the data in Figure 6, where $M = \log_2 I/C$, where I = mean expression level for a gene in auxin-treated samples and C = mean expression level for the same gene in the control samples and $A = \log_2 \sqrt{(I*C)}$.

To extract the genes differentially expressed between two treatments (auxin-treated/control) or two genotypes (*iaa17-6*/wild type, *axr3-1*/wild

type, or *iaa5-1 iaa6-1 iaa19-1*/wild type), the expression values for each gene were averaged within each treatment/genotype. Then the fold change and the standard two-sample *t* statistic (Dudiot et al., 2002) were computed. A gene was considered differentially expressed if it was at least twofold upregulated or downregulated and the *P* value for its *t* statistic was <0.001. This strict criteria limits the type I family-wise error rate and allowed us to consider genes that demonstrated highly reliable auxin-mediated modulation. For comparative analysis, Venn diagrams were drawn using GeneSpring software package version 5.1 (Silicon Genetics). The entire data set is provided in the supplemental data online.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: AY669797 (At1g51950, *IAA18*), AY669798 (At3g15540, *IAA19*), AY669799 (At5g25890, *IAA28*), AY669800 (At4g32280, *IAA29*), AY669801 (At3g62100, *IAA30*), AY669802 (At3g17600, *IAA31*), AY669803 (At2g01200, *IAA32*), and AY669804 (At5g57420, *IAA33*). The microarray data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE629, GSM9595 to GSM9603, and GSM9605 to GSM9619.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Predicted Molecular Mass, Number of Amino Acids, and Isoelectric Points of the *IAA* Gene Family Members.

Supplemental Table 2. Amino Acid Sequence Comparison of the *Arabidopsis* IAA Proteins.

Supplemental Table 3. Summary of *aux/iaa* T-DNA Insertion Mutants Isolated in This Study.

Supplemental Table 4. List of Auxin-Induced Genes.

Supplemental Table 5. List of Auxin-Repressed Genes.

Supplemental Table 6. List of Genes with Reduced Induction in the *axr3-1/iaa17-1* Mutant (among Auxin-Induced Genes).

Supplemental Table 7. List of Genes with Reduced Repression in the *axr3-1/iaa17-1* Mutant (among Auxin-Repressed Genes).

Supplemental Table 8. List of Genes Repressed in the *axr3-1/iaa17-1* Mutant.

Supplemental Table 9. List of Genes Induced in the *axr3-1/iaa17-1* Mutant.

Supplemental Figure 1. Expression Characteristics of Several *Aux/IAA* Genes in Wild-Type, *iaa17-6*, *axr3-1 iaa17-1*, and *iaa5-1 iaa6-1 iaa19-1* Seedlings.

Supplemental Figure 2. Expression of Peroxidase Genes in Wild-Type, *iaa17-6*, *axr3-1 iaa17-1*, and *iaa5-1 iaa6-1 iaa19-1* Seedlings.

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