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RESEARCH PAPER

Genome-wide analysis of the auxin-responsive transcriptome downstream of iaa1 and its expression analysis reveal the diversity and complexity of auxin-regulated gene expression

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Abstract

The AUXIN RESPONSE FACTORs (ARFs) and the Aux/IAA proteins regulate various auxin responses through auxin perception mediated by the F-box proteins TIR1/AFBs. ARFs are transcription factors that modulate expression of auxin response genes and are negatively regulated by the Aux/IAA proteins. To gain insight into the regulatory mechanisms of Aux/IAA-ARF action at the genome level, the transcriptome regulated downstream of iaa1, a stabilized IAA1 mutant protein, was identified using dexamethasone (DEX)-controlled nuclear translocation of iaa1 during the auxin response. The expression of the iaa1-regulated auxin-responsive genes selected from microarray data was analysed with RNA-gel blot analysis and it was shown that auxin-regulated expression of these genes was significantly inhibited by DEX treatment. While cycloheximide-inducible expression of a majority of these genes was also DEX-suppressible, expression of some genes could not be suppressed by treatment with DEX. Expression analysis in a variety of *arf* mutant backgrounds suggested that all iaa1-regulated auxin-response genes examined are controlled by ARFs to different extents and that the same ARF protein can regulate the expression of these genes in response to auxin in a positive or a negative manner. However, *arf* mutations did not affect auxin-mediated down-regulation, indicating that ARFs might not play a critical role in down-regulation. The decrease in auxin-responsive gene expression in *arf7 arf19* mutants was more severe than that of *tir1/afb* quadruple mutants. These results show the diversity and complexity of mechanisms of Aux/IAA-ARF- and auxin-regulated gene expression. These data also provide the opportunity for functional analysis of genes mediating the auxin-response downstream of Aux/IAA-ARFs.

Key words: ARF, Aux/IAA, auxin, auxin response factor, cycloheximide, dexamethasone, gene expression, IAA1, microarray, TIR/AFB.

Introduction

The plant hormone auxin influences almost every aspect of plant growth and development (Davies, 2004). Numerous genetic and biochemical studies have shown that various auxin responses are regulated by two large protein families named the ARF and Aux/IAA proteins (Berleth *et al.*, 2004; Parry and Estelle, 2006). In *Arabidopsis*, there are 23 ARF proteins. Each ARF contains a conserved DNA-binding domain near their N-terminus, a long middle region, and, in most ARFs, a dimerization domain near the C-terminus (Quint and Gray, 2006). The DNA-binding domain of the ARF proteins binds to the conserved auxin-responsive promoter element and, depending on the structure of the middle region, each ARF functions as a transcriptional activator or repressor. ARF5, ARF6, ARF7, and ARF8 contain a Gln-rich middle region and function as transcriptional activators in transient protoplast transfection assays. By contrast, ARF1, ARF2, ARF3, ARF4, and ARF9 contain a proline- and/or serine-rich

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region, or lack a Gln-rich region, and act as transcriptional repressors (Ulmasov *et al.*, 1999*a*; Tiwari *et al.*, 2003).

Different ARFs are involved in various developmental processes ranging from embryogenesis to floral development (Parry and Estelle, 2006). Although many single *arf* mutants lack obvious phenotypes, which is indicative of the functional overlap among the various family members, some *arf* single mutants display overt phenotypes. For example, *ARF2* is implicated in regulating leaf senescence and floral organ abscission (Ellis *et al.*, 2005; Okushima *et al.*, 2005*b*). An *mnt* mutant with dramatically increased seed size and weight was identified as an allele of *ARF2* (Schruff *et al.*, 2006). Mutations in *ARF2* have also been identified as extragenic suppressors of *hookless1* (*hls1*), suggesting that *ARF2* may be regulated downstream of ethylene-regulated *HLS1* (Li *et al.*, 2004). *ARF3*/*ETTIN* is involved in floral organ and gynoecium development (Sessions and Zambryski, 1995; Sessions *et al.*, 1997). *Trans*-acting siRNAs mediate regulation of *ARF3* gene function during developmental timing and patterning (Fahlgren *et al.*, 2006). *ARF5*/*MONOPTEROS* (*MP*) is involved in vascular strand formation and the initiation of the embryo axis (Hardtke and Berleth, 1998). Loss of function mutations in *ARF7*/*NONPHOTOTROPIC HYPOCOTYL4*(*NPH4*)/*MASSUGU1*(*MSG1*)/*TRANSPORT INHIBITOR RESPONSE5*(*TIR5*) resulted in impaired hypocotyl phototropism to blue light (Harper *et al.*, 2000). A mutation in *ARF8* causes parthenocarpic fruit (Goetz *et al.*, 2006). *ARF8* functions as a negative regulator of fruit initiation and is also involved in hypocotyl elongation (Tian *et al.*, 2004; Goetz *et al.*, 2006). MicroRNA (miRNA) 160 targets *ARF10*, *ARF16*, and *ARF17* and miR167 targets *ARF6* and *ARF8* (Rhoades *et al.*, 2002). Plants expressing a miRNA-resistant version of *ARF17* exhibited various developmental defects, demonstrating the importance of miR160-directed *ARF17* regulation (Mallory *et al.*, 2005). Furthermore, *arf* double mutants exhibited enhanced phenotypes or phenotypes that single mutants did not display, suggesting that there are unique and overlapping functions among related ARF family members. For example, although the *arf1* single mutation alone did not confer any phenotype by itself, *arf1* mutations enhanced many *arf2* pleiotropic phenotypes in *arf1 arf2* double mutants, indicating common functions (Ellis *et al.*, 2005). *ARF5*/*MP* and *ARF7*/*NPH4* function redundantly during oriented cell differentiation and synergistically in cell expansion (Hardtke *et al.*, 2004). *ARF6* and *ARF8* function redundantly in flower maturation (Nagpal *et al.*, 2005). While *arf6* and *arf8* single mutant plants have delayed stamen development and decreased fecundity, *arf6 arf8* double mutants have a complete block in flower maturation. Mutations in *ARF19* had a little effect on their own, but in *arf7 arf19* double mutants, several phenotypes not observed in the single mutants were found, including a decrease in lateral and adventitious root formation, abnormal gravitropism in both hypocotyls and roots, and a decrease in leaf cell expansion (Wilmoth *et al.*, 2005; Okushima *et al.*, 2005*b*).

The ARF proteins are negatively regulated by the Aux/IAA proteins. In *Arabidopsis*, there are 29 Aux/IAA proteins that contain four conserved domains (Parry and Estelle, 2006). Domain I is responsible for repression (Tiwari *et al.*, 2004), whereas domains III and IV mediate homo- and hetero-dimerization among the Aux/IAA proteins as well as between Aux/IAA and ARFs (Kim *et al.*, 1997; Ulmasov *et al.*, 1997*a*, *b*). The Aux/IAA proteins act as transcriptional repressors by interacting with ARFs through domains III and IV (Tiwari *et al.*, 2001, 2004). Domain II contains a 13-amino acid degron motif that is responsible for the rapid degradation of the Aux/IAA proteins (Worley *et al.*, 2000; Ramos *et al.*, 2001). So far, 13 different loss-of-function *aux*/*iaa* mutants have been characterized and none of them have shown obvious developmental defects. Double and triple mutants of the *Aux*/*IAA* genes, such as *iaa8 iaa9* or *iaa5 iaa6 iaa19*, within the same clade exhibited wild-type phenotypes, indicating extensive genetic redundancy among *Aux*/*IAA* family members (Overvoorde *et al.*, 2005).

Clues as to the biological function of the *Aux*/*IAA* genes all came from analysis of gain-of-function mutants. Ten gain-of-function *aux*/*iaa* mutants were identified including *IAA1*/*AXR5*, *IAA3*/*SHY2*, *IAA6*/*SHY1*, *IAA7*/*AXR2*, *IAA12*/*BDL*, *IAA14*/*SLR*, *IAA17*/*AXR3*, *IAA18*, *IAA19*/*MSG2*, and *IAA28*, each exhibiting reduced auxin-response in various aspects of development and growth (Berleth *et al.*, 2004; Yang *et al.*, 2004). All of the mutations reside in the 13-amino acid degron motif of domain II. These mutations result in a dramatically increased life span and an increased abundance of the affected protein. This, in turn, leads to inhibition of ARF function, conferring auxin-related phenotypes.

The rapid degradation of the Aux/IAA proteins is proteasome-dependent (Ramos *et al.*, 2001; Gray *et al.*, 2001). Auxin promotes degradation of the Aux/IAA proteins by enhancing the interaction between the ubiquitin-ligase SCF^{TIR1} complex and domain II of the Aux/IAA proteins (Gray *et al.*, 2001; Zenser *et al.*, 2001). The F-box protein TIR1 was shown to be an auxin receptor that mediates Aux/IAA degradation and transcriptional responses to auxin (Dharmasiri *et al.*, 2005*a*; Kepinski and Leyser, 2005). Three additional F-box proteins (AFB1, -2, and -3), homologous to TIR1, interact with the Aux/IAA proteins in an auxin-dependent manner (Dharmasiri *et al.*, 2005*b*). Plants deficient in all four proteins are auxin-insensitive, indicating that the TIR1 and AFB proteins collectively mediate auxin responses throughout plant development. Crystal structures showed that auxin binds to a single surface pocket of TIR1 and on top of auxin, the Aux/IAA peptide occupies the rest of the pocket (Tan *et al.*, 2007). A very recent study showed that TOPLESS (TPL) can interact with IAA12 through the ERF-associated amphiphilic repression motif and can repress transcription *in vivo* (Szemenyei *et al.*, 2008). Also, direct interaction between TPL and ARF5 is regulated by IAA12, causing a loss-of-function *arf5* phenotype. These results demonstrate that TPL is a transcriptional co-repressor for Aux/

IAA-ARF-mediated gene regulation during the auxin response.

While there is extensive knowledge on the signal transduction events from auxin perception to gene regulation, little is known about signalling and function of the genes modulated downstream of the Aux/IAA and ARF proteins for mediating the auxin response. We had previously set out to study signalling downstream of the Aux/IAA and ARF proteins using an inducible system that employed the regulatory mechanism of the mammalian glucocorticoid hormone receptor (Park *et al.*, 2002). Transgenic *Arabidopsis* (*Pro$_{35S}$:iaa1:GR*) has been made that expresses the hormone binding domain of glucocorticoid receptor(GR)-fused stabilized-iaa1 proteins harbouring an amino acid change in domain II. Treatment of DEX to this transgenic *Arabidopsis* evoked dramatic auxin-related phenotypes and repressed auxin induction of various *Aux/IAA* genes, indicating that the iaa1 protein impaired auxin responses by acting as a negative regulator for the auxin-response pathway. DEX-inducible translocation of the iaa1 protein to the nucleus can induce auxin-related phenotypes that can be linked to downstream signalling of the Aux/IAA proteins at the time of treatment. The effects of DEX-inducible iaa1 on auxin-regulated gene expression are examined here, focusing on early genes and using the Affymetrix full genome array. The transcriptome downstream of iaa1 was identified in an effort to understand the auxin response pathway at the genome level. These microarray data provide valuable resources for understanding specific auxin responses and/or a subset of these responses. Expression of representative auxin-regulated genes affected by iaa1 has also been analysed in terms of induction kinetics using the RNA-gel blot method. The effects of cycloheximide and auxin with or without DEX in wild-type, *arf*, and *tir1/afb* mutant backgrounds were examined. The results highlight the complexity and diversity of auxin-regulated gene expression, which cannot be explained by the Aux/IAA-ARF system alone, and it also revealed versatile *ARF* functions during auxin-regulated gene expression.

Materials and methods

Plant growth and tissue treatment

Arabidopsis thaliana seedlings were grown for 7 d with a 16 h photoperiod on 3MM Whatman filter paper on top of agar plates containing half-strength Murashige–Skoog (MS) media salts with vitamins, 1.5% sucrose, 2.5 mM MES, pH 5.7, and 0.8% phytoagar at 23 °C. The filter paper with the seedlings was then transferred to plates containing half-strength MS with plant hormone or chemicals (20 μM IAA, 10 μM DEX, or 50 μM cycloheximide) without agar and incubated for a given period of time with gentle shaking in the light at 23 °C.

Plasmid construction and Arabidopsis *transformation*

The promoter region of *IAA1* encompassing 1702 bp (from –1705 to –4 bp relative to the AUG initiation codon) was isolated by PCR from genomic DNA of *Arabidopsis* Col-0, subcloned into pBI101 (Clontech) in place of the CaMV 35S promoter, and transgenic *Arabidopsis* were made containing this construct (*Pro$_{IAA1}$:GUS*) by *Agrobacterium*-mediated transformation (Bechtold *et al.*, 1993). T_3 homozygous transformants were made and amplified. All constructs were verified by DNA sequencing prior to plant transformation.

Histochemical GUS assays

Histochemical assays for GUS activity were performed by incubating the treated seedlings in 5-bromo-4-chloro-3-indolyl glucuronide (Duchefa, The Netherlands) at 37 °C for 24 h and removing the chlorophyll from green tissues by incubation in 100% ethanol, as previously described by Jefferson and Wilson (1991).

Confirmation of Arabidopsis *ARF T-DNA insertion mutants and* tir1/afb *mutants*

Arabidopsis ARF T-DNA insertion mutants from ABRC were verified by PCR with the primers designed by the T-DNA primer design program available from the Salk Institute Genomic Analysis Laboratory (SIGNAL) (http://signal.salk.edu/) (see Supplementary Table S1 at *JXB* online). Triple and quadruple *tir1/afb* mutants were verified by PCR with the primers shown in Supplementary Table S1 at *JXB* online.

RNA isolation, RT-PCR, and RNA-gel blot analysis

Following treatment, *Arabidopsis* plants were immediately frozen in liquid nitrogen and stored at –80 °C. Total RNA was isolated from frozen *Arabidopsis* using the TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA). Total RNA was separated on 1.2% agarose gels, transferred to nylon membranes, and hybridized with ^{32}P-labelled DNA probes at 68 °C for 3 h using 10 ml of QuickHyb solution (Stratagene, La Jolla, CA, USA) and then washed. The blot was then exposed to X-ray film. For RT-PCR analysis, total RNA was isolated using an RNeasy plant mini kit (Qiagen, Hilden, Germany) and subject to RT-PCR analysis with the Access RT-PCR System (Promega, Madison, WI, USA) according to the manufacturer's instruction. The DNA probes for RNA gel-blot analysis were amplified by RT-PCR, subcloned into the pGEM®-T Easy vector (Promega), and verified by DNA sequencing. RT-PCR conditions, primer sequences, and other DNA vector probes are shown in Supplementary Table S1 at *JXB* online.

Microarray analysis

For Affymetrix GeneChip analysis, *Pro$_{35S}$:iaa1:GR* seedlings (Park *et al.*, 2002) were grown for 7 d on half-strength MS media, treated with 20 μM IAA, 20 μM IAA and 10 μM DEX, or mock-treated for 2 h, as described above in 'Plant growth and tissue treatment', and total RNA was isolated with an RNeasy plant mini kit (Qiagen). Five micrograms of RNA were used to make biotin-labelled cRNA products. The

Affymetrix *Arabidopsis* ATH1 genome array GeneChip, which contains >22,500 probe sets representing approximately 20,000 genes, was used. Probe synthesis from total RNA samples, hybridization, detection, and scanning were performed according to standard protocols from Affymetrix, Inc. (Santa Clara, CA, USA). Expression profiles were analysed using the GeneChip Operating Software (Affymetrix, CA, USA). GeneChip Operating Software (Affymetrix) was used to determine the absolute analysis metrics (Detection, Detection *P*-value) using the scanned probe array data and the different treatment group signals were compared to generate the change, change *P*-value, and signal log ratio (fold change). The experimental data from the microarray analysis were normalized by global scaling (Statistical Algorithms Reference Guide, Technical Report, Affymetrix, 2001). Differentially regulated genes in response to auxin were selected based on the criteria: $|\log_2(\text{fold-change})| > 1$ and P-value < 0.05 with Welch's *t*-test (Welch, 1947). The microarray data were analysed using GenPlex™ version 2.6 software (ISTECH, Seoul, Korea). Gene function analysis was performed using the gene ontology mining software, High-Throughput GoMiner (http://discover.nci.nih.gov/gominer/htgm.jsp). Specification of the many gene annotations was also supplemented by further online database searches such as http://www.arabidopsis.org/tools/bulk/go/index.jsp. Microarray data were deposited into ArrayExpress with the accession number E-MEXP-1256 at http://www.ebi.ac.uk/at-miamexpress.

Results

GUS expression of Pro_{IAA1}:GUS

To get an insight into the physiological and functional relevance of iaa1 in auxin-related phenotypes, including gene regulation, it was determined precisely where *IAA1* is expressed in *Arabidopsis*. The 1.7 kbp promoter region of the *IAA1* gene was fused to *GUS* and five independent homozygous transgenic *Arabidopsis* lines containing the corresponding construct (*Pro_{IAA1}:GUS*) were generated. As shown in representative histochemical analysis of GUS expression (Fig. 1A), strong GUS staining was detected in various tissues including primary roots, lateral roots, petioles, veins, hydathodes, and hypocotyls of 7-d-old seedlings before auxin treatment. The treatment with auxin, indole-3 acetic acid, greatly enhanced the degree of GUS staining throughout the whole seedling (Fig. 1B). Similar expression patterns of GUS staining were observed in 14-d-old plants (data not shown). GUS expression was also observed in the flower of the mature plants (G-I), particularly in the stigma, ovary, filament, and petal. These GUS expression patterns of the *IAA1* promoter suggest a role for *IAA1* in a variety of tissues during auxin response at early developmental stages.

Identification of early auxin-regulated genes affected by iaa1 using microarrays

To identify the transcriptome downstream of iaa1, genome-wide analysis was performed on the effects of the DEX-inducible nuclear localization of iaa1 on auxin-regulated gene expression using the Affymetrix ATH1 *Arabidopsis* full genome array. A 2 h time point was selected following auxin treatment to investigate the early auxin response. For microarray analysis, *Arabidopsis* transgenic plants (*Pro_{35S}:iaa1:GR*) were used that overexpressed the iaa1 mutant protein fused to GR under the CaMV 35S promoter (Park *et al.*, 2002). *Pro_{35S}:iaa1:GR* seedlings were incubated with mock, IAA, or IAA and DEX. Total RNA was then isolated and the validity of auxin-regulated gene expression was verified by RNA-gel blot analysis using an *IAA2* DNA probe (data not shown). This experiment and microarray analysis were performed in triplicate and genes were extracted that satisfied the criteria of both a >2-fold difference on average between auxin- and mock-treated plants and $P < 0.05$ using Welch's *t* test (Welch, 1947) as a cutoff value (Tables 1, 2). Further, the genes that express the transcripts showing absent calls (A) in all arrays were eliminated. In the case of down-regulated genes, only the genes that express the transcripts showing present calls (P) before auxin treatment in all three hybridizations were included. It has previously been demonstrated that the treatment with DEX for 2 h on wild-type plants and wild-type *IAA1:GR* plants did not affect expression levels of the *IAA* genes tested (Park *et al.*, 2002). Also, no phenotypic change in wild-type plants or in wild-type *IAA1:GR* plants when treated with DEX was observed.

To gain an insight into the function of the auxin-regulated genes displaying both >2-fold differences, on average, between auxin- and mock-treated plants and $P < 0.05$, these genes were classified into 12 functional groups using Gene Ontology (GO) annotation with some manual modifications (Tables 1, 2). It was found that 148 genes were up-regulated and 59 genes were down-regulated more than 2-fold following treatment with auxin and many of these auxin-regulated genes were negatively affected by DEX treatment. Almost all of the *Aux*/*IAA* genes, except for a few *Aux*/*IAA* members due to late auxin-induced gene expression, were induced by auxin treatment and this auxin-induction was reduced by DEX treatment in triplicate experiments (Table 1; Fig. 2A). These data were similar to the previously reported RNA-gel blot data (Park *et al.*, 2002), confirming validity of our microarray analysis. In addition, a 2 kbp promoter region upstream of the start codon was analysed for the auxin response elements (AuxREs), TGTCnC or GnGACA sequence (Ulmasov *et al.*, 1995, 1997*a*, *b*). It was found that most of the auxin-regulated genes affected by iaa1 have the AuxREs in the 2 kbp region (Tables 1, 2).

Genes encoding a number of auxin-responsive proteins and GH3 are also up-regulated by auxin (Fig. 2B). Development-related genes including *lateral organ boundaries domain16* (*LBD16*), *LBD18*, *LBD29*, and *LBD33*, and *PIN1* are up-regulated. *ACC synthase* (*ACS*)*4, ACS6, ACS8, ACS9,* and *ACS11* and *ethylene response regulator* (*ERS*)*2* as well as *cytokinin oxidase* (*CKX*)*7* are up-regulated (Table 1; Fig. 2C). Many genes involved in signal transduction and transcriptional control are also

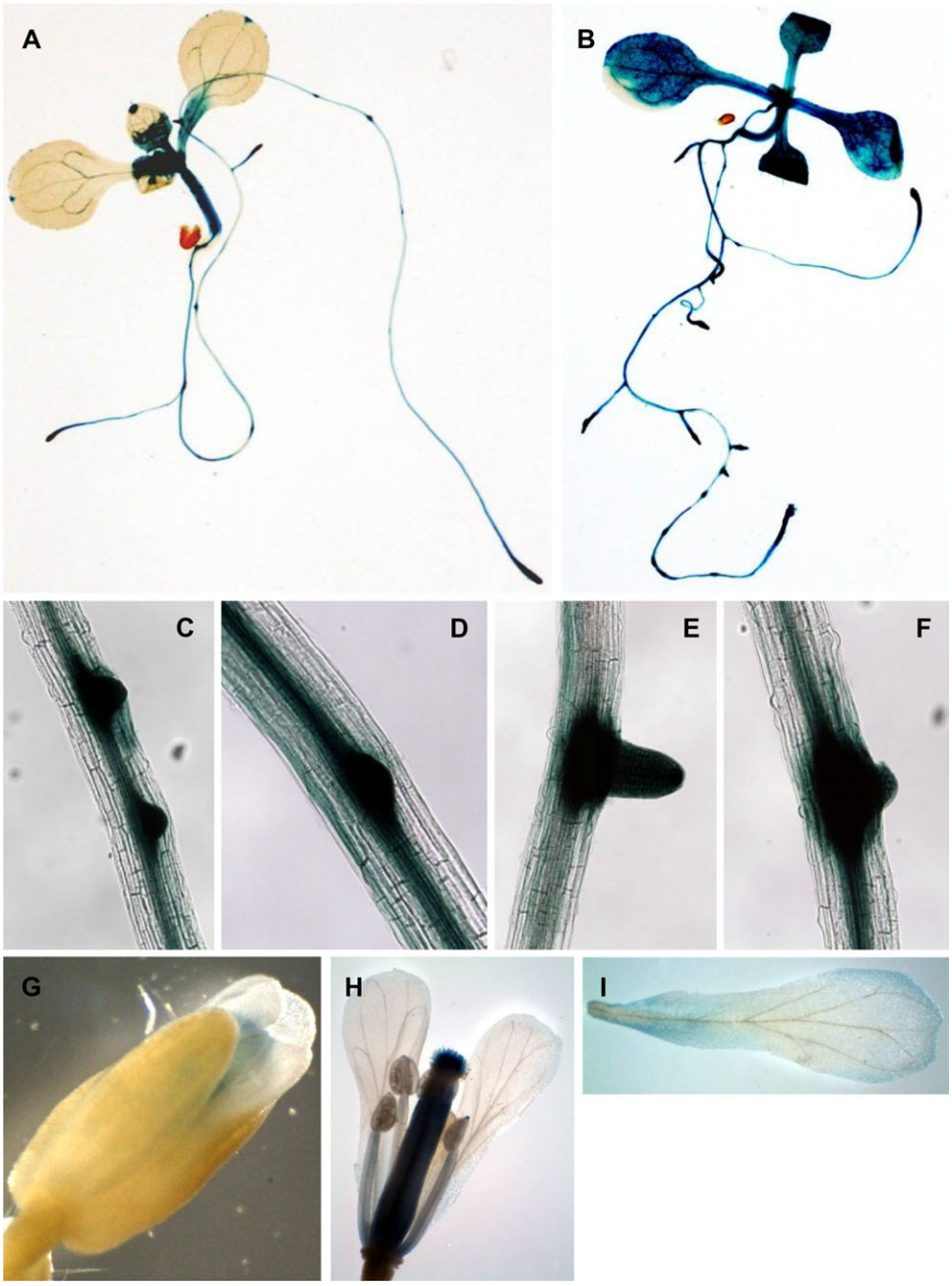

Fig. 1. Expression of GUS in Pro_{IAA1}:*GUS* transgenic plants. (A, B) Seven-day-old light-grown seedling without (A) or with 20 μM of IAA (B) for 2 h. (C–F) Emerging (C, D) or emerged (E, F) lateral roots of 7-d-old light grown seedlings without (C, E) or with (D, F) IAA treatment for 2 h. (G, H) Flower of 7-week-old light-grown seedling. (I) Petal of 7-week-old light-grown seedling.

up-regulated by auxin. A variety of transcription factors are up-regulated, including bHLH proteins, AP2 domain-containing proteins, homeobox leucine-zipper proteins, zinc-finger proteins (ZFPs), MYB108, ethylene response factor (ERF)11, and ethylene-responsive element binding protein (EREBP). A large number of genes encoding metabolic enzymes and unknown proteins are also up-regulated. A number of genes encoding metabolic enzymes and unknown proteins are down-regulated by auxin (Table 2). In particular, the genes encoding various cytochrome P450 proteins are down-regulated. ROTUNDIFOLIA(ROT)3, which catalyses the conversion of teasterone to castasterone in the brassinosteroids (BRs) biosynthetic pathway, is down-regulated, whereas phyB activation-tagged suppressor1-dominant (BAS)1, which inactivates brassinolide by oxidation, is up-regulated by auxin. The type-A response regulator genes such as *Arabidopsis response regulator* (*ARR*)*4*, *ARR5*, *ARR6*, *ARR7*, and *Arabidopsis histidine kinase* (*AHK*)*4* (the components of the cytokinin two-component signalling system) are co-ordinately down-regulated. Simultaneous treatment of auxin and DEX suppressed the auxin-inducible expression

Table 1. List of genes up-regulated 2 h after treatment with IAA

Category	At no.	Probe ID	Gene title	IAA		IAA + Dex		Impaired in *arf* mutants[e]	AuxRE	
				FC[a]	*P*-value[b]	FC[c]	*P*-value[d]		A[f]	B[g]
Auxin-regulated	At1g15580	261766_at	IAA5 (Aux2-27)	92.3	0.002	21.9	0.031	√	1	0
	At1g29490	259785_at	Auxin-responsive protein	69.2	0.017	17.9	0.260	√	0	0
	At2g23170	245076_at	GH3.3	60.4	0.008	22.5	4.8E-04	√	3	2
	At4g37390	262099_s_at	GH3.2 (YDK1)	39.2	0.015	4.3	2.2E-05		2	3
	At2g14960	266611_at	GH3.1	31.5	7.2E-05	8.9	1.1E-03	√	3	0
	At3g15540	258399_at	IAA19	25.7	0.007	10.9	0.024	√	6	1
	At4g32280	253423_at	IAA29	22.0	0.039	4.4	1.2E-03	√	2	0
	At4g27260	253908_at	GH3.5	14.4	0.011	5.5	1.4E-03	√	2	1
	At1g52830	260152_at	IAA6	11.6	0.031	4.9	0.302	√	1	0
	At3g62100	251246_at	IAA30	10.8	0.006	6.0	0.058	√	2	0
	At2g18010	265806_at	Auxin-responsive protein	8.6	0.043	3.5	0.247	√	0	2
	At4g22620	254323_at	Auxin-responsive protein	8.3	0.002	2.9	0.002	√	1	4
	At5g54510	248163_at	Dwarf in light 1 (DFL-1, GH3.6)	8.0	0.002	4.5	0.015	√	2	1
	At4g12410	254809_at	Auxin-responsive protein	6.0	0.015	2.3	0.033	√	1	0
	At3g23030	257766_at	IAA2	5.5	0.008	1.9	0.037	√	2	1
	At4g36110	253103_at	Auxin-responsive protein	4.5	0.033	1.8	0.091	√	1	2
	At1g29500	259773_at	Auxin-responsive protein	4.3	0.005	1.3	0.033		4	0
	At2g33310	255788_at	IAA13	4.1	0.022	2.4	0.038	√	1	0
	At5g20820	246000_at	Auxin-responsive protein-related	3.9	0.004	2.1	0.064	√	0	0
	At5g43700	249109_at	IAA4 (Aux2-11)	3.5	0.003	1.9	0.010	√	2	4
	At4g28640	253791_at	IAA11	3.3	0.003	1.5	0.012	√	0	1
	At4g34770	253207_at	Auxin-responsive protein	3.1	0.006	1.6	0.055	√	0	1
	At1g04240	263656_at	IAA3, Short hypocotyl 2 (SHY2)	2.7	0.030	1.7	0.222		3	4
	At1g19220	256010_at	ARF19	2.6	0.015	1.1	0.022	√	1	1
	At4g14550	245593_at	IAA14, Solitary root (SLR)	2.6	0.020	1.4	0.012	√	0	0
	At3g07390	259018_at	Auxin-responsive protein (AIR12)	2.1	0.003	1.4	0.059	√	0	2
Biotic and abiotic	At4g38410	252988_at	Dehydrin	3.1	0.032	2.8	0.809	√	2	0
Development	At3g58190	251565_at	LBD29	123.7	0.015	18.7	0.039	√	1	1
	At5g06080	250709_at	LBD33	14.7	6.3E-04	8.0	0.067		3	1
	At2g45420	245140_at	LBD18	6.3	0.007	1.7	0.009	√	1	1
	At2g42430	265856_at	LBD16	5.6	0.003	1.6	0.025	√	1	0
	At3g15370	258388_at	EXP12	4.3	0.023	2.6	0.021		1	0
	At1g73590	259845_at	Auxin efflux carrier protein (PIN1)	3.1	0.002	2.2	0.073		0	1
	At2g39700	267590_at	EXP4	2.4	0.013	1.8	0.295	√	0	1
	At3g60550	251423_at	Cyclin	2.2	0.026	1.4	0.081		1	2
Energy	At1g72230	259801_at	Plastocyanin-like domain-containing protein	2.4	0.010	1.9	0.324	√	1	1
	At1g30760	264527_at	FAD-binding domain-containing protein	2.1	0.032	1.1	0.158		1	1
Hormone responsive	At4g08040	255177_at	ACS11	36.4	1.0E-03	7.9	0.022		1	1
	At2g22810	266830_at	ACS4	17.9	0.007	5.6	0.164	√	1	1
	At4g37770	253066_at	ACS8	5.7	0.015	2.2	0.077	√	3	3
	At3g49700	252279_at	ACS9	5.2	0.033	2.5	0.016	√	0	1
	At3g63440	251178_at	Cytokinin oxidase (CKX7)	4.2	0.010	2.6	0.110	√	1	0
	At2g41510	245108_at	Cytokinin oxidase (CKX1)	3.7	0.018	3.0	0.494	√	1	2
	At1g04310	263653_at	Ethylene response sensor 2 (ERS2)	3.5	0.026	2.2	0.091	√	1	0
	At4g11280	254926_at	ACS6	2.6	0.012	1.6	0.150	√	3	1
Metabolism	At1g74110	260376_at	CYP78A10	38.4	0.002	1.9	0.004		1	1
	At1g78970	264100_at	Lupeol synthase (LUP1)	4.8	0.010	2.6	0.139		0	3
	At3g26760	258253_at	Short-chain dehydrogenase/reductase	4.7	0.008	2.8	0.134	√	2	2
	At5g19530	245947_at	Spermidine synthase (ACL5)	3.9	0.009	2.4	0.031		5	1
	At5g18930	249951_at	Adenosylmethionine decarboxylase	3.3	0.008	2.5	0.288		1	3
	At2g26710	267614_at	CYP72B1 (BAS1)	3.3	0.035	1.4	0.056		1	2
	At1g02660	260915_at	Lipase class 3	2.8	0.021	1.4	0.015	√	0	2
	At3g53450	251940_at	Decarboxylase	2.7	0.007	2.7	0.972		2	0
	At5g48175	248717_at	Thioglucoside glucohydrolase	2.7	0.013	2.3	0.252	√	1	0
	At1g06080	260957_at	Δ-9 desaturase (ADS1)	2.6	0.032	3.9	0.215		1	0
	At2g47130	266761_at	Short-chain dehydrogenase/reductase	2.5	0.008	1.2	0.027	√	1	0

Table 1. *Continued*

Category	At no.	Probe ID	Gene title	IAA		IAA + Dex		Impaired in *arf* mutants[e]	AuxRE	
				FC[a]	*P*-value[b]	FC[c]	*P*-value[d]		A[f]	B[g]
	At2g47550	245151_at	Pectin esterase	2.3	0.012	1.3	0.035	√	2	1
	At5g64250	247283_at	2-nitropropane dioxygenase	2.3	0.011	1.3	0.073	√	1	0
	At5g54500	248162_at	Quinone reductase 1	2.1	0.012	1.5	0.090	√	0	2
Protein metaboism	At1g62770	262643_at	Invertase/pectin methylesterase inhibitor	4.2	1.9E-05	1.5	0.002	√	1	0
	At5g09800	250493_at	U-box domain-containing protein	2.7	0.016	1.6	0.177		4	1
	At4g00080	255695_at	Invertase/pectin methylesterase inhibitor	2.3	0.003	0.7	0.057	√	0	2
	At4g36880	246250_at	Cysteine proteinase	2.3	0.009	2.3	0.909	√	0	1
Signal transduction	At5g02760	251017_at	PP2C family protein	6.3	0.012	1.5	0.010	√	1	2
	At5g54490	248164_at	Pinoid-binding protein 1	5.7	0.002	2.4	0.038	√	0	0
	At1g78100	260058_at	F-box protein	4.4	0.015	1.5	0.040		3	3
	At3g20830	257975_at	Protein kinase	3.7	0.015	1.9	0.007	√	2	4
	At1g51170	265144_at	Protein kinase	3.5	0.009	2.1	0.013	√	2	3
	At2g47860	266507_at	NPH3 protein	3.5	0.033	1.8	0.004		1	4
	At2g30040	266832_at	Protein kinase (MAPKKK14)	3.5	8.3E-04	1.4	0.084	√	1	1
	At2g25790	266663_at	LRR protein kinase	3.0	0.015	1.9	0.015		0	0
	At1g77280	264479_at	Protein kinase	2.8	0.025	1.7	0.121	√	1	5
	At2g41820	260494_at	LRR protein kinase	2.7	0.004	1.9	0.009		1	1
	At5g59010	247743_at	Potein kinase-related	2.5	0.029	1.5	0.033		2	0
	At3g13380	256981_at	LRR protein kinase	2.4	0.028	1.1	0.041	√	2	1
	At3g50310	252212_at	MAPKKK20	2.2	0.012	1.6	0.130		0	1
	At3g14710	258118_at	F-box protein	2.1	0.034	1.3	0.151		0	0
	At5g12940	250277_at	LRR protein kinase	2.1	0.023	1.3	0.083		5	2
	At2g26290	267372_at	Protein kinase	2.0	0.029	1.6	0.401	√	1	3
	At4g37590	253062_at	NPH3 protein	2.0	0.015	1.2	0.052		2	0
	At3g54030	251922_at	Protein kinase	2.0	0.008	1.6	0.103		0	0
Transcription factor	At5g26930	246798_at	ZFP	10.8	0.009	3.1	0.024		2	0
	At5g67060	247023_at	bHLH protein (HEC1)	5.4	8.2E-04	1.4	3.5E-04	√	1	1
	At5g18560	249992_at	AP2 domain-containing transcription factor	4.9	1.1E-03	2.0	0.009	√	1	2
	At5g47370	248801_at	Homeobox-leucine zipper protein 2 (HAT2)	4.6	0.009	2.0	0.023	√	1	3
	At1g65920	261917_at	Regulator of chromosome condensation protein	4.5	0.031	1.8	0.122	√	1	1
	At5g25190	246932_at	Ethylene-responsive element-binding protein	4.5	0.018	2.6	0.172	√	1	4
	At1g44830	261327_at	AP2 domain-containing transcription factor (TINY)	3.2	0.027	1.8	0.205	√	1	3
	At1g18400	261717_at	bHLH protein (BEE1)	3.1	0.029	1.3	0.066		0	1
	At1g28370	261470_at	ERF domain protein 11 (ERF11)	2.9	0.038	1.9	0.295	√	0	2
	At5g40590	249364_at	DC1 domain-containing protein	2.6	0.030	3.5	0.095	√	0	1
	At2g01430	266346_at	Homeobox-leucine zipper protein 17 (HB-17)	2.6	0.022	2.5	0.908		0	0
	At5g15160	250155_at	bHLH protein (PRE2)	2.5	0.014	1.8	0.167		0	0
	At4g37890	253011_at	C3HC4-type RING finger protein	2.4	0.033	1.5	3.9E-04	√	1	1
	At3g25710	257642_at	bHLH protein	2.4	0.042	1.9	0.227		1	2
	At1g73830	260070_at	bHLH protein (BEE3)	2.2	0.031	1.6	0.196		0	1
	At4g32880	253402_at	Homeobox-leucine zipper protein (HB-8)	2.2	0.033	1.8	0.056		3	1
	At5g58620	247795_at	Zinc finger (CCCH-type)	2.0	0.032	1.2	0.082		1	4
	At3g06490	258516_at	MYB108	2.0	0.034	1.2	0.027		0	1
Transferase	At5g67430	246992_at	GCN5-related N-acetyltransferase (GNAT)	19.8	0.043	3.9	0.050	√	0	0
	At5g55250	248104_at	SAM:carboxyl methyltransferase	3.8	0.001	3.1	0.205		2	1
	At2g39980	267337_at	Transferase	3.7	0.030	2.0	0.019	√	1	2
	At5g01210	251144_at	Transferase	2.6	0.043	1.7	0.128		0	0
	At1g21980	255959_at	AtPIP5K1	2.4	0.046	1.2	0.017		1	0
	At4g15550	245277_at	UDP-glucose:IAA beta-D-glucosyltransferase	2.3	0.024	2.0	0.584	√	1	0
Transporter	At3g06370	258907_at	Sodium proton exchanger (NHX3)	3.7	0.007	2.7	0.312	√	1	1
	At1g58340	256024_at	MATE efflux protein-related (ZF14)	3.6	0.022	2.4	0.047	√	2	0
	At1g59740	262912_at	Proton-dependent oligopeptide transport protein	3.5	0.005	2.8	0.160	√	0	4

Table 1. *Continued*

Category	At no.	Probe ID	Gene title	IAA		IAA + Dex		Impaired in *arf* mutants[e]	AuxRE	
				FC[a]	*P*-value[b]	FC[c]	*P*-value[d]		A[f]	B[g]
	At2g21050	264025_at	Amino acid permease	3.2	0.022	2.9	0.126	√	2	0
	At5g27000	246802_at	Kinesin 4 (ATK4)	2.7	0.040	1.7	0.069		0	1
	At5g52890	248278_at	AT hook motif-containing protein	2.2	0.041	0.7	0.141		1	2
Unknown	At2g39370	266974_at	Expressed protein	15.2	6.E-05	4.8	1.4E-03	√	3	1
	At5g62280	247474_at	Expressed protein	9.0	0.020	1.8	0.003	√	3	3
	At5g50335	248509_at	Expressed protein	6.8	0.045	2.3	0.130	√	1	1
	At2g28690	263436_at	Expressed protein	6.5	0.004	1.9	0.046	√	0	2
	At1g64405	259735_at	Expressed protein	6.4	2.9E-04	1.9	0.016	√	2	2
	At3g19200	257026_at	Hypothetical protein	5.9	0.010	4.2	0.031	√	0	1
	At3g28420	257900_at	Expressed protein	5.5	0.003	4.1	0.361		2	2
	At4g35210	253180_at	Hypothetical protein	5.3	0.019	0.3	0.048		4	3
	At5g17340	250091_at	Expressed protein	5.2	0.007	1.6	0.022	√	0	0
	At4g17350	245416_at	Expressed protein	5.1	0.006	1.7	0.005	√	0	1
	At5g52900	248282_at	Expressed protein (MXC20_13)	4.4	0.018	0.9	0.012	√	2	0
	At4g37295	253047_at	Expressed protein	3.9	0.004	1.5	0.025	√	0	0
	At3g29370	256743_at	Expressed protein	3.8	0.017	1.9	0.006	√	3	0
	At3g50340	252204_at	Expressed protein	3.7	1.3E-03	1.6	0.006	√	1	0
	At3g59900	251436_at	Expressed protein	3.7	0.007	2.3	0.027	√	3	1
	At3g15250	257049_at	Expressed protein	3.6	0.002	1.7	0.049		2	2
	At4g35200	253179_at	Hypothetical protein	3.5	5.8E-04	1.2	0.001	√	3	4
	At5g57760	247878_at	Expressed protein	3.4	0.012	1.3	0.009	√	1	1
	At5g12050	250327_at	Expressed protein	3.3	0.027	1.2	0.019		1	4
	At4g22530	254318_at	Embryo-abundant protein-related	3.3	0.017	1.8	0.102		1	0
	At4g13195	254761_at	Expressed protein	3.3	0.010	1.5	0.026	√	3	3
	At3g55720	251751_at	Expressed protein	3.1	0.006	0.9	0.043		1	1
	At1g80240	262045_at	Expressed protein (ATGDI1)	2.8	0.012	1.3	0.099	√	4	1
	At1g03820	265083_at	Expressed protein	2.8	0.046	2.0	0.361	√	1	1
	At3g03170	258878_at	Expressed protein	2.6	0.006	1.1	0.016	√	1	1
	At5g22310	249887_at	Expressed protein	2.6	0.016	1.7	0.014		2	2
	At4g09890	255028_at	Expressed protein	2.5	0.035	1.9	0.363		2	1
	At3g54000	251925_at	Expressed protein	2.4	0.011	1.3	0.022	√	3	1
	At1g23340	263042_at	Expressed protein	2.4	0.012	1.9	0.231		0	2
	At5g51670	248423_at	Expressed protein	2.4	0.016	2.0	0.266	√	1	0
	At1g29195	260841_at	Expressed protein	2.3	0.016	1.0	0.081	√	2	0
	At1g23060	264902_at	Expressed protein	2.2	0.029	1.4	0.039		1	1
	At2g40000	267357_at	Expressed protein	2.2	0.016	1.2	0.026		1	2
	At5g49170	248623_at	Expressed protein	2.2	0.025	1.6	0.209		0	2
	At1g60010	263737_at	Expressed protein	2.1	0.010	1.3	0.015	√	3	1
	At3g60520	251372_at	Expressed protein	2.1	0.002	1.6	0.012		2	0
	At3g47510	252419_at	Expressed protein	2.0	0.007	1.7	0.251	√	0	1

[a] Fold change of gene expresion in auxin-treated 7-d-old *iaa1:GR* transgenic plants compared to that of mock-treated plants.
[b] *P*-value calculated from auxin-induced gene expression and mock expression using Welch's *t*-test.
[c] Fold change of gene expresion in auxin and DEX-treated 7-d-old *iaa1:GR* transgenic plants compared to that of mock-treated plants.
[d] *P*-value calculated from auxin-induced gene expression in the presence of DEX and auxin-induced gene expression using Welch's *t*-test.
[e] Genes showing auxin-induced expression impaired in *nph4-1* and/or *arf19-1* and/or *nph4-1 arf19-1* mutants by microarray analysis (Okushima *et al.*, 2005).
[f] Number of AuxRE, TGTCnC sequences in the 2 kb fragments upstream of the start codon. N, any nucleotide.
[g] Number of AuxRE, GnGACA sequences in the 2 kb fragments upstream of the start codon.

of most of these genes and increased expression of the auxin-down-regulated genes toward their initial levels. Taken together, these results suggest that the iaa1 protein is involved in regulating expression of many early auxin-responsive genes responsible for transcriptional control, signal transduction, and metabolism. It has also been found that auxin-regulated expression of some genes is not affected by the iaa1 protein, indicative of a portion of auxin-regulated genes independent of iaa1 regulation. These include genes encoding dehydrin, CKX1, decarboxylase, cysteine proteinase, homeobox-leucine zipper protein 17 (HB-17), and UDP-glucose:IAA β-D-glucosyltransferase in the case of auxin up-regulation, and pathogenesis-related protein, dehydrodolichyl diphosphate synthase, and ARR7 in the case of auxin down-regulation (Tables 1, 2).

Table 2. List of genes downregulated 2 h after treatment with IAA

Category	At no.	Probe ID	Gene title	IAA		IAA + Dex		Impaired in *arf* mutants[e]	AuxRE	
				FC[a]	*P*-value[b]	FC[c]	*P*-value[d]		A[f]	B[g]
Auxin-regulated	At4g31320	253515_at	Small auxin up RNA (SAUR_C)	0.28	0.034	1.18	0.012	√	1	0
	At4g17280	245412_at	Auxin-responsive protein	0.45	0.013	0.73	0.036		0	1
Boitic and abiotic	At5g45210	248990_at	Disease resistance protein	0.28	5.46E-04	0.52	0.157		0	1
	At4g39030	252921_at	Salicylic acid induction deficient 1 (SID1)	0.49	0.007	0.78	0.070		1	1
	At1g73620	260077_at	Pathogenesis-related protein	0.49	0.039	0.56	0.637		0	0
Development	At2g20750	265443_at	β-expansin (EXPB1)	0.27	0.024	0.73	0.066		3	0
	At4g36380	246216_at	Rotundifolia3 (ROT3)(CYP90C1)	0.35	0.002	0.68	0.002	√	1	2
Metabolism	At1g05660	263229_s_at	Polygalacturonase	0.03	0.022	1.13	0.014		3	0
	At1g64590	261956_at	Short-chain dehydrogenase (SDR)	0.22	0.006	0.97	0.008	√	1	1
	At1g67110	264470_at	Cytochrome P450 protein (CYP735A2)	0.27	1.80E-04	0.62	0.068		1	0
	At4g13310	254767_s_at	Cytochrome P450 protein (CYP71A19)	0.27	0.038	0.54	0.118		0	1
	At5g47990	248727_at	Cytochrome P450 protein (CYP705A5)	0.29	0.040	0.76	0.101		3	0
	At2g43880	267222_at	Polygalacturonase	0.34	0.010	0.82	0.038		0	3
	At4g21840	254385_s_at	Methionine sulfoxide reductase	0.35	0.019	0.67	0.061		0	2
	At1g78090	260059_at	Trehalose-6-phosphate phosphatase	0.39	0.017	0.85	0.029		2	1
	At5g42590	249203_at	Cytochrome P450 protein (CYP71A16)	0.39	0.050	1.35	0.060		2	2
	At4g39950	252827_at	Cytochrome P450 protein (CYP79B2)	0.41	0.003	0.62	0.041	√	0	2
	At4g11290	254914_at	Peroxidase	0.45	0.040	0.72	0.178		3	0
	At5g58784	247781_at	Dehydrodolichyl diphosphate synthase	0.48	0.047	0.55	0.359		1	0
	At2g23560	267123_at	Hydrolase	0.49	0.004	0.94	0.077		0	0
Protein metabolism	At3g20015	256626_at	Aspartyl protease	0.43	0.028	0.84	0.060		0	0
Signal transduction	At5g62920	247406_at	ARR6	0.22	0.017	0.71	0.038		1	0
	At3g48100	252374_at	ARR5	0.24	0.039	0.57	0.080		1	3
	At5g24100	249768_at	LRR protein kinase	0.25	0.017	0.56	0.092		3	1
	At1g19050	259466_at	ARR7	0.43	0.038	0.47	0.689		3	1
	At1g10470	263236_at	ARR4	0.49	0.011	0.74	0.038		0	2
Transcription factor	At1g10480	263208_at	ZFP5	0.21	0.007	0.96	0.005		1	1
	At3g46130	252534_at	MYB111	0.31	0.042	1.09	0.080		0	4
	At5g25160	246933_at	ZFP3	0.34	0.003	0.83	7.52E-04		1	3
	At1g72200	259854_at	Zinc finger (C3HC4-type) protein	0.50	0.013	0.95	0.020	√	2	0
Transferase	At2g18800	266066_at	Xyloglucan:xyloglucosyl transferase	0.08	0.020	0.56	0.035		1	0
	At4g12510	254820_s_at	Lipid transfer protein (LTP)	0.15	0.008	0.54	0.010	√	0	0
	At5g47980	248725_at	Transferase	0.17	0.004	0.65	0.010		1	2
	At1g77530	259758_s_at	*O*-methyltransferase 2	0.21	0.008	0.69	0.021		0	1
	At5g47950	248723_at	Transferase	0.34	1.48E-04	0.82	0.038		0	2
	At1g80050	262039_at	Adenine phosphoribosyltransferase 2 (APT2)	0.39	0.004	0.66	0.016	√	1	0
	At1g53680	259964_at	Glutathione *S*-transferase (AtGSTU28)	0.39	0.023	0.74	0.026	√	0	0
	At3g45070	252605_s_at	Sulphotransferase	0.40	0.021	1.06	0.066		1	0
	At3g29680	257281_s_at	Transferase	0.40	0.007	0.75	0.076		2	0
	At3g08860	258983_at	Alanine-glyoxylate aminotransferase	0.44	0.045	1.02	0.060		0	0
	At2g23410	267137_at	*cis*-Prenytransferase	0.46	0.016	0.26	0.213		3	1
Transporter	At5g47450	248790_at	Major intrinsic protein (TIP2;3)	0.27	0.020	0.84	0.032	√	1	2
	At5g60660	247586_at	PIP2:4	0.28	0.007	0.73	0.025	√	0	0
	At3g45680	252594_at	Oligopeptide transporter	0.31	9.04E-04	0.70	0.017		0	2
	At1g31770	246580_at	ABC transporter	0.35	0.007	0.76	0.002		2	1
	At3g23430	258293_at	Phosphate transporter (PHO1)	0.42	0.005	0.89	6.51E-04		0	0
Unknown	At4g26320	253957_at	Arabinogalactan-protein (AGP13)	0.16	0.007	0.46	0.071	√	1	1
	At5g60520	247634_at	Late embryogenesis abundant protein	0.19	0.027	0.55	0.133	√	0	0
	At5g53250	248252_at	Arabinogalactan-protein (AGP22)	0.29	0.018	0.53	0.116	√	2	2
	At4g02850	255450_at	PhzC/PhzF family protein	0.33	0.019	0.61	0.065		0	0
	At3g21680	258178_at	Expressed protein	0.34	0.038	1.12	0.016		1	2
	At4g30460	253619_at	Glycine-rich protein	0.37	0.031	0.84	0.003		0	1
	At2g21560	263545_at	Expressed protein	0.37	0.019	0.88	0.029		0	2
	At5g28610	255945_at	Expressed protein	0.38	0.031	0.56	0.457		1	2
	At3g46880	252501_at	Expressed protein	0.39	0.004	0.65	0.036		0	2

Table 2. *Continued*

Category	At no.	Probe ID	Gene title	IAA		IAA + Dex		Impaired in *arf* mutants[e]	AuxRE	
				FC[a]	*P*-value[b]	FC[c]	*P*-value[d]		A[f]	B[g]
	At5g03960	250872_at	Calmodulin-binding protein	0.42	0.015	1.01	0.015		1	2
	At5g19970	246142_at	Expressed protein	0.42	0.003	0.86	0.005		1	0
	At1g31050	265160_at	Expressed protein	0.44	0.035	0.89	0.055		2	2
	At2g09970	260453_s_at	Expressed protein	0.47	0.007	0.93	0.023		0	1

[a] Fold change of gene expresion in auxin-treated 7-d-old *iaa1:GR* transgenic plants compared to that of mock-treated plants.
[b] *P*-value calculated from auxin-down-regulated gene expression and mock expression using Welch's *t*-test.
[c] Fold change of gene expresion in auxin and DEX-treated 7-d-old *iaa1:GR* transgenic plants compared to that of mock-treated plants.
[d] *P*-value calculated from auxin-down-regulated gene expression in the presence of DEX and auxin-down-regulated gene expression using Welch's *t*-test.
[e] Genes showing auxin-induced expression impaired in *nph4-1* and/or *arf19-1* and/or *nph4-1 arf19-1* mutants by microarray analysis (Okushima et al., 2005).
[f] *Number* of AuxRE, TGTCnC sequences in the 2 kb fragments upstream of the start codon.
[g] Number of AuxRE, GnGACA sequences in the 2 kb fragments upstream of the start codon.

RNA-gel blot analysis of the selected auxin-response genes affected by iaa1

RNA-gel blot analysis was performed with selected auxin-up-regulated gene probes to confirm these microarray results. It should be noted that 5% of the genes listed that changed more than 2-fold in the microarray data are due to change variation and thus a relatively relaxed stringency has been used for the microarray data that set up the RNA-gel blot analyses. To represent the auxin-regulated transcriptome, an attempt was made to pick a range of gene probes that exhibit the highest and lowest auxin-regulated expression as well as genes in between. Also, genes were included with functions related to auxin response, transcription, and development to gain some insights into functional meaning of gene regulation in response to auxin. To study the expression characteristics of the iaa1-regulated auxin-responsive genes, the focus was on the genes showing auxin-regulated expression that were significantly affected by DEX treatment. For the analysis of auxin-up-regulated genes, 18 genes have been chosen in which 15 genes show $P < 0.05$ and P calls in all three hybridizations after auxin treatment, as well as three genes (At3g24240, At5g57520, and At1g29440) that show P calls in all three hybridizations even with $P > 0.05$. Expression of these selected genes was first examined over a time-course in response to auxin and the effects of DEX treatment. *Pro$_{35S}$:iaa1:GR* transgenic *Arabidopsis* was incubated with auxin for varying amounts of time, without auxin for 8 h (–8), or with auxin and DEX, followed by RNA-gel blot analysis (Fig. 3). Quantitative analyses of the RNA-gel blots were performed with Phosphoimager (see Supplementary Figs S1 and S2 at *JXB* online). The results showed that auxin-induced expression of these genes can be divided into two groups, in which one group shows transient expression patterns and the other group shows a gradually increasing expression pattern without decay of expression until 8 h. These genes are also grouped into early or late genes based on the timing of gene expression in response to auxin. Three genes (At3g24240, At5g57520, and At1g29440) showing P calls in all three hybridizations but with $P > 0.05$, and thus are not listed in the Tables 1 and 2, still exhibited significant auxin-inducible expression patterns. This result indicates the significance of P calls for extracting differentially regulated genes in response to auxin from our microarray data. Expression of all the genes examined in response to auxin was effectively repressed by DEX treatment. Similarly, expression of seven auxin-down-regulated genes was significantly down-regulated at 10 min (the earliest case) or within 2 h. DEX treatment suppressed the auxin-mediated down-regulation of those genes (Fig. 3; see Supplementary Fig. S2 at *JXB* online). These results show a negative regulation of iaa1 on various early auxin-responsive genes and in part, verify the data obtained by microarray analysis. However, the effect of DEX on auxin-mediated down-regulation of *ARR5* was marginal except for the 8 h incubation.

Expression of iaa1-regulated auxin-response genes in the presence of cycloheximide

The protein synthesis inhibitor, cycloheximide, has been shown to induce expression of the primary auxin-responsive *Aux/IAA* genes and pea *Aux/IAA* genes, *PS-IAA4/5* and *PS-IAA6* (Abel *et al.*, 1995; Koshiba *et al.*, 1995). According to the current model for auxin action, cycloheximide seems to inhibit the continued supply of labile Aux/IAA repressors being degraded via the proteasome. This results in a release of transcriptional repression that, in turn, allows ARF proteins to modulate auxin-response genes (Quint and Gray, 2006). Translocation of the iaa1 proteins to the nucleus by DEX treatment can suppress auxin- or cycloheximide-inducible *Aux/IAA* expression (Park *et al.*, 2002). These characteristics were used to gain further insight into how these early auxin-response genes are regulated by iaa1. *Pro$_{35S}$:iaa1:GR* transgenic seedlings were incubated with mock treatment, auxin, cycloheximide, auxin and DEX, auxin and cycloheximide, cycloheximide and DEX, or auxin and cycloheximide and DEX for 2 h. The total RNAs were subject to RNA-gel blot analysis using DNA probes against

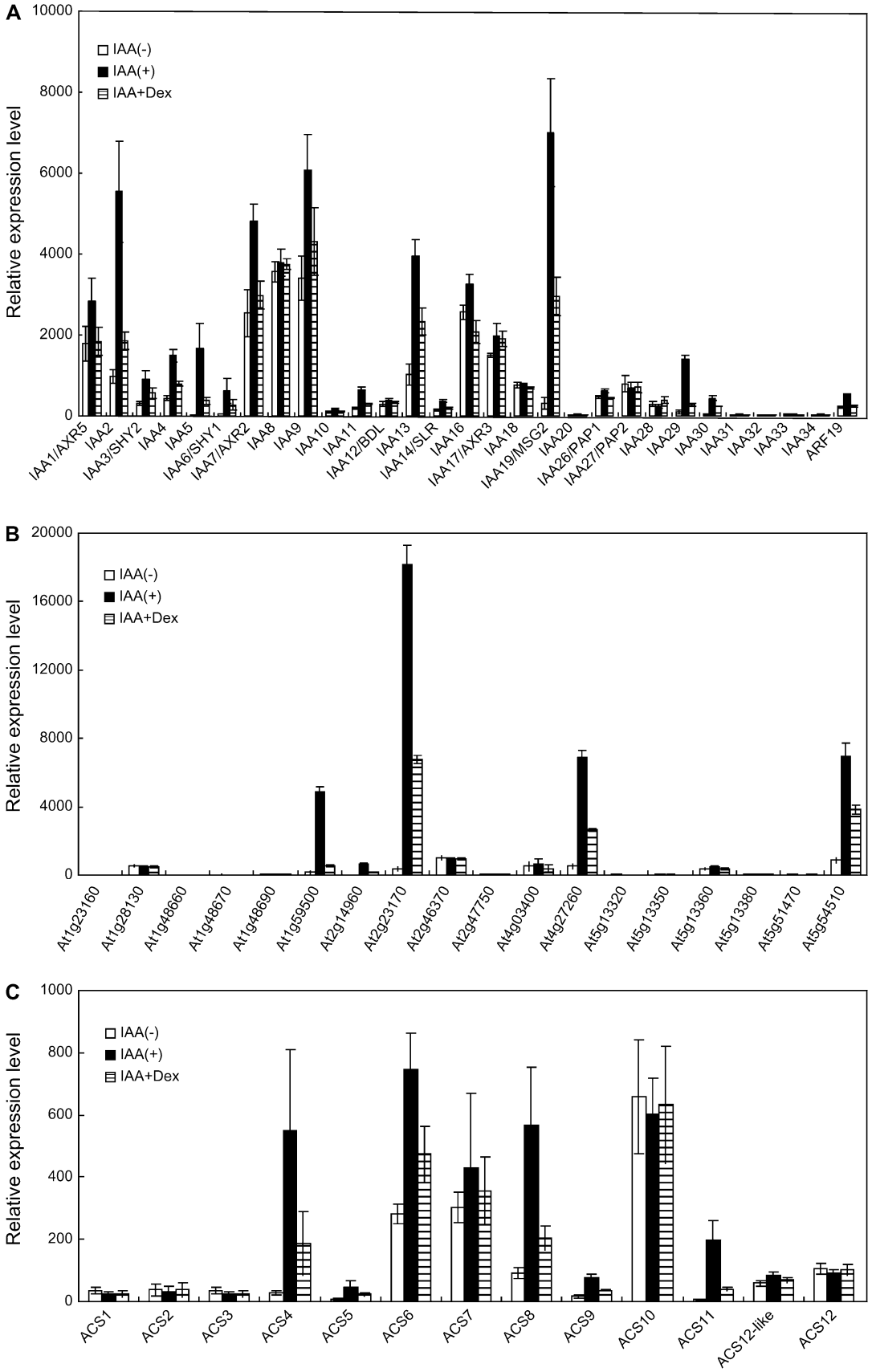

Fig. 2. The expression profiles of representative auxin-up-regulated gene families and the effects of DEX on auxin-induced gene expression. (A) *Aux/IAA* gene family. The data represent the average of the relative expression levels of the samples treated with mock (open bar), treated with auxin (black bar), or treated with auxin in the presence of DEX (shaded bar) from triplicate experiments. Bars indicate SE of the average. (B) *GH3* gene family. The legend is the same as (A). (C) *ACS* gene family. The legend is the same as (A).

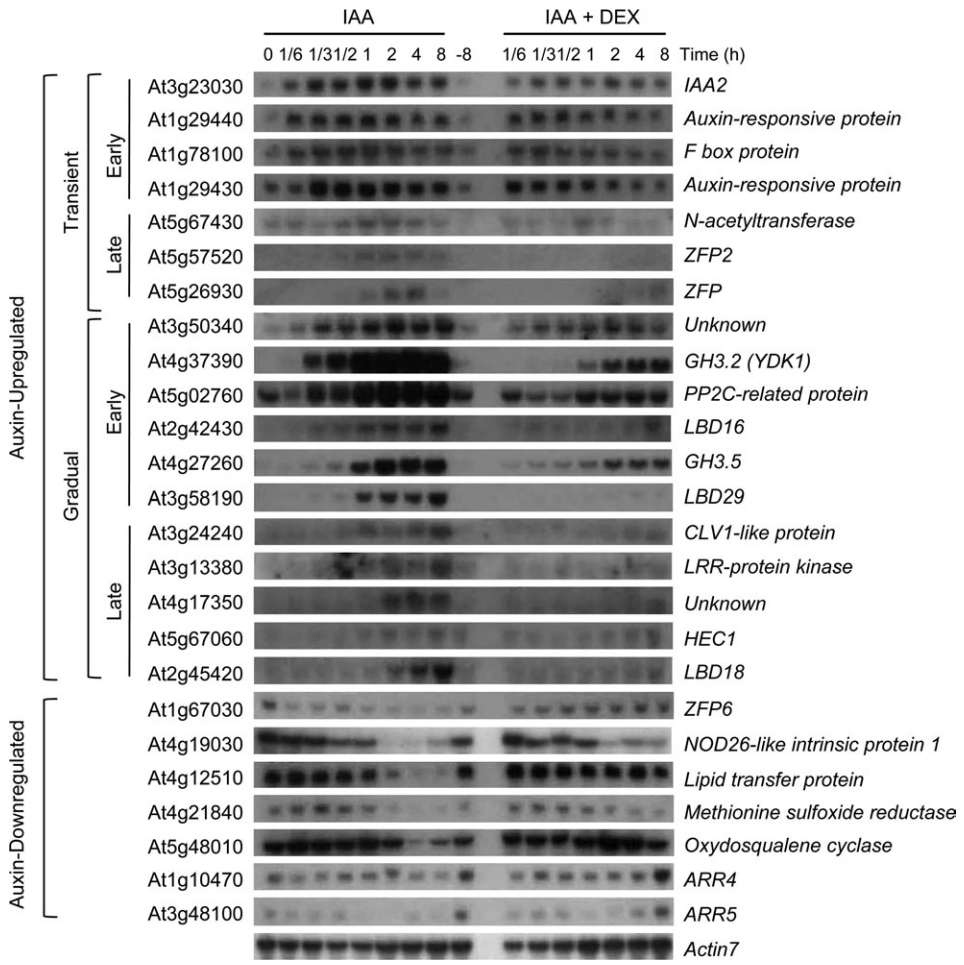

Fig. 3. Time-course expression analysis of representative auxin-up or down-regulated genes with auxin or auxin and DEX. *Pro$_{35S}$:iaa1:GR* seedlings were incubated with auxin (IAA) or auxin and DEX (IAA+DEX) for the indicated time in the light and subject to RNA-gel blot analysis with the given DNA probes. –8 h indicates the samples incubated with mock for 8 h. Representative blots are shown from at least two independent biological replicates. Auxin-induced expression of these genes are divided into two groups, a transient expression group (Transient) and a gradually increasing expression group (Gradual). 'Early' or 'Late' indicates the genes expressing the transcripts detectable within (Early) or after (Late) 30 min in response to exogenous auxin treatment. The blots were displayed with timing of auxin-responsive gene expression.

the 18 indicated genes for auxin-up-regulation and the four indicated genes for auxin-down-regulation (Fig. 4). Quantitative analyses of the RNA-gel blots were performed with Phosphoimager (see Supplementary Fig. S3 at *JXB* online).

Treatment of cycloheximide to *Pro$_{35S}$:iaa1:GR* transgenic seedlings induced most of the up-regulated genes to equal or higher levels than the expression levels of those genes when treated with auxin. As expression of *LBD18* and *CLV1-like protein* was not clear due to the low levels of the transcripts, a longer incubation followed by RT-PCR analysis was performed (see Supplementary Fig. S4 at *JXB* online). The effect of cycloheximide on the expression of these genes was marginal, indicating that the expression of these genes is not under the direct control of labile repressors or it may be a secondary response as they are late genes (Fig. 3). Treatment of DEX effectively suppressed the expression of most of the iaa1-regulated genes in response to auxin or by cycloheximide. However, cycloheximide-induced expression of some genes encoding proteins such as N-acetyltransferase, LRR-protein kinase, and unknown (At4g17350) could not be suppressed by DEX treatment, even though auxin induction of these genes can be suppressed by DEX. A summary of this result is shown in Fig. 5A. This result indicates that an additional pathway or components might exist, thus effecting the auxin-regulated expression of these genes.

DEX treatment inhibited auxin-mediated down-regulation of the four genes tested, as expected. Interestingly, genes encoding ZFP6, methionine sulphoxide reductase, and ARR5 were super-induced by cycloheximide treatment alone, indicative of the repression of these genes by labile repressors. Moreover, DEX treatment could not prevent cycloheximide-induced up-regulation of these auxin-down-regulated genes. DEX treatment did not have any effect on either auxin or cycloheximide-induced expression of all four auxin-down-regulated genes. A summary of this result is shown in Fig. 5B. This result suggests that non-Aux/IAA

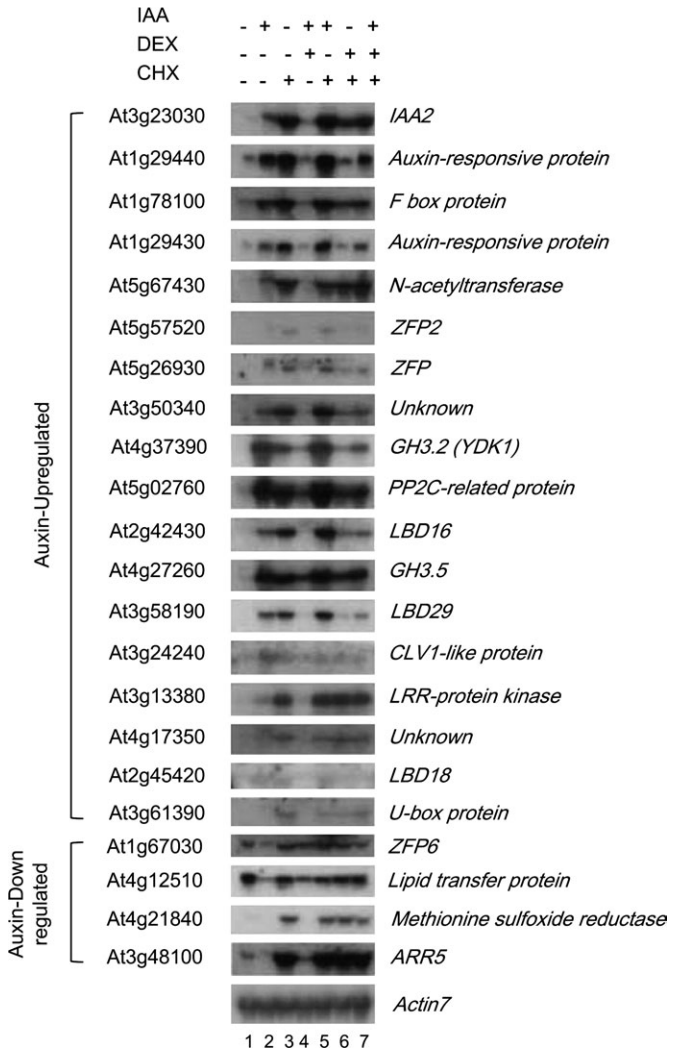

Fig. 4. Expression analysis of representative auxin-response genes in response to cycloheximide, auxin, or DEX treatment. *Pro$_{35S}$:iaa1:GR* seedlings were incubated with mock (lane 1), auxin (lane 2), cycloheximide (lane 3), auxin and DEX (lane 4), auxin and cycloheximide (lane 5), cycloheximide and DEX (lane 6), or auxin, DEX and cycloheximide (lane 7) for 2 h in the light, followed by RNA-gel blot analysis with the indicated DNA probes. The transcript levels were determined as described in Fig. 3 legend.

proteins functioning as labile repressors might be involved in the auxin-mediated down-regulation of these genes.

Analysis of iaa1-regulated auxin-response genes in a variety of arf mutants

To determine if the auxin response genes identified in the present study are controlled by ARFs, their expression in response to auxin was analysed further in a variety of *arf* mutant backgrounds including ten single T-DNA insertion mutants and two double mutants previously isolated (Okushima *et al.*, 2005*b*), and compared with wild-type plants using RNA-gel blot methods (Figs 6, 7). Quantitative analyses of the RNA-gel blots were performed with Phosphoimager (see Supplementary Figs S5 and S6 at *JXB* online). All single *arf* mutations affected auxin regulation of iaa1-regulated genes to varying degrees. A summary of these results are shown in Fig. 8. The *arf3* mutation resulted in enhanced auxin-induced gene expression, whereas the rest of the *arf* mutations decreased auxin-induced gene expression (when they demonstrated any effect). Expression of auxin-induced genes is differently affected by *arf* mutations. For example, *arf2-8*, *arf9-2*, *arf13-1,* or *arf14* weakly inhibited auxin-induced expression of most iaa1-regulated genes tested when compared with the other *arf* mutations. By contrast, *arf4*, *arf6-1*, *arf10-1,* or *arf19-2* mutations significantly inhibited auxin-induced expression of a broad range of genes encoding IAA2, auxin-responsive protein, F-box protein, ZFP2, ZFP, unknown (At3g50340), PP2C-related protein, LBD16, LBD29, LRR-protein kinase, unknown (At4g17350), and LBD18, collectively. To gain an insight into the functional relevance of *arf* mutation effects on iaa1-downstream genes, organ-specific expression patterns of a select set of genes were analysed along with *IAA1*. Some genes such as *LBD16* showed preferential expression patterns in particular organs, while others such as *CLV1-like protein* showed general expression throughout various tissues (see Supplementary Fig. S7 at *JXB* online). All the genes examined showed significant expression in the aerial parts as well as in the roots of 14-d-old plants.

The *arf7-1* mutation most significantly inhibited the auxin-regulated expression of all 16 auxin-up-regulated genes and the three down-regulated genes tested. The *arf7-1* mutation greatly or even completely inhibited the auxin-induced expression of *F-box protein*, *unknown* (At3g50340), *GH3.2*, *GH3.5*, *LBD29*, *LRR-protein kinase*, and *LBD18* when compared with the other *arf* mutations, indicating that ARF7 preferentially regulates the expression of theses genes to auxin more so than the other ARFs do. Auxin-induced expression of the *LBD16* and *LRR-protein kinase* are partially inhibited by the *arf7-1* or *arf19-2* mutations, but are completely inhibited by double mutations, indicating co-operative action between ARF7 and ARF19 for regulating expression of these two genes. On the other hand, expression of *ZFP* and *ZFP2* to auxin is completely silent in *arf7-1* or *arf19-2* single mutants, suggesting independent roles for ARF7 and ARF19 in regulating expression of these genes. *Auxin-responsive protein* (At1g29440), *F-box protein*, *ZFP2*, *ZFP*, *PP2C-related protein*, *LBD29*, *unknown* (At4g17350), and *LBD18* are also rendered completely unresponsive to auxin by the *arf7-1* mutation. Both *arf7-1 arf19-1* mutants and *arf19-1 nph4-1* mutants exhibited greatly reduced auxin-induced expression of all auxin-regulated genes tested compared with their corresponding single mutants, except for one auxin-down-regulated gene (At2g44940). Expression of many genes is completely unresponsive to auxin in these two double mutations including *unknown* (At3g50340), *LBD16*, *GH3.5*, *CLV1-like protein*, *LRR-protein kinase*, and *HECTATE* (*HEC*)*1*. Expression of various genes is unresponsive to auxin by a single *arf* mutation as summarized in Fig. 8. Auxin-responsive expression of some genes such as *auxin-responsive protein* (At1g29440) and *ZFP2* are completely

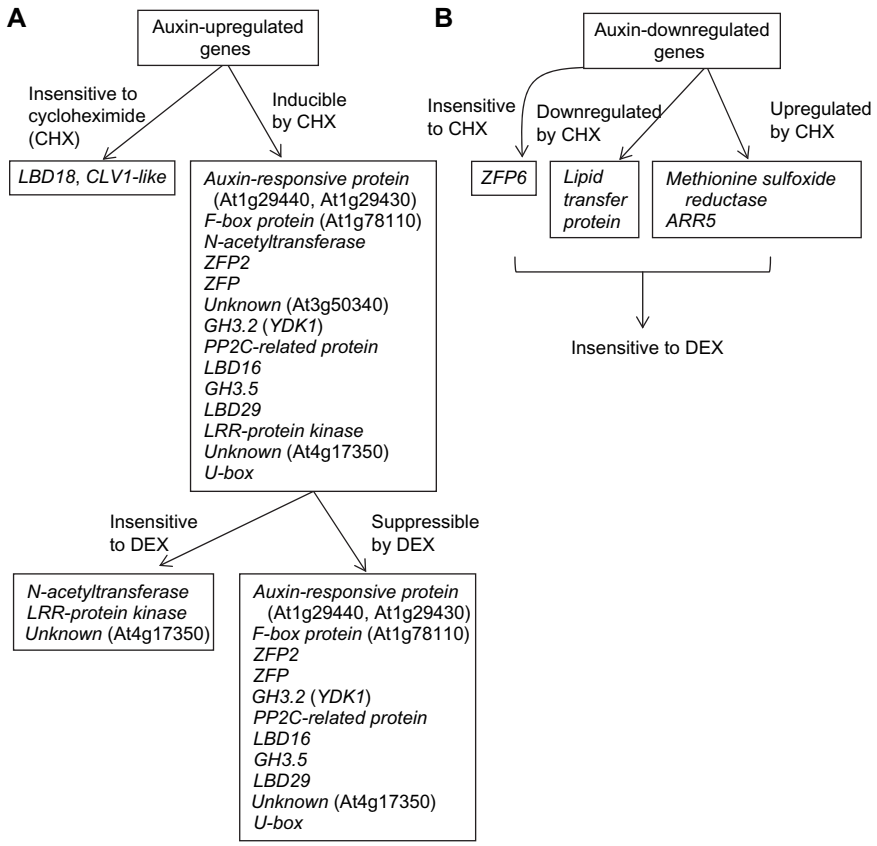

Fig. 5. Schematic summary of expression patterns of representative auxin-response genes in the presence of cycloheximide and/or DEX. (A) Expression pattern of auxin-up-regulated genes. (B) Expression pattern of auxin-down-regulated genes. The results obtained by the expression analysis in Fig. 4 were schematically summarized.

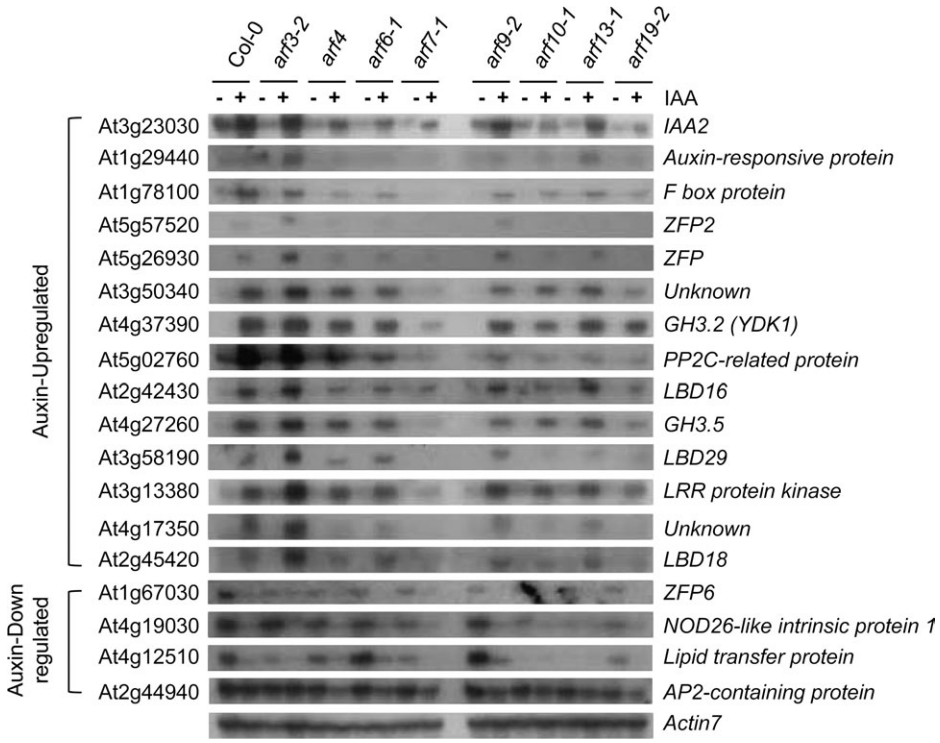

Fig. 6. Expression profiling of auxin-responsive genes regulated by iaa1 in various *arf* mutant backgrounds. *arf* mutant seedlings were incubated with mock (–) or auxin (+) for 2 h in the light, followed by RNA-gel blot analysis with the indicated DNA probes. The transcript levels were determined as described in the Fig. 3 legend.

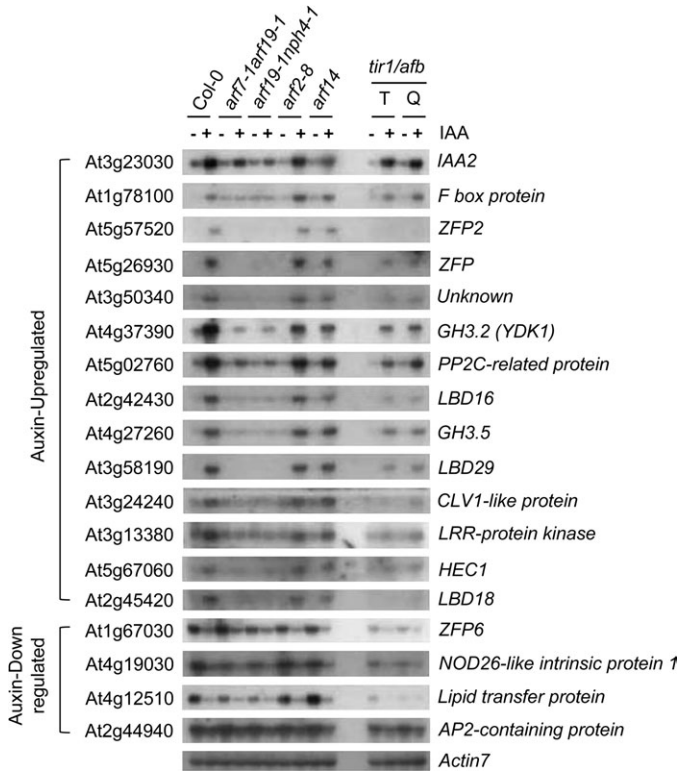

Fig. 7. Expression profiling of auxin-responsive genes regulated by iaa1 in various *arf* and *tir1/afb* mutant backgrounds. *arf* mutant seedlings were incubated with mock (–) or auxin (+) for 2 h in the light, followed by RNA-gel blot analysis with the indicated DNA probes. The transcript levels were determined as described in the Fig. 3 legend. T and Q indicate *tir1 afb2 afb3* triple mutants and *tir1-1 afb1 afb2 afb3* quadruple mutants, respectively.

suppressed to the basal levels by various *arf* mutations, indicating redundant role of these *ARF*s in auxin-regulated expression of these genes.

Interestingly, *arf6-1*, *arf9-2,* and *arf14* mutations up-regulated the basal levels of the auxin-down-regulated gene encoding lipid transfer protein, while auxin-mediated down-regulation was normal. By contrast, *arf4*, *arf6-1*, *arf7-1*, *arf10-1, arf13-1*, and *arf19-2* mutations resulted in greatly reduced expression of *NOD26-like intrinsic protein1* without auxin. *arf3-2*, *arf7-1*, *arf10-1, arf13-1*, and *arf19-2* mutations resulted in greatly reduced expression of *lipid transfer protein* without auxin. However, treatment with auxin decreased their expression levels as in wild-type plants. Similarly, auxin-mediated down-regulation is normal in *arf7 arf19* double mutants, whereas basal levels of auxin-down-regulated genes are decreased in the *arf7-1* mutant or *arf7 arf19* double mutants. The basal levels of *ZFP6* were greatly reduced by all *arf* mutations tested even including *arf3*. *arf7 arf19* double mutations did not have additive effects compared with that of single mutations, suggesting that *ARF7* and *ARF19* might play an independent role in the regulation of *ZFP6* expression level. Normal auxin-mediated down-regulation in *arf* mutants indicates that ARFs might not play a critical role in mediating gene down-regulation in response to auxin.

Expression of iaa1-regulated auxin-response genes in tir1/afb *auxin receptor mutants*

TIR1/AFB F-box proteins are auxin receptors mediating auxin responses throughout plant development (Dharmasiri *et al.*, 2005*a*, *b*; Kepinski and Leyser, 2005). It was tested whether auxin response of the Aux/IAA-ARF-modulated genes, which had been identified in our microarray analysis, became insensitive by triple or quadruple mutations of these *TIR1/AFB* genes. It was found that most of these genes exhibited significant levels of auxin responsiveness except for *ZFP2* and *LBD18* (Fig. 7). Moreover, quadruple *tir1-1 afb1 afb2 afb3* mutants had less effect on auxin-regulated gene expression than the *arf* double mutants, *arf7-1 arf19-1* or *arf19-1 nph4-1*, and was comparable with that of *arf7-1* single mutants.

Discussion

Microarray analysis of the effect of iaa1 on early auxin-regulated gene expression

Numerous studies have firmly established the functional importance of the Aux/IAA-ARF proteins during auxin response and their mechanism of action in auxin-mediated gene regulation (Berleth *et al.*, 2004; Woodward and Bartel, 2005; Parry and Estelle, 2006). A stabilized iaa1 had previously been constructed by introducing a point mutation in domain II and expressing it under the CaMV 35S promoter in order to produce auxin-related phenotypes (Park *et al.*, 2002). *AXR5* later turned out to be *IAA1*, whose gain-of-function mutation caused some phenotypes to be comparable with DEX-treated *Pro$_{35S}$:iaa1:GR* transgenic plants (Yang *et al.*, 2004). In the present study, the transcriptome downstream of iaa1 was analysed during the auxin response on a genome-wide level by using GR-fused iaa1, which can be induced to translocate to the nucleus following DEX treatment. Putative target genes for iaa1 were determined by identifying the genes that were regulated by auxin and negatively affected by the DEX-inducible translocation of iaa1. The iaa1-regulated auxin-responsive transcriptome was further supported by the analysis showing the presence of AuxREs (TGTCnC or GnGACA) in the promoter regions of most of the genes affected by iaa1 (Tables 1, 2; see Supplementary Table S2 at *JXB* online). Several exceptions to this rule were noted (see Supplementary Table S2 at *JXB* online), indicating that other AuxRE variants might exist in the promoter region of auxin-regulated genes.

The constitutive CaMV 35S promoter was used to find any potential auxin-responsive targets regulated downstream of Aux/IAA-ARFs. The putative Aux/IAA-ARF targets were investigated to reveal biological roles in auxin

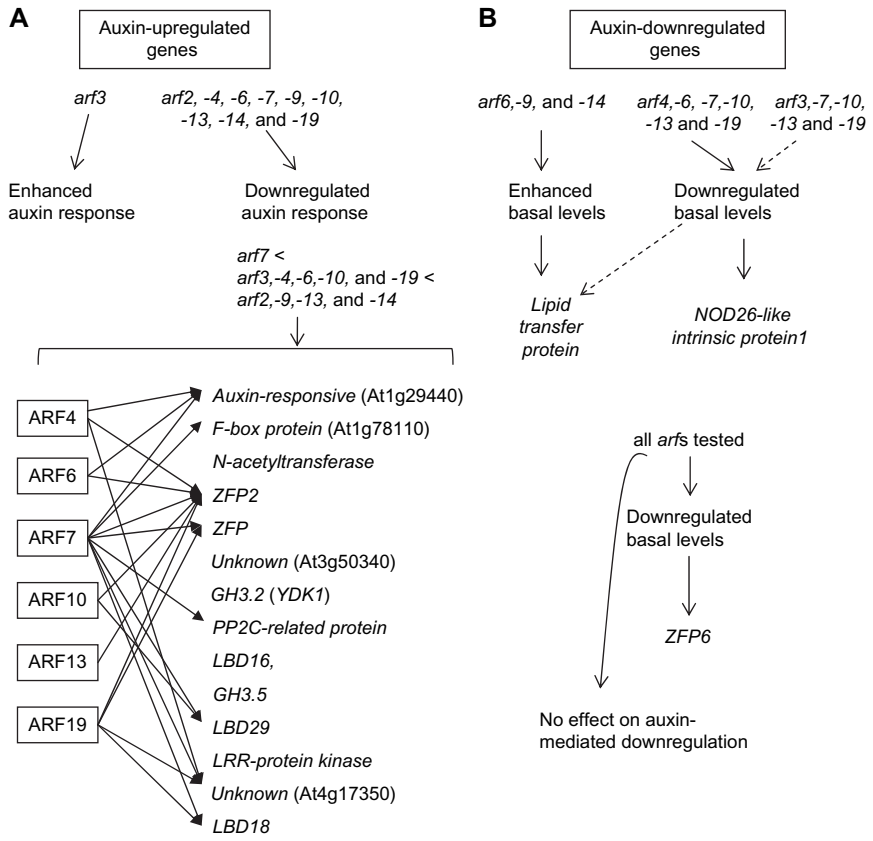

Fig. 8. Schematic summary of expression patterns of iaa1-regulated auxin-responsive genes in various *arf* mutant backgrounds. (A) Expression pattern of auxin-up-regulated genes in *arf* mutants. (B) Expression pattern of auxin-down-regulated genes in *arf* mutants. The results obtained by the expression analysis in Figs 6 and 7 were schematically summarized.

signalling and response. Some of the molecular targets could be due to activation of the mutant iaa1 due to translocation into the nucleus by DEX treatment, as a DEX-triggered appearance of auxin-related phenotypes had been observed by Park *et al.* (2002). Others might be due to the expression of iaa1 in cells that normally do not express this protein. Expression of GUS of Pro_{IAA1}:*GUS* in a variety of tissues (Fig. 1) as well as constitutive expression of *IAA1* in various organs (Abel *et al.*, 1995) suggest that significant portions of the targets identified by microarray could be IAA1-targets, although further molecular characterization is necessary for confirmation of the individual target genes. In addition, cross-regulation and extensive genetic redundancy between Aux/IAA family members (Park *et al.*, 2002; Overvoorde *et al.*, 2005) intrinsically render the identification of the target genes of the individual Aux/IAA proteins complex, especially without any phenotypic changes to be shown in multiple loss-of-function *aux*/*iaa* mutants.

The genes regulated by auxin, which have been identified in the present microarray analysis, are generally consistent with previous reports (Tian *et al.*, 2002; Pufky *et al.*, 2003; Goda *et al.*, 2004; Redman *et al.*, 2004; Okushima *et al.*, 2005*b*; Overvoorde *et al.*, 2005). For example, auxin-regulated expression of a variety of *Aux*/*IAA* genes, *GH3* genes, and *ACS* genes has been observed. However, our microarray analysis highlights different aspects of the auxin-responsive transcriptome, as DEX-inducible iaa1 was used to study the auxin response. This approach eliminates permanent effects of constitutive expression or gain-of-function mutations on plant growth and development throughout the whole plant life cycle and allows us to focus on the effect of iaa1 during auxin action. Thus our data have some similarities as well as differences when compared with microarray analysis from gain-of-function *aux*/*iaa* mutants. In *axr3-1*/*iaa17-1* seedlings, 108 and 78 genes have been identified as repressed or induced, respectively (Overvoorde *et al.*, 2005). For example, the expression of *ARR3*, *ARR5*, *ARR6*, and *ARR7* was suppressed by this mutation. However, the expression profiles of the genes that encode enzymes involved in cytokinin biosynthesis and catabolism were not significantly different from those of the wild type, indicating that a new equilibrium of gene expression, seemingly unrelated to auxin response, is established in the *axr3-1*/*iaa17-1* mutants (Overvoorde *et al.*, 2005). There are significant differences in auxin-regulated gene expression profiles between our present results and these previous reports, and this is possibly because of differences in growth conditions and treatment systems. Similarities as well as significant differences in the negative effects of the *axr3-1*/*iaa17-1* mutation and *iaa1* overexpression have also been observed in auxin-regulated

gene expression profiles. The genes with reduced auxin-induction shared between *axr3-1/iaa17-1* mutants and *Pro$_{35S}$:iaa1:GR* are *IAA5, IAA6*, *IAA11*, *GH3.1*, *GH3.5*, *ARF19*, *LBD16*, *LBD18*, *LBD29*, *EXP4*, *ACS4*, *ACS6*, *ACS8*, *ERS2*, *pectin esterase* (At2g47550), *protein kinase* (At1g77280), *ERF11*, and *GNAT* (At5g67430). The genes with reduced repression shared are *SAUR* (At4g31320), *TIP2;3* (At5g47450), *PIP2;4* (At5g60660), *AGP13* (At4g26320), and *AGP22* (At5g53250). A large number (94 out of 148 genes) of DEX-suppressible auxin-induced genes has been detected in which their expression is also impaired in *nph4-1*, *arf19-1*, and/or *nph4-1 arf19-1* mutants (Okushima *et al.*, 2005*b*) (Table 1). Such shared expression patterns might contribute to the common phenotypes observed among these mutant plants (Leyser *et al.*, 1996; Park *et al.*, 2002; Okushima *et al.*, 2005*b*). Likewise, unshared expression patterns could explain part of their distinct phenotypes or varying degree of phenotypes.

Recent results provide evidence for the involvement of some genes identified by our microarray analysis, particularly transcription factors, in auxin response and plant development. *HEC1* (At5g67060) and other *HEC* genes (*HEC2* and *HEC3*) encoding basic helix-loop-helix (bHLH) proteins were shown to be involved in auxin-mediated control of gynoecium patterning (Gremski *et al.*, 2007). *HEC1* was also found to be up-regulated by auxin (Overvoorde *et al.*, 2005). The AP2 domain-containing transcription factor (At5g18560) named *PUCHI* has been shown to be required for morphogenesis in the early lateral root primordium of *Arabidopsis* (Hirota *et al.*, 2007). Overexpression of *HAT2* (At5g47370), encoding a homeobox-leucine zipper protein, caused the opposite effects on the shoot and root tissues in regulating auxin-mediated morphogenesis (Sawa *et al.*, 2002). Two (At1g18400 and At1g73830) of the three brassinosteroid early response genes, *BR ENHANCED EXPRESSION1*, *-2*, and *-3* (*BEE1*, *BEE2*, and *BEE3*), encoding bHLH proteins, are up-regulated by auxin in our microarray analysis. These genes were found to be functionally redundant positive regulators of BR signalling and also were shown to be up-regulated by other hormones including auxin (Friedrichsen *et al.*, 2002), indicating auxin-mediated positive regulation of BR signalling. PACLOBUTRAZOLE RESISTANCE2 (PRE2) (At5g15160), homologous to PRE1 that has been proposed to play a regulatory role in gibberellin-dependent plant development (Lee *et al.*, 2006), is up-regulated, indicating crosstalk between auxin and gibberellin signalling. Auxin-inducible MYB77 has been shown to modulate auxin signal transduction via an interaction between MYB77 and ARFs (Shin *et al.*, 2007). MYB108 was identified in the present study and might be a possible MYB transcription factor involved in auxin signalling. Genes related to lateral organ development, *LBD16*, *LBD18*, *LBD29*, and *LBD33*, are up-regulated. Previously, *LBD16*, *LBD17*, *LBD18* or *LBD29* have been reported to be up-regulated by auxin (Redman *et al.*, 2004; Okushima *et al.*, 2005*b*; Overvoorde *et al.*, 2005). A recent study showed that lateral root formation of *Arabidopsis* is regulated by ARF7 and ARF19 via direct activation of *LBD16* and *LBD29* (Okushima *et al.*, 2007). The expression of all these genes in response to auxin was suppressed by iaa1, suggesting that these putative transcription factors are likely to be modulated downstream of the Aux/IAA-ARF proteins during plant development. Up-regulation of *PIN1*, encoding an auxin efflux carrier, by auxin (Table 1) has also been reported (Heisler *et al.*, 2005). The rest of the genes that encode transcription factors and signal transduction components need to be investigated further for their potential functional roles in the auxin response.

Four *ACS* genes encoding the key enzymes in ethylene biosynthesis are up-regulated by auxin, consistent with a recent report (Stepanova *et al.*, 2007). Up-regulation of *ACS4, ACS6,* and *ACS8* by auxin has previously been reported (Tian *et al.*, 2002; Goda *et al.*, 2004). We and another group (Okushima *et al.*, 2005*b*) found that *ERS2*, encoding a second subfamily of the ethylene receptors, is also up-regulated. Up-regulation of these *ACS* genes and *ERS2* might contribute to an auxin-mediated response to ethylene effects. Up-regulation of *CKX1* and *CKX7,* responsible for the degradation of cytokinins, and down-regulation of various cytokinin-inducible A-type response regulator genes (*ARR4*, *ARR5*, *ARR6*, and *ARR7*) and a cytokinin receptor gene (*AHK4*) have been observed. In addition to *CKX1* and *CKX7*, *CKX6* is up-regulated by auxin (Overvoorde *et al.*, 2005). Down-regulation of *CKX4* has also been reported (Goda *et al.*, 2004). Thus, auxin plays a complex role in the regulation of cytokinin signal transduction and cytokinin action. It was found that ROT3, involved in the biosynthesis of BRs, is down-regulated, whereas BAS1 that inactivates BRs is up-regulated by auxin. This is consistent with previous observations (Goda *et al.*, 2004) and indicative of an antagonistic regulation of auxin in BR action. The expression of all these hormone-related genes in response to auxin was suppressed by iaa1. These results provide additional evidence for crosstalk between auxin and other plant hormones, ethylene, cytokinin, and brassinosteroids, by auxin-mediated transcriptional response via the Aux/IAA-ARF system.

It is clear that these microarray data will be valuable resources for investigations towards understanding specific auxin responses or a subset of auxin responses as well as hormonal crosstalk. Loss-of-function studies on single or multiple mutants of those identified target genes will also be important, as many auxin responses are constitutively affected by mutations in *Aux/IAA*s or *ARF*s (Okushima *et al.*, 2005*b*; Overvoorde *et al.*, 2005), or can be affected in an inducible way (Park *et al.*, 2002).

Verification of microarray data by RNA-gel blot analysis

RNA-gel blot analysis was used to confirm our microarray data and to analyse the expression characteristics under various conditions so as to further our understanding of auxin-regulated gene expression. Transient expression patterns have been observed from a group of these genes in response to auxin (Fig. 3), indicating a negative feedback

loop on their expression which is reminiscent of the response of A-type *ARR* genes to cytokinin (D'Agostino *et al.*, 2000). Expression of all 18 auxin-up-regulated genes tested from the iaa1-regulated transcriptome was suppressed by DEX treatment. Similarly, auxin-mediated down-regulation was also suppressed by DEX. These results suggest that iaa1 might have promiscuous interactions with a variety of ARFs, resulting in inhibiting ARF functions. Auxin-induced expression of *LBD16*, *LBD18*, and *LBD29* are effectively suppressed by DEX. *LBD16* and *LBD29* have been shown to be involved in lateral root formation (Okushima *et al.*, 2007). DEX treatment greatly reduced lateral root formation in *Pro$_{35S}$:iaa1:GR* plants (Park *et al.*, 2002). Thus simultaneous suppression of those three *LBD* genes to auxin might contribute to a reduction in lateral root numbers. DEX treatment effectively suppressed auxin-inducible expression of two *GH3* genes, typical marker genes for early auxin response, demonstrating the validity of the microarray data.

Expression analysis of iaa1-regulated genes in the presence of cycloheximide indicates the complexity and diversity of auxin-regulated gene expression

Cycloheximide-induced expression of many of the auxin-up-regulated genes examined were suppressed by DEX, consistent with the current model in which labile Aux/IAA repressors mediate transcriptional repression for the ARF proteins that regulate auxin-response genes. Interestingly, cycloheximide-induced expression of some of the iaa1-regulated genes, *N-acetyltransferase*, *LRR-protein kinase*, and *unknown* (At4g17350) was not suppressible by DEX (Figs 4, 5). It has been reported that optimized pairs of interacting Aux/IAA-ARF proteins generate developmental specificity (Knox *et al.*, 2003; Weijers *et al.*, 2005: Muto *et al.*, 2007). It is thus possible that there might be specificity in the protein–protein interactions between Aux/IAA and ARF, and iaa1 might not be able to interact with ARFs that are involved in this gene regulation. This result might also indicate the existence of an additional pathway for auxin-regulated gene expression in addition to the Aux/IAA-ARF pathway. One possibility is that some auxin-responsive genes downstream of Aux/IAA-ARF proteins might require a labile co-repressor for their regulation. If this is true, then iaa1 alone may not suppress cycloheximide-induced gene expression because of the lack of this hypothetical corepressor. The recent identification of the TPL corepressor function in Aux/IAA-ARF-mediated gene regulation during the auxin response (Szemenyei *et al.*, 2008) might support this hypothesis.

ARR5, *methionine sulphoxide reductase*, and *ZFP6*, which are auxin-down-regulated, were super-induced by cycloheximide treatment alone, indicative of the repression of these genes by labile repressors. However, super-induction of these genes by cycloheximide could not be repressed by DEX, suggesting involvement of non-Aux/IAA proteins as the labile repressors in auxin-regulated down-regulation of this gene expression. These results show the complexity and diversity of auxin-regulated gene expression that cannot be explained by the Aux/IAA-ARF system alone.

Expression analysis of the iaa1-regulated genes in arf *mutants suggests iaa1-regulated transcriptome as ARF targets and versatile ARF functions in auxin-regulated gene expression*

The representative iaa1-target genes were auxin-responsive and auxin induction of these genes was repressible by DEX-triggered nuclear translocation of iaa1, suggesting that these genes are regulated by Aux/IAA-ARFs. This proposal is further supported by the analysis showing the presence of AuxREs in the promoter regions of theses iaa1-target genes where ARFs directly bind (Table 1; see Supplementary Table S2 at *JXB* online). To confirm that these genes are regulated by ARFs in the auxin response, the effect of auxin on gene regulation at the early time of 2 h was examined in various *arf* mutants. Expression analysis of the iaa1-regulated genes in a large set of *arf* mutants showed that expression of all the genes tested in response to auxin was affected by any of the *arf* mutations (with varying impacts depending on the genes and *arf* mutations), indicating that each of these ARFs has a role in modulating the expression of auxin-regulated genes in plants. Mutation in *ARF3* caused a super-induction of gene expression to the auxin treatment compared with that of wild-type Col-0, but the rest of the *arf* mutations, *arf2*, *-4*, *-6*, *-7*, *-9*, *-10*, *-13*, *-14*, and *-19*, reduced auxin-induced gene expression, suggesting that ARF3 might function as a negative component and the other ARFs tested might function as positive components in regulating auxin-responsive gene expression. Mutations in *ARF*s greatly overexpressed or reduced the basal levels of *lipid transfer protein* and *NOD26-like intrinsic protein1*, respectively, which are both auxin-down-regulated. Expression analysis of these genes in *arf* mutants indicates that ARF9 and ARF14 function as negative components, while ARF3, -4, -7, -10, -13, and -19 as positive components. Moreover, ARF6 might act as a negative component for *lipid transfer protein* and as a positive component for *NOD26-like intrinsic protein1* in maintaining their basal expression levels. Therefore, ARFs could act either as a transcriptional activator or as a repressor depending on the nature of expression of the auxin-responsive genes. These results suggest that although several steps of positive and/or negative regulation may be involved at any point, ARFs could have versatile roles in auxin-regulated gene expression. This analysis was based on the differences in steady-state RNA levels being monitored. Thus further molecular studies are needed to draw conclusions about the roles of ARFs for the regulation of these genes in response to auxin.

ARF1 to *ARF9* mRNA are each present in roots, rosette leaves, cauline leaves and flowers (Ulmasov *et al.*, 1999*b*). In *Pro$_{ARF1}$:GUS* and *Pro$_{ARF2}$:GUS* lines, GUS staining appeared throughout 8-d-old seedlings and in rosette leaves, as well as in the sepals and carpels of young flower buds (Ellis *et al.*, 2005). GUS expression of *Pro$_{ARF2}$:GUS* was also detected in the vascular tissues and the initiation sites

of lateral roots (Okushima *et al.*, 2005*a*). *ETTIN* (*ARF3*) is expressed in floral organs (Sessions *et al.*, 1997). Low levels of *MP* (*ARF5*) were detected in all major organs in RNA-gel blot analyses (Hardtke and Berleth, 1998). GUS expression of both Pro_{ARF6}*:GUS* and Pro_{ARF8}*:GUS* was detected in flowers at multiple stages (Nagpal *et al.*, 2005). Pro_{ARF7}*:GUS* was expressed in petioles and blades of cotyledons and leaves, and in roots (Wilmoth *et al.*, 2005). The root expression was strongest in the vasculature and in more mature parts of the root, and also appeared in root meristems. Pro_{ARF19}*:GUS* was expressed in vasculature of cotyledons and leaves, stems and roots. Both GUS fusion genes were also expressed in flower organs. Another study has shown that the expression patterns of these two GUS fusion genes are distinct, with partial overlap (Okushima *et al.*, 2005*b*). Strong GUS expression was observed in the hypocotyls and petioles of Pro_{ARF7}*:GUS* seedlings, whereas Pro_{ARF19}*:GUS* expression was restricted to the vascular tissue in the aerial parts. In root tissue, Pro_{ARF19}*:GUS* is strongly expressed throughout the meristematic region, root cap, root hair, and the sites of newly forming lateral toots (Okushima *et al.*, 2005*b*; Li *et al.*, 2006). Pro_{ARF7}*:GUS* expression in the primary root is restricted to the vascular tissues. The GUS expression of various *ARF-GUS* fusions detected in the early stages of seedlings, as well as throughout various tissues, and overlapping expression of iaa1-regulated genes (see Supplementary Fig. S1 at *JXB* online) and *ARF*s further strengthen the argument that various ARFs might be involved in regulating representative iaa1-target genes.

Mutations in the *ARF* genes did not affect auxin-mediated down-regulation *per se*. However, those mutations had significant effects on the basal levels of the down-regulated genes, indicating that these ARFs are involved in maintaining basal levels as positive components or negative components depending on the ARFs and/or target genes, but not in the process of down-regulation itself.

Mutations in *ARF2*, *-9*, *-13*, and *-14* had weak effects on auxin-induced gene expression, whereas mutations in *ARF3*, *-4*, *-6*, *-7*, *-10*, and *-19* had strong effects, suggesting that some ARFs play a major role in auxin-induced gene expression, but others may play an auxiliary or redundant role at the young seedling stage. In particular, *arf7* mutation greatly reduced auxin-inducible expression of a majority of genes examined (Fig. 6). *Auxin-responsive protein* (At1g29440), *F-box protein*, *ZFP2*, *ZFP*, *PP2C-related protein*, *LBD29*, *unknown* (At4g17350), and *LBD18* were completely unresponsive to auxin by *arf7* mutation, suggesting that these genes might be the primary targets for ARF7. Double mutations in ARF7 and ARF19 dramatically reduced auxin-induced expression of all 14 genes examined. The obvious growth phenotype of *arf3* (Sessions *et al.*, 1997), *arf7* (Harper *et al.*, 2000), or *arf7 arf19* (Okushima *et al.*, 2005*b*) mutants correlates with the degree of inhibition of auxin-induced gene expression. Our results showing impaired auxin-induced gene expression in *arf7, arf19,* and *arf7 arf19* mutants generally agree with the global gene expression profiling of these mutants. In the case of *arf19*, RNA-gel blot analysis of a selected set of genes in the present study showed more significant inhibition of auxin-induced gene expression compared with global expression profiling. Noticeable difference in RNA-gel blot data might be due to the sensitive auxin-induced expression of the chosen genes or might be due to different growth conditions and tissue treatment methods. Auxin-induction of 11 out of the 14 auxin-induced genes used for RNA-gel blot analysis in the present study was found to be impaired in *nph4-1*, *arf19-1,* and/or *nph4-1 arf19-1* mutants based on microarray data (Okushima *et al.*, 2005*b*). Expression analysis of auxin-induced genes in *arf7 arf19* mutants using RNA-gel blot analysis in the present study thus confirms the previous microarray data. Three genes (At5g57520, At1g78100, and At4g37390) are absent from the list of genes with reduced auxin induction, and this might be due to the fold-change cut-off used to make the gene list in their microarray analysis.

No obvious growth-phenotype for *arf4*, *-6*, *-10*, or *-19* single mutants had previously been reported (Okushima *et al.*, 2005*b*), although these mutations significantly impaired auxin-induced gene expression in the present study. *arf6* had delayed stamen development and decreased fecundity (Nagpal *et al.*, 2005). *arf19* showed an altered phototropic response and a mild but significant resistance to exogenous auxin in the roots (Okushima *et al.*, 2005*b*). Thus these ARFs may function in specific aspects of the auxin response.

Potential mechanisms for an ARF to act as a positive or a negative component in auxin-regulated gene expression

Our expression analysis in the *arf* mutant backgrounds suggests that ARFs have a positive or a negative function (or both) in auxin-regulated gene expression depending on the target gene. Previous studies with transient gene expression assays in protoplasts showed that, depending on the structure of the middle region, each ARF functions as a transcriptional activator or a repressor (Ulmasov *et al.*, 1999*a*; Tiwari *et al.*, 2003). While the molecular function of ARFs could be revealed in that protoplast reporter gene system, it is still poorly understood how the ARFs regulate their target gene expression in plants. For example, the *arf3* mutation caused enhanced auxin-responsive expression of auxin-induced genes, while expression of auxin-down-regulated genes, *ZFP6* and *lipid transfer protein*, was reduced by this mutation (Fig. 5), suggesting dual functions for ARF3 in its target gene regulation. Another example is the observation that the *arf6* mutation up-regulated *lipid transfer protein,* but down-regulated *NOD26-like intrinsic protein1* in their basal expression levels. It is not without precedent that transcription factors have dual functions as activators or repressors. A variety of studies on transcription factors of mammalian cells such as hormone receptors, Pit-1, and Oct-1 transcription factors have demonstrated that, although some factors are pure activators or repressors, many others can both activate and repress transcription

in a manner that is dependent on the particular situation (Latchman, 2001). For example, Pit-1 can act as either an activator or a repressor of cell-type specific genes depending on the nature of its binding sites. This distinction depends on a two-base-pair hormone promoter site. The allosteric effect on Pit-1, in combination with other DNA binding factors, results in the recruitment of a corepressor N-CoR for active repression of the growth hormone gene in the lactotrope, while it activates growth hormone gene expression in the other cell type, the somatotrope (Scully *et al.*, 2000). It is therefore possible that interaction between ARFs and coactivators or corepressors depending on the sequence variation on the promoters may determine whether the same ARFs activates or represses transcription of a given target gene.

arf7 arf19 *mutants exhibit additionally reduced auxin-induction relative to* tir1/afb *quadruple mutants*

The *arf7 arf19* double mutants exhibited stronger auxin-related phenotypes than those of *arf7* and *arf19* single mutants (Okushima *et al.*, 2005*b*). Consistently, global expression profiling of the *arf7 arf19* double mutants displayed severely impaired auxin-induced gene expression. Likewise, it was found that all of the iaa1-regulated genes tested via RNA-gel blot analyses had severely inhibited auxin-induced gene expression. *tir1 afb2 afb3* triple and *tir1-1 afb1 afb2 afb3* quadruple mutants of auxin receptor genes had reduced auxin-induced gene expression, but significantly less than that of the *arf7 arf19* double mutants. The quadruple mutant showed an extremely strong phenotype in plant growth and development from embryos to adults (Dharmasiri *et al.*, 2005*b*). Even so, the degree of impairment to auxin-induced expression of Aux/IAA-ARF-regulated genes was much milder than that of the *arf7 arf19* double mutants, and was comparable to that of *arf7* or *arf19* single mutants. This may be because two other additional TIR1-like proteins, AFB4 and AFB5, might play significant roles during auxin signalling mediated by the Aux/IAA-ARF proteins. This could also be due to some of the receptor mutants utilized not being complete nulls (Dharmasiri *et al.*, 2005*b*). The other explanation is that the lack of TIR1/AFBs or ARF7 and ARF19 might result in very distinct patterns of gene expression being established and the effect of auxin might then be superimposed onto these patterns.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Quantification of time-course expression of representative auxin-up-regulated genes selected from microarray analysis with auxin or with auxin and DEX.

Supplementary Fig. S2. Quantification of time-course expression of representative auxin-down-regulated genes selected from microarray analysis with auxin or with auxin and DEX.

Supplementary Fig. S3. Quantification of expression analysis of representative auxin-response genes selected from microarray analysis in response to cycloheximide, auxin, and DEX treatment.

Supplementary Fig. S4. Expression analysis of *LBD18* and *CLV1-like protein* in response to cycloheximide, auxin, or DEX treatment.

Supplementary Fig. S5. Quantification of expression profiling of auxin-responsive putative target genes regulated by iaa1 in various *arf* mutant backgrounds.

Supplementary Fig. S6. Quantification of expression profiling of auxin-responsive putative target genes regulated by iaa1 in various *arf* and *tir1/afb* mutant backgrounds.

Supplementary Fig. S7. Organ-specific expression profiles of auxin-responsive genes regulated by iaa1.

Supplementary Table S1. Oligonucleotides and PCR conditions used for RT-PCR and genotyping and DNA probes for RNA-gel blot analysis.

Supplementary Table S2. List of genes examined for AuxREs beyond 2 kbp promoter region or whole promoter region.

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