Protein–protein interactions among the Aux/IAA proteins

(auxin signaling/yeast two-hybrid system/protein cross-linking/β-sheet DNA binding motif/combinatorial interactions

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ABSTRACT The plant hormone indoleacetic acid (IAA) transcriptionally activates early genes in plants. The *Aux/IAA* family of early genes encodes proteins that are short-lived and nuclear-localized. They also contain a putative prokaryotic βαα DNA binding motif whose formation requires protein dimerization. Here, we show that the pea PS-IAA4 and *Arabidopsis* IAA1 and IAA2 proteins perform homo- and heterotypic interactions in yeast using the two-hybrid system. Gel-filtration chromatography and chemical cross-linking experiments demonstrate that the PS-IAA4 and IAA1 proteins interact to form homodimers *in vitro*. Deletion analysis of PS-IAA4 indicates that the βαα containing acidic C terminus of the protein is necessary for homotypic interactions in the yeast two-hybrid system. Screening an *Arabidopsis* λ-ACT cDNA library using IAA1 as a bait reveals heterotypic interactions of IAA1 with known and newly discovered members of the *Arabidopsis Aux/IAA* gene family. The new member IAA24 has similarity to ARF1, a transcription factor that binds to an auxin response element. Combinatorial interactions among the various members of the *Aux/IAA* gene family may regulate a variety of late genes as well as serve as autoregulators of early auxin-regulated gene expression. These interactions provide a molecular basis for the developmental and tissue-specific manner of auxin action.

The plant growth hormone auxin typified by indoleacetic acid (IAA) regulates various aspects of plant growth and development (1, 2). Auxin transcriptionally activates early genes that are thought to be responsible for mediating the various auxin effects (3–5). Four different families of early genes have been identified in various plant species during the last ten years (for review, see ref. 6). The members of the first family encode glutathione *S*-transferases. The second family, represented by GH3, may encode proteins that affect auxin homeostasis (G. Hagen and T. Guilfoyle, personal communication). The *SAUR*s genes are members of the third family, and the function of their encoded polypeptides is unknown. Finally, the *Aux/IAA* gene family, represented by *PS-IAA4*, the most thoroughly studied family member, encodes short-lived nuclear proteins. These proteins contain a putative βαα DNA recognition motif found in the prokaryotic repressors of the Arc family (7). Based on this motif, it has been proposed that the *Aux/IAA* gene products may be transcriptional regulators of late genes responsible for mediating the various auxin responses (7). Structural analysis of the Arc and MetJ repressors has shown that the antiparallel β-sheet responsible for DNA binding is formed by protein homodimerization and that the second helix of the motif is responsible for this interaction (8). There is no evidence to indicate either that the Aux/IAA gene products form homodimers or that they bind DNA. Here we show that the Aux/IAA proteins have the ability to form homodimers *in vivo* using the yeast two-hybrid system (9) and *in vitro* using biochemical experiments. Furthermore, a two-hybrid screen with *IAA1* as bait and a Gal4 transactivation domain-tagged *Arabidopsis* cDNA library reveals that heterotypic interaction occurs among highly divergent Aux/IAA gene products.

MATERIALS AND METHODS

Bacterial, Yeast Strains, and Plant Material. *Escherichia coli* DH5α was used for plasmid propagation. *E. coli* MH4 was used to recover the Gal4 activation domain (Gal4AD) plasmid from yeast (10). Yeast Y190 was used as the host strain for the yeast two-hybrid system (11). *Arabidopsis thaliana* (ecotype Columbia) was germinated and grown in liquid culture as described (12).

Plasmid Construction. Full-length cDNAs of *IAA1* and *IAA2* (*Arabidopsis*) and *PS-IAA4* (pea) were generated by PCR and subcloned in frame into the yeast vectors pGBT9.BS and pGAD.GH giving rise to pGal4BD-*IAA1* (-*IAA2* and -*PS-IAA4*) and pGal4AD-*IAA1* (-*IAA2* and -*PS-IAA4*), respectively. The following deletions of *PS-IAA4* were made by PCR and fused in frame into the Gal4 DNA binding domain (Gal4BD) of pGBT9.BS and into Gal4AD of pGAD.GH; deletion 2 (aa 1–85); deletion 3 (aa 1–137); deletion 4 (aa 86–137); deletion 5 (aa 138–189); and deletion 6 (aa 86–189). All subcloned PCR products were verified by sequencing. The plasmids of Gal4BD-*SNF4* in pAS and Gal4AD-*SNF1* in pACT were the gifts from S. Elledge (13).

Quantitation of β-Galactosidase (β-gal) Activity. β-Gal activity was assayed using chlorophenyl-red-β-D-galactopyranoside (Boehringer Mannheim) as substrate as described (14, 15). β-Gal activity (units) was calculated as follows: OD_{574} of the supernatants $\times$ 1,000/reaction time (min) $\times$ culture volume used for the assay (ml) $\times OD_{600}$ of the culture.

Construction of *Arabidopsis* cDNA Library in λ-ACT. The construction of cDNA library was done essentially as described (13, 16). Poly(A) RNA was isolated from 3-day-old etiolated *Arabidopsis*. First-stranded cDNA was synthesized at 37°C from 5 μg of poly(A) RNA with oligo(dT) and 1,000 units of Superscript (BRL). After the second-strand reaction, the cDNA was precipitated with spermine and washed with spermine wash buffer. The cDNA was dissolved in 40 μl of TE buffer and made flush with T4 DNA polymerase according to the supplier's instructions (New England Biolabs). After inactivation of the enzyme by the addition of 5 μl of 0.5 M EDTA, samples were extracted with phenol/chloroform and precipitated with ethanol. The cDNA was resuspended in 12 μl TE buffer and then ligated with 3 μl of equal mixture of phosphorylated adapters (100 μM) in a total volume of 20 μl at 4°C overnight (see ref. 13 for the sequence of the adapter). The adapters were annealed by heating

for 2 min at 88°C, 10 min at 65°C, 10 min at 37°C, and 5 min at room temperature (17). After ligation, the samples were precipitated by the addition of spermine and washed as described (13). The adapted cDNA was resuspended in 20 μl of TE and electrophoresed on a 1% low melting temperature agarose gel. cDNAs of more than 570 bp in length were gel-purified for ligation into λ-ACT arms. The cDNA prepared from 0.5 μg of poly(A) RNA was ligated to 2 μg of T-filled λ-ACT plasmid DNA (11) in a volume of 5 μl at 4°C overnight and packaged using Gigapack Gold packaging extract (Stratagene). Automatic subcloning conversion of the *Arabidopsis* cDNA library into the plasmid library was performed as described (11). A total of 36 million independent recombinants were obtained. The phage library was amplified in *E. coli* LE392 and stored at −80°C in the presence of 7% dimethyl sulfoxide.

Yeast Transformation and Two-Hybrid Screening of the λ-ACT cDNA Library. Yeast transformation was performed using the PEG/LiAcetate method as described (18). Yeast strain Y190 was first transformed with pGal4BD-*IAA1* followed by transformation with the λ-ACT cDNA library. The transformants were plated on synthetic complete (SC) medium-Trp, Leu, His containing 25 mM 3-aminotriazole (3-AT) (Sigma) and incubated at 30°C for 6–7 days. 3-AT was used to repress the basal activity of the *His3* reporter gene resulting in unspecific background growth in the absence of exogenous histidine (19). His⁺ colonies were assayed for β-gal activity by filter-lift assay (20) and His⁺,LacZ⁺ yeast cells were colony-purified. pACT cDNA plasmids were rescued into *E. coli* MH4 and cDNAs were identified by digestion analysis with *Xho*I. To eliminate the false positives, the rescued pACT cDNAs were retransformed into Y190 containing pGal4BD-*IAA1* or pGal4BD and again tested for *His3* and *LacZ* gene expression. The cDNAs were sequenced using *Taq* FS dye terminator chemistry (Applied Biosystems).

Expression and Purification of (His)₆-Tagged PS-IAA4 and IAA1 Proteins. The full-length cDNAs of *PS-IAA4* and *IAA1* generated by PCR were subcloned into pQE plasmids (Qiagen, Chatsworth, CA) to produce PS-IAA4 and IAA1 with C-terminal (His)₆ tags. The recombinant proteins were expressed in *E. coli* M15[pREP] (Qiagen) at 30°C for 4 h by induction with 1.5 mM isopropyl β-D-thiogalactopyranoside. The bacterial pellets were extracted in 5 ml of ice-cold buffer [50 mM K_2HPO_4/KH_2PO_4, pH 8.0/1 M KCl/0.1 mM DTT/5 mM benzamidin·HCl/1 mM ε-aminocaproic acid/8 mM phenylmethylsulfonyl fluoride/0.1% (wt/vol) leupeptin and pepstatin] per g fresh weight with a French press. The extracts were cleared by centrifugation at $20000 \times g$ for 30 min at 4°C. The supernatants were incubated with 125 μl of Ni-nitrilotriacetic acid (NTA) agarose (Qiagen) per ml at 4°C for 1.5 h. The Ni-NTA agarose was washed with buffer A (50 mM K_2HPO_4/KH_2PO_4, pH 8.0/1 M KCl/0.1 mM DTT/40 mM imidazole) until the flow-through at A_{280} was less than 0.02. The proteins were eluted with the buffer A containing 0.4 M imidazole.

Gel Filtration, Cross-Linking, SDS/PAGE, and Western Blot Analysis. Gel-filtration was performed at 4°C at the flow rate of 0.5 ml/min with buffer containing 50 mM K_2HPO_4/KH_2PO_4, pH 8.0/1 M KCl/0.1 mM DTT using a Superdex 200 column (Pharmacia) connected to fast protein liquid chromatography. The column was calibrated with 250 μl of calibration standard solution containing chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), BSA (67-kDa, monomer; 134-kDa dimer) according to the manufacturer's instruction (Pharmacia) and run with 250 μl of Ni-NTA purified PS-IAA4 and IAA1 proteins (0.1 mg/ml each). For cross-linking experiments, Ni-NTA purified PS-IAA4 (0.1 mg/ml), IAA1 (0.1 mg/ml), or ribonuclease A (0.5 mg/ml) were incubated in buffer A with 0.5 mM bi(sulfosuccinimidyl)suberate (BS³; (Pierce) at 25°C for 1 min in a total volume of 50 μl. Control reactions were carried out without BS³. The reactions were terminated by adding 25 μl of SDS

sample buffer. SDS/PAGE, Coomassie staining, Western blot analysis, and immunodetection of the proteins with a (His)₆ tag-specific mAb (Dianova, Hamburg, Germany) were performed as described (21).
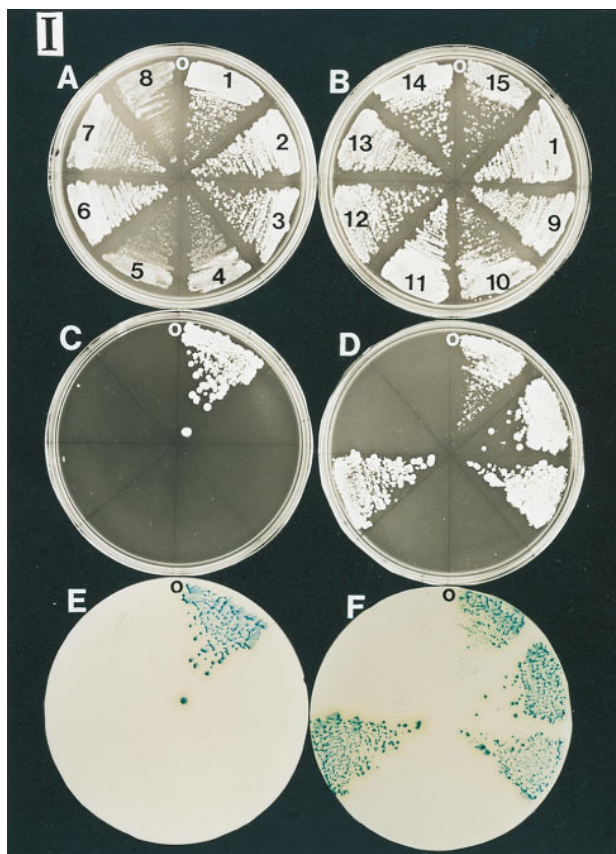
Gal4 DNA Binding Domain (BD)	Gal4 DNA Activating Domain (AD)	SC-His+3-AT 25mM	SC-His+3-AT 100mM	Colony Color	β-Galactosidase Activity (Units)
1. BD-IAA1	AD-IAA1	+++	+++	Blue	296.1
2. BD	--	--	--	White	0.013
3. --	AD	--	--	White	0.010
4. BD	AD	--	--	White	0.039
5. BD-IAA1	--	--	--	White	0.314
6. --	AD-IAA1	--	--	White	0.054
7. BD-IAA1	AD	--	--	White	0.184
8. BD	AD-IAA1	--	--	White	0.061
9. BD-IAA2	AD-IAA2	+++	+++	Blue	102.6
10. BD-IAA2	AD	--	--	White	0.080
11. BD	AD-IAA2	--	--	White	0.044
12. BD-PS-IAA4	AD-PS-IAA4	+++	++	Blue	100.7
13. BD-PS-IAA4	AD	--	--	White	0.056
14. BD	AD-PS-IAA4	--	--	White	0.096
15. BD-SNF4	AD-SNF1	+++	+	Blue	48.4
16. BD-SNF4	AD	--	--	White	0.118
17. BD	AD-SNF1	+/-	--	Pale Blue	0.733
Gal4 (1-881)					11774.3

FIG. 1. Homotypic interactions of IAA1, IAA2, and PS-IAA4 in yeast. (*I*) Growth of yeast transformants on SC (*A* and *B*), SC-His containing 25 mM 3-AT (*C* and *D*), and the colony color of the transformants determined by the filter-lift assay (*E* and *F*). *A*, *C*, and *E* show the homotypic interaction of IAA1. *B*, *D*, and *F* show homotypic interactions of IAA2 and PS-IAA4. The numbers shown on *A* and *B* correspond to the numbers on the left column of *II*. O indicates the orientation of each plate. (*II*) The relative growth, colony color, and β-gal activity of the yeast transformed with the indicated plasmids. Growth on SC-His plates containing 25 and 100 mM 3-AT, respectively, was scored as follows: +++, strong growth; ++, good growth; +, poor growth; +/−, very little growth; −, no growth. The color of the colonies was determined by the filter-lift assay, and β-gal activity was quantified enzymatically (see *Material and Methods*). The enzyme activities are the average of three independent yeast colonies.
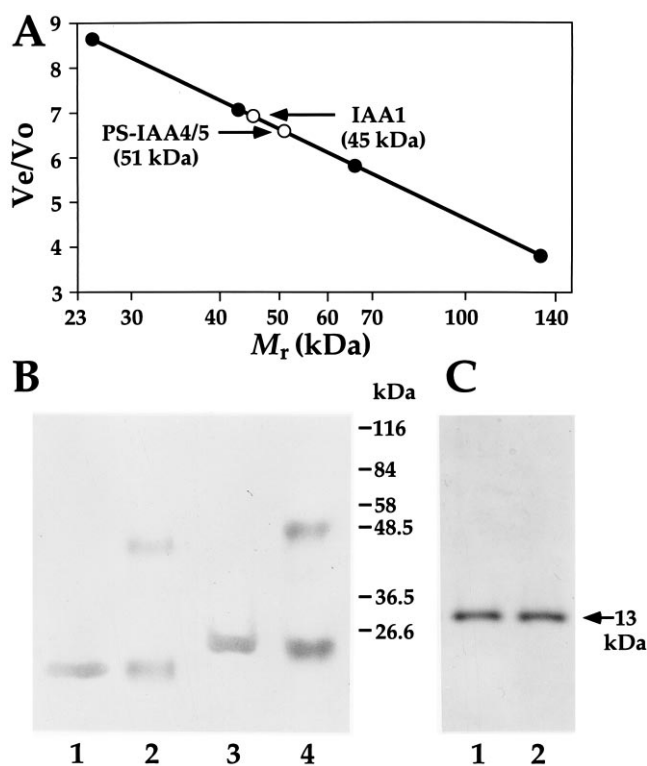
FIG. 2. Homodimerization of IAA1 and PS-IAA4 *in vitro*. (*A*) Elution positions (○) of (His)$_6$-tagged IAA1 and (His)$_6$-tagged PS-IAA4 after gel filtration relative to those of the molecular weight standard (●). Ve/Vo, Elution volume/void volume. The *x* axis is in log scale. (*B*) Cross-linking of (His)$_6$-tagged IAA1 and (His)$_6$-tagged PS-IAA4. Ni-NTA purified (His)$_6$-tagged IAA1 (lanes 1 and 2) and (His)$_6$-tagged PS-IAA4 (lanes 3 and 4) were incubated in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of BS^3 for 1 min at 25°C. After terminating the reactions, the proteins were run on SDS/PAGE gel, transferred to membrane, and immunodetected using a (His)$_6$ tag-specific mAb. Numbers on the right of the blot indicate the size of the molecular weight standards. (*C*) Cross-linking of ribonuclease A. Ribonuclease A, a monomeric protein, was incubated with (lane 2) or without (lane 1) BS^3, run on SDS/PAGE gel, and stained with Coomassie blue. The arrow indicates the position of ribonuclease A (molecular weight, 13 kDa).

RESULTS

Homotypic Interactions of the Aux/IAA Gene Products in the Yeast Two-Hybrid System. We used the yeast two-hybrid system (9) to study homotypic interactions between representative members of the *Aux/IAA* gene family. *IAA1* and *IAA2* from *Arabidopsis* and *PS-IAA4* from pea were fused to the Gal4BD and Gal4AD, respectively, and transformed into the yeast reporter strain Y190 harboring two reporter genes, *His3* and *LacZ*, integrated into the chromosome. SNF4 and SNF1 were used as a positive control (13). The *His3* reporter activity was assayed by testing the growth of the transformants on SC medium lacking histidine and containing 3-AT (see *Material and Methods*). *LacZ* reporter gene expression was monitored by filter-lift assay and quantitative enzymatic assay of β-gal. As shown in Fig. 1 *IC1* and *III1*, the yeast strain that contains the Gal4BD-*IAA1* and Gal4AD-*IAA1* constructs is able to grow on SC medium lacking histidine and containing 100 mM of 3-AT. None of the negative controls is able to grow on SC-His+3-AT (Fig. 1 *IC2–8* and *D10, 11, 13, 14* and *II2–8, 10, 11, 13, 14*). In addition, the color of the colonies in the yeast strain containing Gal4BD-*IAA1* and Gal4AD-*IAA1* is blue in the filter-lift assay (Fig. 1 *IE1* and *III1*), whereas all of the negative controls fail to stain. Furthermore, the β-gal activity is 1,000- to 10,000-fold higher than the activity of the negative controls (Fig. 1 *III1, IE2–8, D10, 11, 13, 14* and *II2–8, 10, 11, 13, 14*). The same results are found for IAA2 and PS-IAA4 (Fig. 1

ID9, 12 and *F9, 12* and *II9, 12*) suggesting that IAA1, IAA2, and PS-IAA4 can perform homotypic interactions in yeast.

Homodimerization of IAA1 and PS-IAA4 *in Vitro*. To test the physical association and the oligomerization of IAA1 and PS-IAA4, the corresponding (His)$_6$-tagged proteins were expressed in *E. coli*, purified on an Ni-NTA column, and analyzed by gel filtration and chemical cross-linking. Fig. 2*A* shows the elution positions of IAA1-(His)$_6$ and PS-IAA4-(His)$_6$ after gel filtration on a Superdex 200 column precalibrated with molecular weight standards (see *Materials and Methods*). IAA1-(His)$_6$ as well as PS-IAA4-(His)$_6$ elute as single peaks of an apparent molecular weight of 45 kDa and 51 kDa, respectively, corresponding to their dimeric size. To confirm the gel filtration data the IAA1-(His)$_6$ and PS-IAA4-(His)$_6$ proteins were cross-linked with the non-cleavable chemical cross-linker BS^3. Cross-linked protein bands of the dimeric size appear when BS^3 is present during incubation

Gal4 DNA Binding Domain (BD)	Gal4 DNA Activating Domain (AD)	SC-His+3-AT 25mM	SC-His+3-AT 100mM	Colony Color	β-Galactosidase Activity (Units)
BD-1	AD-1	+++	++	Blue	100.7
BD-2	AD-2	--	--	White	0.124
BD-1	AD-2	--	--	White	0.033
BD	AD-2	--	--	White	0.054
BD-2	AD	--	--	White	0.027
BD-3	AD-3	--	--	White	0.034
BD-1	AD-3	+/-	+/-	White	0.039
BD	AD-3	--	--	White	0.004
BD-3	AD	--	--	White	0.004
BD-4	AD-4	--	--	White	0.010
BD-1	AD-4	--	--	White	0.042
BD	AD-4	--	--	White	0.051
BD-4	AD	--	--	White	0.019
BD-5	AD-5	++	+	Blue	10.3
BD-1	AD-5	--	--	White	0.049
BD	AD-5	--	--	White	0.056
BD-5	AD	++	+	Blue	32.3
BD-6	AD-6	++	++	Blue	4.08
BD-1	AD-6	++	++	Blue	11.48
BD	AD-6	- -	- -	White	0.054
BD-6	AD	- -	- -	White	0.069

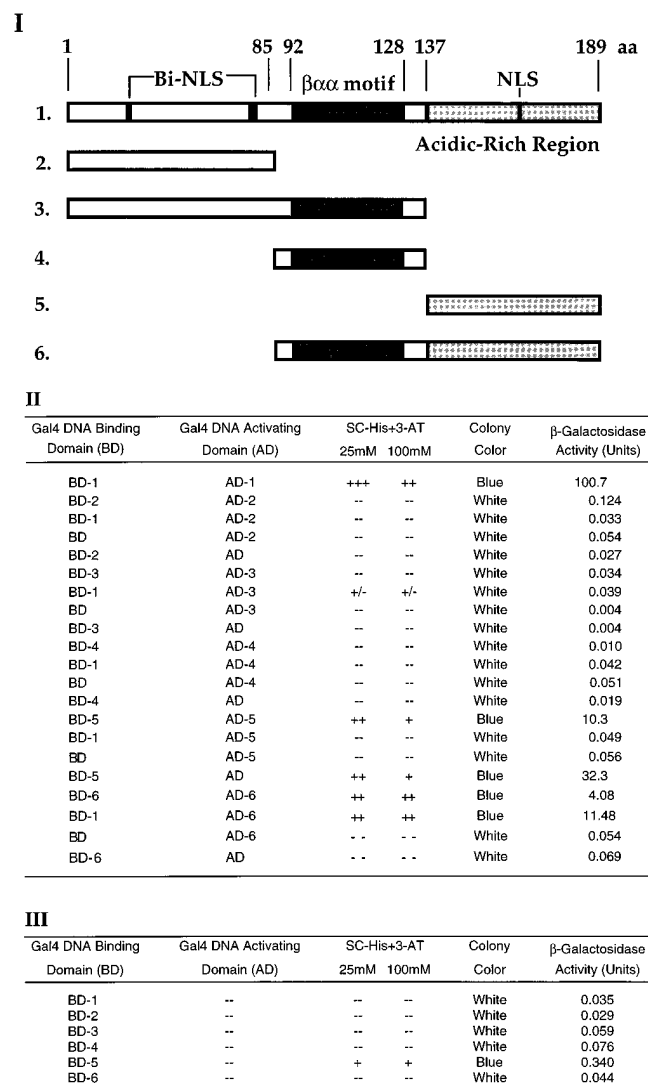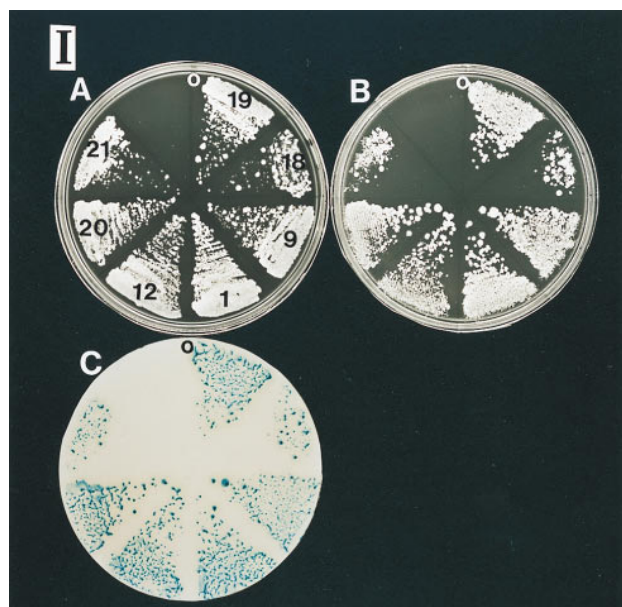| Gal4 DNA Binding Domain (BD)	Gal4 DNA Activating Domain (AD)	SC-His+3-AT 25mM	SC-His+3-AT 100mM	Colony Color	β-Galactosidase Activity (Units)
BD-1	--	--	--	White	0.035
BD-2	--	--	--	White	0.029
BD-3	--	--	--	White	0.059
BD-4	--	--	--	White	0.076
BD-5	--	+	+	Blue	0.340
BD-6	--	--	--	White	0.044

FIG. 3. Interaction domain analysis of PS-IAA4 in the yeast two-hybrid system. (*I*) Schematic diagram showing the deletion constructs of PS-IAA4 (constructs 1–6). The numbers indicate the positions of the deletions (constructs 2–6) with regard to full-length PS-IAA4 (construct 1). The DNA sequences encoding the different peptides were fused in-frame to the DNA binding domain and transactivation domain of Gal4, respectively, and the constructs were transformed into Y190. Cells were grown on SC-His plates containing 25 and 100 mM 3-AT, respectively, and assayed for colony color and β-gal activity. (*II* and *III*) The tables show the relative growth, colony color, and β-gal activity of yeast cells containing the indicated plasmids.

II

Gal4 DNA Binding Domain (BD)	Gal4 DNA Activatiion Domain (AD)	SC-His+3-AT 25mM	100mM	Colony Color	β-Galactosidase Activity (Units)
18. BD-IAA1	AD-IAA2	+++	++	Blue	68.3
19. BD-IAA1	AD-IAA1	+++	+++	Blue	140.1
20. BD-IAA1	AD-PS-IAA4	+++	+++	Blue	84.2
21. BD-PS-IAA4	AD-IAA1	++	+	Blue	32.5

FIG. 4. Heterotypic interaction of IAA1 with IAA2 and PS-IAA4. (*I*) Growth of yeast cells transformed with the indicated plasmids on SC plates (*A*), SC-His plates containing 25 and 100 mM 3-AT, respectively (*B*), and the colony color of the transformants determined by filter-lift assay (*C*). The numbers shown on *A* correspond to the numbers of the plasmids shown on the left column of *II* in this figure and in Fig. 1. (*II*) The table shows the relative growth, colony color, and β-gal activity of the yeast cells transformed with the indicated plasmids.

(Fig. 2*B*, compare lanes 2 and 4 with lanes 1 and 3, respectively). The monomeric protein ribonuclease A does not form any oligomeric structures in the presence of BS^3 indicating the specificity of the cross-linking reaction for the Aux/IAA proteins (Fig. 2*C*, compare lane 2 with 1). These experiments are in agreement with the *in vivo* ones and suggest that IAA1-(His)$_6$ and PS-IAA4-(His)$_6$ physically interact and form stable homodimers.

Dimerization Domain Analysis of PS-IAA4. According to the structure of the $\beta\alpha\alpha$ domain in the MetJ and Arc repressors of *E. coli*, the β-strands pair to form an antiparallel DNA-binding β-sheet. The second helix of the $\beta\alpha\alpha$ domain is involved in dimerization whereas the first helix mediates cooperative binding of dimer units (8). The putative $\beta\alpha\alpha$ domain of the Aux/IAA proteins may also be responsible for their *in vivo* and *in vitro* dimerization properties. To test this possibility, we constructed different deletions of PS-IAA4 (Fig. 3*I*) and analyzed them in the yeast two-hybrid system. As shown in Fig. 3*II*, the $\beta\alpha\alpha$ motif alone (peptide 4)and the N-terminal peptide containing the $\beta\alpha\alpha$ motif (peptide 3) do not show any reporter gene activity. In contrast, the C-terminal peptide containing the $\beta\alpha\alpha$ motif (peptide 6) is capable of interacting with itself as well as with the full-length protein. The C-terminal peptide without the $\beta\alpha\alpha$ domain (peptide 5) also is capable of reporter gene expression. However, this activity is not due to specific protein–protein interaction but to intrinsic transcriptional activity which seems to be masked in the full-length PS-IAA4 protein (Fig. 3 *II* and *III*). To exclude the possibility that the differential lack of reporter gene activity may be due to differential stability of the tested peptides in yeast we carried out immunoprecipitation and Western blot analyses using yeast crude extracts and a polyclonal antibody against the

Gal4BD. Although the expression of full-length PS-IAA4 as well as of peptides 5 and 6 could easily be demonstrated, neither the $\beta\alpha\alpha$ peptide 4 nor peptide 3 (data not shown) could be detected. Thus, although the $\beta\alpha\alpha$ domain is present in all interacting peptides, we cannot conclude from the data of Fig. 3 that this domain is necessary and sufficient for the dimerization of PS-IAA4 (see *Discussion*).

Heterotypic Interaction of IAA1, IAA2, and PS-IAA4. Because all the characterized Aux/IAA gene products contain the postulated $\beta\alpha\alpha$ motif (22), we tested putative heterotypic interactions of IAA1, IAA2, and PS-IAA4 in the yeast two-hybrid system. These studies show that heterotypic interactions occur between IAA1 and IAA2 as well as between IAA1 and PS-IAA4 (Fig. 4). When the cDNA fusion is switched from the Gal4BD to Gal4AD or *vice versa*, the *His3* and *LacZ* reporters are still activated. These results using such reciprocal hybrid proteins indicate that reporters reflect the physical associations between different AUX/IAA proteins (23).

Generation of a λ-ACT Gal4AD-Tagged cDNA Library from *Arabidopsis* and Two-Hybrid Screening Using IAA1 As Bait. To explore the possibility that novel proteins may interact with the Aux/IAA gene products and therefore might be involved in auxin signaling, a Gal4 transactivation domain-tagged cDNA library from 3-day-old etiolated *Arabidopsis* seedling was constructed using the λ-ACT system. Because the λ-ACT system utilizes *cre–lox*-mediated site-specific recombination to convert the Gal4AD DNA from phage to plasmid form, it allows the generation of large cDNA libraries with a high percentage of inserts (16). Our *Arabidopsis* library contains 3.6×10^7 recombinants with 95% of the clones containing inserts. The library was amplified, converted to plasmid form, and used for a yeast two-hybrid screening with the IAA1 as a "bait." From a total of 3.6 million transformants, 123 His^+-$LacZ^+$ clones were obtained. Among the 123 clones, 89 pACT cDNA plasmids were rescued in *E. coli*. After retransformation of the pACT cDNA plasmids into yeast reporter strain containing Gal4BD-*IAA1* or Gal4BD, and

Table 1. Proteins encoded by the cDNAs isolated from the yeast two-hybrid screening using IAA1 as bait

Group	cDNA	No. of clones*	GenBank accession no.	Colony color BD-IAA1	BD
Group I	*IAA2*	3	L15449	Blue	White
	IAA3	6	U18406	Blue	White
	IAA4	8	L15450	Blue	White
	IAA8	7	U18410	Blue	White
	IAA9	2	U18411	Blue	White
Group II	*IAA16*	20	U49072	Blue	White
	IAA17	16	U49073	Blue	White
	IAA18	2	U49074	Blue	White
	IAA19	1	U49075	Blue	White
	IAA20	11	U49076	Blue	White
	IAA21	2	U49077	Blue	White
	IAA22	4	U53672	Blue	White
	IAA23	1	U79555	Blue	White
	IAA24	1	U79557	Blue	White
	IAA25	1	U79556	Blue	White

The λ-ACT *Arabidopsis* cDNA library was screened with IAA1 as bait using the two-hybrid system. Plasmids of clones which gave a His^+ and $LacZ^+$ phenotype were retransformed into yeast strains containing either Gal4BD-*IAA1* or Gal4BD and tested again for *His3* and *LacZ* reporter gene expression. The cDNAs of constructs which produced a His^+ and $LacZ^+$ phenotype with BD-*IAA1* but not with Gal4BD were sequenced and classified into two groups. Group I includes known Aux/IAA homologues and group II includes previously unidentified members of the Aux/IAA gene family. IAA17 is identical to the F19P19.31 gene recently identified by genomic sequencing of BAC F19P9 (GenBank accession no. AC0000104).
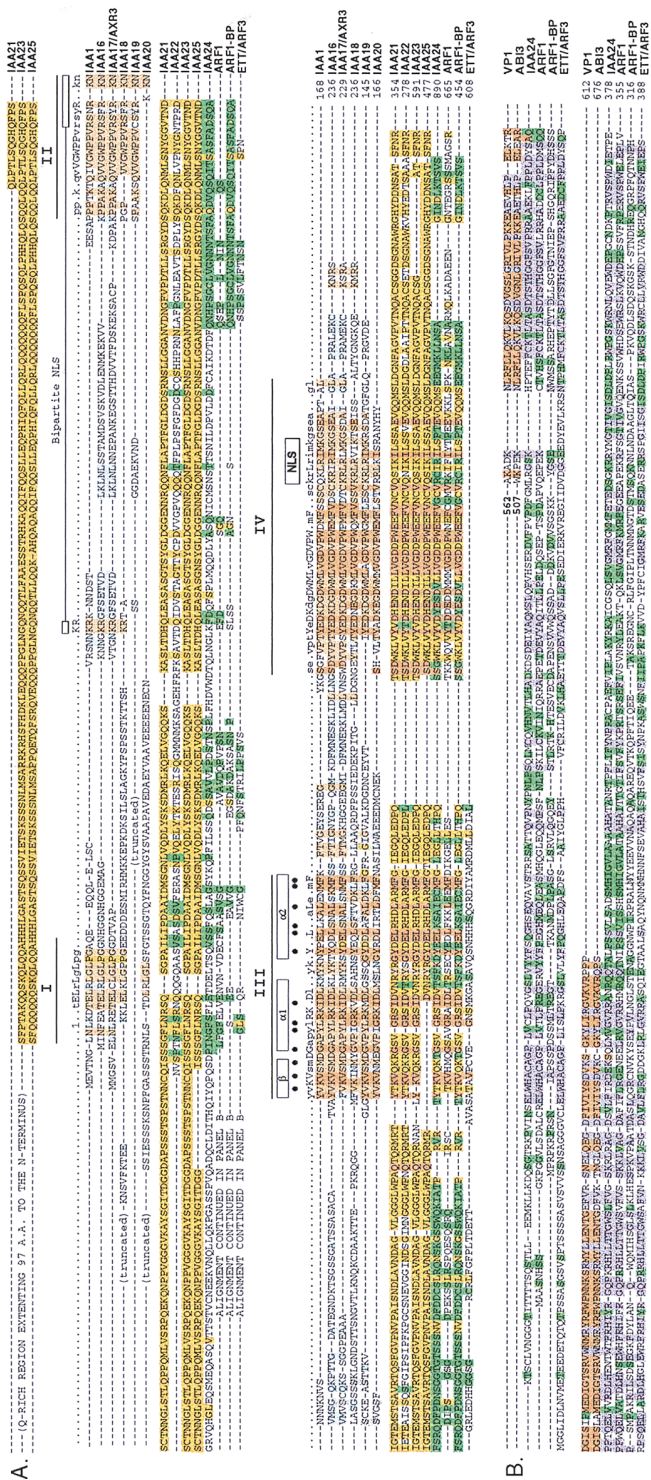*Number of independently isolated clones encoding identical Aux/ IAA polypeptides.

A.

B.

again monitoring for the His$^+$-LacZ$^+$ phenotype, 85 clones were considered true positives and sequenced. Table 1 shows the isolated cDNAs can be classified based on their sequences into two groups. Group I contains previously identified *IAA* genes, such as *IAA2, IAA3, IAA4, IAA8,* and *IAA9*. All these cDNAs were independently isolated multiple times and contain different 5′ ends. The detection of *IAA2, 3,* 4, 8, and 9 with *IAA1* as a bait strongly indicates that heterotypic interactions among the early auxin-induced Aux/IAA gene products are prevalent in *Arabidopsis*. Group II contains 10 previously unidentified *Aux/IAA*-like cDNAs, named *IAA16–25*. Fig. 5*A* shows their amino acid alignment compared with that of IAA1, a "classical" Aux/IAA protein. The recent discovery that mutations in domain II of IAA17 are responsible for the *AXR3* mutant (O. Leyser, personal communication) offers genetic evidence for the functional significance of the AUX/IAA proteins in auxin signaling. The IAA16–19 proteins contain all the structural and functional elements described for the classical Aux/IAA proteins (23), except for IAA20, which does not have domain II [bipartite nuclear localization signal (NLS)] and has a less-conserved simian virus 40-type NLS. IAA21–25 are larger and highly divergent in the N terminus compared with the other Aux/IAA proteins. The N-terminal conserved domains I and II are absent and their N termini do not have any homology to the Aux/IAA proteins. Furthermore, both NLSs are missing and the putative $\beta\alpha\alpha$ motif is present in all of them except for IAA25 where the β-sheet is absent (Fig. 5*A*). The N termini of the IAA23–25 proteins are rich in glutamic acid. Sequence alignment shows that IAA21, 23, and 24 are quite similar in the N terminus (Fig. 5*A*) and may constitute members of a new class of transcriptional regulators. The absence of the glutamate-rich domain in IAA21 may be due to a truncated cDNA. The N terminus of IAA22 is quite different from all the newly discovered members and may also be a member of another class of transcription factors. It remains to be determined whether these newly discovered Aux/IAA-like gene products IAA16–25 are nuclear localized and auxin-regulated. Recently, it was discovered that the IAA24 protein shares extensive amino acid identity with the auxin response factor 1 (ARF1) (Fig. 5 *A* and *B*), a transcription regulator that binds to an early auxin responsive element (37). In addition, IAA24 has amino acid identity with the N termini of the ARF1, ARF1-BP, and ETT/ARF3 gene products (37, 38). The C terminus of IAA24 is similar to the C termini of ARF1 and its binding protein ARF1-BP where the $\beta\alpha\alpha$ containing domains III and IV are located (Fig. 5*A*). In addition, the N terminus of IAA24 is similar to the N termini of the $\beta\alpha\alpha$-containing ARF1 and ARF1-BP proteins and also to the non-$\beta\alpha\alpha$ containing ETT/ARF3 protein (ref. 38; Fig. 5*B*). This N-terminal region is similar to the C terminus of the maize transcriptional activator Viviparous-1 (VP1; ref. 39) and its *Arabidopsis* homolog ABI3 (40). Thus, IAA24, ARF1, and ARF1-BP proteins consist of a new class of transcription factors that contain the prokaryotic $\beta\alpha\alpha$ and the eukaryotic VP1/ABI3 motifs. The ETT/ARF3 protein is responsible for patterning *Arabidopsis* floral meristems and reproductive organs (38).

DISCUSSION

In this communication we describe the homo- and heterotypic interactions of Aux/IAA proteins from *Arabidopsis* and pea in yeast. Cross-linking and gel filtration experiments also demonstrate the formation of stable dimers *in vitro* suggesting that these proteins may also form dimers *in planta*. The deletion analysis of PS-IAA4 as well as the structure of the *Aux/IAA* cDNAs obtained by the two-hybrid screen indicate that the postulated $\beta\alpha\alpha$ domain is a prerequisite for their stable dimer formation. Due to the instability of some of the tested fusion peptides in yeast, it is not clear whether the $\beta\alpha\alpha$ domain is sufficient for

FIG. 5. (*A*) Sequence alignment and domain structure of Aux/IAA proteins isolated by two-hybrid screening compared with those of ARF1, ARF1-BP, and ETT/ARF3 (37, 38). Identical and conserved amino acid residues (at least 50% matches) are shaded (light brown) and appear in the consensus (capital and small letters, respectively). Conserved domains are underlined and indicated by Roman numerals. Amino acid residues conserved within a distinct lineage are colored. Amino acid residues that may form hydrophobic surfaces in the predicted conserved amphipathic $\beta\alpha\alpha$ motif are indicated by (●). NLS indicates the nuclear localization signal. The numbers at the right of the sequences indicate the number of amino acid residues for the corresponding coding region of the cDNA. The consensus sequence shown at the top of the alignment was obtained from the IAA1–15 proteins (34). IAA1, the prototype Aux/IAA protein, is shown below the consensus. (*B*) Similarity of the N-terminal region of IAA24,

ARF1, ARF1-BP, and ETT/ARF3 with a portion of the C-terminal region of VP1 (39) and ABI3 (40).

proper *in vivo* dimerization. However, gel filtration experiments performed with a set of purified recombinant (His)$_6$-tagged PS-IAA4 peptides similar to those tested in yeast reveals that the putative $\beta\alpha\alpha$ domain is responsible for stable *in vitro* dimerization (K.H. and A.T., unpublished data). These results are in agreement with our proposition that the $\beta\alpha\alpha$ domain of the Aux/IAA gene products is structurally related to that of the dimeric prokaryotic transcriptional repressors MetJ and Arc (8). Furthermore, because IAA24 lacks the β-sheet motif and a substantial portion of the $\alpha1$ helical region, but still is capable of forming a dimer with IAA1, the α_2 helical region of the $\beta\alpha\alpha$ domain may be critical for dimerization. This finding is consistent with the crystal structures of MetJ and Arc where the α_2 helix from each monomer packs against each other and forms direct contacts in the dimer. In addition*, in vitro* and *in vivo* binding site selection assays reveals that the full-length IAA1 protein and its $\beta\alpha\alpha$ domain have sequence-specific DNA-binding activity (S. Abel and A.T., unpublished data). PS-IAA4 also contains a C-terminal acidic domain that is able to transactivate the *His3* and *LacZ* reporter genes when fused to the Gal4 DNA-binding domain (Fig. 3). However, this activity is masked when the intact protein is expressed in yeast (Fig. 3). These data strongly indicate that the nuclear localized Aux/IAA gene products may be transcriptional regulators capable of forming homo- and heterodimers.

The finding that the *Aux/IAA* gene family is comprised of homo- or heterodimeric transcription factors has important implications for their putative role in auxin signal transduction. Intensive studies of the basic leucine zipper (bZIP), basic helix-loop-helix (bHLH), and the ligand-dependent nuclear transcription factor families suggest that dimerization between different members can provide an enormous repertoire of different regulatory proteins (24–26). First, homo- and heterodimeric complexes between different polypeptides can bind with significantly different efficiency to DNA (27). Second, dimer formation can influence the DNA-binding specificity of homo- or heterodimeric complexes (25, 28). Third, a missing or altered DNA-binding domain in one of the polypeptides of the heterodimeric complex can produce a dominant negative regulator (29–31). Fourth, homo- and heterodimeric complexes can have different affinities to corepressors or cotransactivators (32). Our results suggest a similar regulatory potential by dimerization/heterodimerization of the Aux/IAA proteins, though these need to be confirmed by *in planta* studies. The possibility exists that the Q-rich region of IAA23–25 may be involved in direct protein interaction with the basal transcriptional machinery allowing them to act as strong transcriptional activators in response to auxin for certain subsets of late genes. This proposition is supported by the recent finding that IAA24 is similar to ARF1, a transcriptional regulator that binds to an early auxin responsive element (37).

The large size of the *Aux/IAA* gene family (at least 25 members) and the ability of its members to homo- and heterodimerize constitutes a vast potential for regulation of late or downstream genes. This proposition is supported by a simple calculation using the formula, $C = n(n + 1)/2!$, where $n = 25$ (the number of different Aux/IAA proteins) and $C =$ the number of dimers. It indicates that 325 different homo- and heterodimers can be formed. If the Aux/IAA products bind to DNA as tetramers like the Arc family of prokaryotic repressors (8), then 20,475 different tetramers can be formed as indicated by the formula, $C = n(n + 1)(n + 2)(n + 3)/4!$. In addition, based on their differential mode of expression with respect to timing, hormone concentration, and spatially restricted expression pattern (33–36), we propose that tissue- and cell-specific subsets of homo- and heterodimeric Aux/IAA transcription factors are responsible for the differential gene expression pattern of late auxin-regulated genes. These interactions therefore establish the highly pleiotropic and complex physiological and morphological auxin responses during plant development.

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