

The *PS-IAA4/5*-like Family of Early Auxin-inducible mRNAs in *Arabidopsis thaliana*

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The plant hormone auxin transcriptionally activates early genes. We have isolated a 14-member family of DNA sequences complementary to indoleacetic acid (IAA)-inducible transcripts in *Arabidopsis thaliana*. The corresponding genes, *IAA1* to *IAA14*, are homologs of *PS-IAA4/5* and *PS-IAA6* from pea, *Aux22* and *Aux28* from soybean, *ARG3* and *ARG4* from mungbean, and *AtAux2-11* and *AtAux2-27* from *Arabidopsis*. The members of the family are differentially expressed in mature *Arabidopsis* plants. Characterization of *IAA* gene expression in etiolated seedlings demonstrates specificity for auxin inducibility. The response of most family members to IAA is rapid (within 4 to 30 minutes) and insensitive to cycloheximide. Cycloheximide alone induces all the early genes. Auxin-induction of two late genes, *IAA7* and *IAA8*, is inhibited by cycloheximide, indicating requirement of protein synthesis for their activation. All *IAA* genes display a biphasic dose response that is optimal at 10 μ M IAA. However, individual genes respond differentially between 10 nM and 5 μ M IAA. Expression of all genes is defective in the *Arabidopsis* auxin-resistant mutant lines *axr1*, *axr2* and *aux1*.

The encoded polypeptides share four conserved domains, and seven invariant residues in the intervening regions. The spacers vary considerably in length, rendering the calculated molecular mass of IAA proteins to range from 19 kDa to 36 kDa. Overall sequence identity between members of the family is highly variable (36 to 87%). Their most significant structural features are functional nuclear transport signals, and a putative $\beta\alpha\alpha$ -fold whose modeled three dimensional structure appears to be compatible with the prokaryotic β -ribbon DNA recognition motif. The data suggest that auxin induces in a differential and hierarchical fashion a large family of early genes that encode a structurally diverse class of nuclear proteins. These proteins are proposed to mediate tissue-specific and cell-type restricted responses to the hormone during plant growth and development.

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Introduction

The growth hormone auxin typified by IAA, appears to control a plethora of processes during the life-cycle of a plant such as apical dominance, tropisms, vascular tissue differentiation, or lateral

and adventitious root formation (Went & Thimann, 1937). The hormone profoundly effects cell turgor, cell elongation, cell division and cell differentiation, the shaping forces in morphogenesis and oncogenesis (Went & Thimann, 1937; Estelle, 1992). At the molecular level, auxin exerts its regulatory role most likely by modulating gene expression (Guilfoyle, 1986; Theologis, 1986) and membrane function (Blatt & Thiel, 1993; Assmann, 1993). Despite its fundamental role in plant growth and development, the molecular mechanisms of auxin action are not understood.

Auxin-mediated cell elongation is one of the fastest hormonal responses known (with a lag period of 15 to 25 minutes) and is associated with rapid changes

Abbreviations used: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylate; α -NAA, naphthalene-1-acetic acid; BA, benzyl adenine; CHX, cycloheximide; EST, expressed sequence tag; GA, gibberellic acid; GUS, β -glucuronidase; IAA, indole-3-acetic acid; NLS, nuclear localization signal; ORF, open reading frame; PAA, phenylacetic acid; PCR, polymerase chain reaction; SAUR, small auxin up RNA; 2,4-D, 2,4-dichlorophenoxyacetic acid.

in the expression of a select set of early genes (Guilfoyle, 1986; Theologis, 1986). We have been focusing our efforts to understand how auxin promotes plant cell growth using two early auxin-induced genes, *PS-IAA4/5* and *PS-IAA6*, from pea. Early genes have successfully been used as bifocal molecular probes to elucidate signal transduction pathways of growth factors in animal cells (Herschman, 1991). Their promoter response elements provide a platform for exploring the intervening steps between gene activation and signal perception, whereas their encoded products most likely play a crucial role in downstream signaling events (Herschman, 1991).

The *PS-IAA4/5* and *PS-IAA6* genes qualify as primary response genes (Theologis *et al.*, 1985). Both genes have been structurally characterized (Oeller *et al.*, 1993), and the auxin-responsive promoter element of *PS-IAA4/5* has been functionally identified (Ballas *et al.*, 1993, 1995). The encoded proteins, PS-IAA4 and PS-IAA6, share similar physical properties and extensive amino acid sequence identity in four domains, which are conserved in polypeptides encoded by related early auxin-inducible genes, *Aux22* and *Aux28* from soybean (Ainley *et al.*, 1988), *ARG3* and *ARG4* from mung bean (Yamamoto *et al.*, 1992), and *AtAux2-11* and *AtAux2-27* from *Arabidopsis* (Conner *et al.*, 1990). PS-IAA4 and PS-IAA6 are short-lived nuclear proteins that contain functional NLS and a putative $\beta\alpha\alpha$ -fold reminiscent of the β -ribbon DNA recognition motif of prokaryotic repressor polypeptides (Abel *et al.*, 1994; Abel & Theologis, 1995; Oeller & Theologis, 1995). These properties suggest a regulatory function of PS-IAA4-like polypeptides in auxin-induced events responsible for plant cell growth. However, no direct evidence has been obtained thus far.

As for any protein, the most appropriate approach to study the biological function of PS-IAA4-like polypeptides is complete inhibition of their expression by targeted gene replacement (Scherer & Davis, 1979). Since this technique is not applicable yet for plants, reverse genetic approaches such as construction of mutants by overexpressing sense or antisense RNA to manipulate levels of gene expression (Gray *et al.*, 1992; Napoli *et al.*, 1990) or by insertional mutagenesis (Osborne & Baker, 1995) are the current methods used. To study the function of *PS-IAA4/5*-like gene products by genetic and reverse genetic means, *Arabidopsis* is the plant of choice (Meyerowitz, 1989).

As a first step toward this long-term goal, we describe the isolation, structure and expression characteristics of a large family of *PS-IAA4/5*-like transcripts in *Arabidopsis thaliana*, designated *IAA1* to *IAA14* for IAA-inducible. This family provides a source of diverse probes to elucidate the function of PS-IAA4-like proteins in auxin-regulated growth processes by reverse genetic, molecular and biochemical approaches. The putative function of this divergent family of nuclear proteins in auxin signal transduction is discussed.

Results

Identification of a *PS-IAA4/5*-like gene family in *Arabidopsis*

A recent immunochemical study indicates that PS-IAA4-like proteins are encoded by a small multigene family in various plant species (Oeller & Theologis, 1995). To identify additional *PS-IAA4/5*-like genes in *Arabidopsis*, we applied a PCR-based approach using degenerate oligonucleotides, DC2 and DC4, corresponding to discrete and highly conserved regions in domain II and domain IV of PS-IAA4-like polypeptides (see Figure 10).

Amplification of PS-IAA4/5-like sequences from various plant species

A complex but similar pattern of multiple DNA fragments is amplified in a PCR with genomic DNA from various plant species (Figure 1, panel IA). The PCR products of *Arabidopsis*, cauliflower, pea, tomato, maize and rice range in size from 240 bp to about 1 kb (lanes 2 to 7). The product pattern is notably similar between *Arabidopsis* and cauliflower, members of the Cruciferae family (compare lane 2 with lane 3), or between maize and rice, members of the Graminae family (compare lane 6 with lane 7). Southern blot hybridization was performed with a degenerate oligomer, DC3, which corresponds to the motif KVSM/V/I/DG in domain III of PS-IAA4-like proteins (see Figure 10). The probe recognizes a distinct set of amplified DNA fragments for each plant species tested (Figure 1, panel IB, lanes 2 to 7). This indicates that PS-IAA4-like polypeptides are encoded by multigene families in monocotyledonous and dicotyledonous plants.

Classification of the Arabidopsis PCR products

Twelve major DNA fragments ranging in size from 250 bp to 550 bp, were reproducibly amplified in a PCR with *Arabidopsis* genomic DNA (Figure 1, panel IA, lane 2). DNA inserts of 72 transformants were characterized by cross-hybridization at high stringency, revealing 12 classes of non-cross-hybridizing DNA fragments. DNA sequencing identified 11 unique fragments related to *PS-IAA4/5*-like genes. Using a pool of these 11 genomic fragments as a probe, subtractive screening by colony hybridization of an additional 280 transformants identified another three unique DNA fragments of the *PS-IAA4/5*-type. The amino acid sequence deduced from each of the 14 genomic DNA products is similar to the region between domain II and domain IV of PS-IAA4-like proteins (see Figure 10). Five *PS-IAA4/5*-type genomic fragments contain a single ORF, whereas a putative intron interrupts the coding region of the other nine gene fragments at a conserved position (Table 1; and see Figure 10).

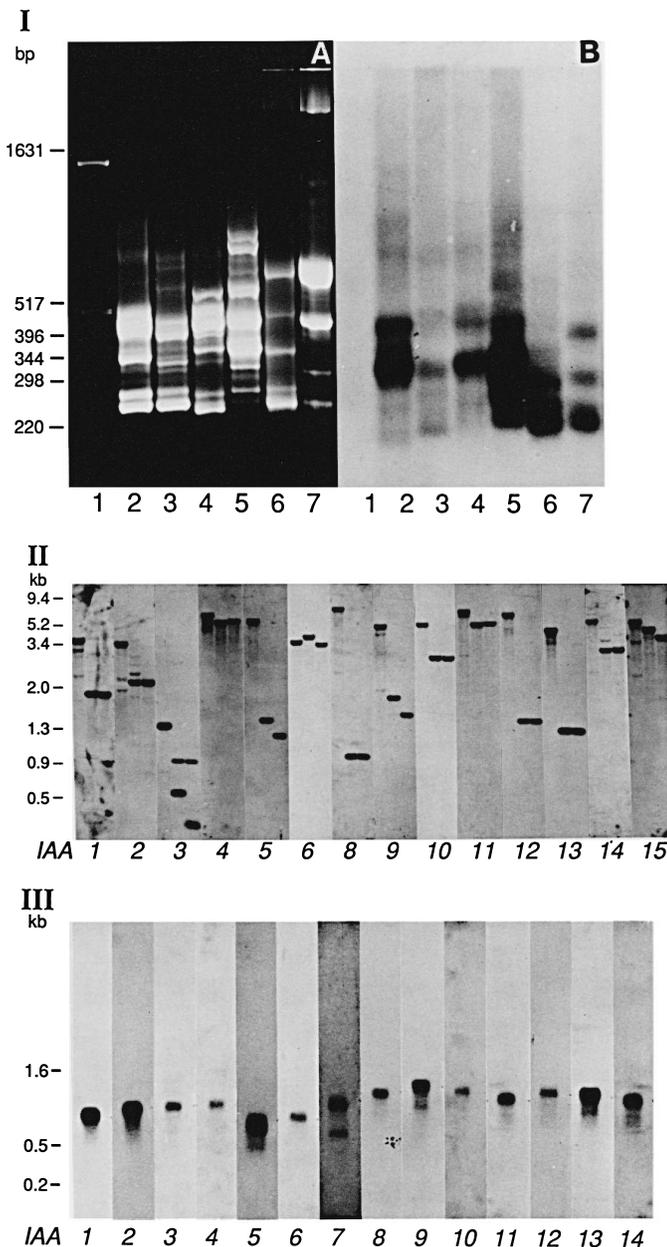


Figure 1. Identification of PS-IAA4/5-like genes in *A. thaliana*. Panel I. Products of the degenerate PCR. A, Amplified gene fragments from *Arabidopsis* (lane 2), cauliflower (lane 3), pea (lane 4), tomato (lane 5), maize (lane 6) and rice (lane 7) were separated in 2% agarose and stained with ethidium bromide. A pBR322 *Hinf*I digest was electrophoresed in lane 1. B, Autoradiogram of the gel in A after Southern transfer and hybridization with 32 P-labeled degenerate oligonucleotide DC3. Panel II, Authenticity of PS-IAA4/5-like *Arabidopsis* gene fragments. For each probe, *Arabidopsis* genomic DNA aliquots (10 μ g) were digested with *Eco*RI (left lane), *Hind*III (central lane) or *Eco*RI/*Hind*III (right lane), separated in a 0.8% agarose gel, transferred to a nylon membrane and hybridized with 32 P-labeled PS-IAA4/5-like DNA inserts. The size markers shown are λ DNA digested with *Hind*III/*Eco*RI. Panel III, Northern blot analysis. Total RNA (25 μ g) from auxin-treated (50 μ M IAA for six hours) etiolated *Arabidopsis* seedlings were separated in 1% agarose, transferred to a nylon membrane and hybridized with 32 P-labeled PS-IAA4/5-like DNA inserts. A denatured pBR322 *Hinf*I digest was used as size markers.

Table 1. Characteristics of the *Arabidopsis* PS-IAA4/5-like gene fragments

| PCR clone | Insert size (bp) | Intron size (bp) | Identity ^a (%) | Genomic locus |
|------------------|------------------|------------------|---------------------------|-------------------|
| 12/1 | 252 | None | 100 | IAA1 |
| 8/15 | 246 | None | 83 | IAA2 |
| R15 | 279 | None | 76 | IAA3 |
| 8/11 | 273 | None | 80 | IAA4 ^b |
| 8/9 | 366 | 117 | 65 | IAA5 ^c |
| R6 | 447 | 192 | 61 | IAA6 |
| N/A ^d | N/A | N/A | N/A | IAA7 |
| 5/8 | 435 | 96 | 76 | IAA8 |
| 5/1 | 451 | 112 | 75 | IAA9 |
| 1/6 | 537 | 97 | 74 | IAA10 |
| 3/6 | 464 | 92 | 71 | IAA11 |
| 5/5 | 461 | 81 | 72 | IAA12 |
| R5 | 381 | None | 71 | IAA13 |
| 3/5 | 473 | 137 | 72 | IAA14 |
| 8/1 | 359 | 116 | 68 | IAA15 |

^a Nucleotide sequence of coding region compared with nucleotide sequence of 12/1.

^b Genomic PCR product is identical with respective region in *Arabidopsis* gene *AtAux2-11*, and

^c *AtAux2-27* (Conner *et al.*, 1990).

^d No genomic PCR fragment cloned.

Authenticity and specificity of the *Arabidopsis* gene fragments

Each PS-IAA4/5-type DNA insert recognizes a unique set of *Arabidopsis* genomic DNA restriction fragments (Figure 1, panel II). As expected from the initial classification scheme, essentially no cross-hybridization is observed at the high stringency hybridization conditions used. The corresponding genes have been designated IAA1 to IAA6 and IAA8 to IAA15 (Table 1). A genomic PCR product corresponding to IAA7 failed to be cloned but a cDNA was isolated upon library rescreening (see below). All corresponding genes except IAA15 are expressed in etiolated *Arabidopsis* seedlings (Figure 1, panel III). The sizes of the IAA gene transcripts differ significantly and range from about 0.75 kb to 1.5 kb, indicating that PS-IAA4-like proteins are encoded by a diverse multi-gene family in *Arabidopsis*. The analysis in Figure 1 (panels II and III) demonstrates authenticity of the PS-IAA4/5-like *Arabidopsis* gene fragments and corroborates their use as specific probes in the following analysis of gene expression.

Organ-preferential expression of IAA genes

Northern blot analysis was used to examine the relative abundance of IAA1 to IAA15 mRNAs in *Arabidopsis* (Figure 2; Table 2). All genes except IAA15 are expressed to readily detectable mRNA levels in untreated etiolated seedlings and in mature plants. For all IAA genes expressed, the mRNA level in seedlings is comparable with the mRNA level in at least one of the five organs tested of mature plants,

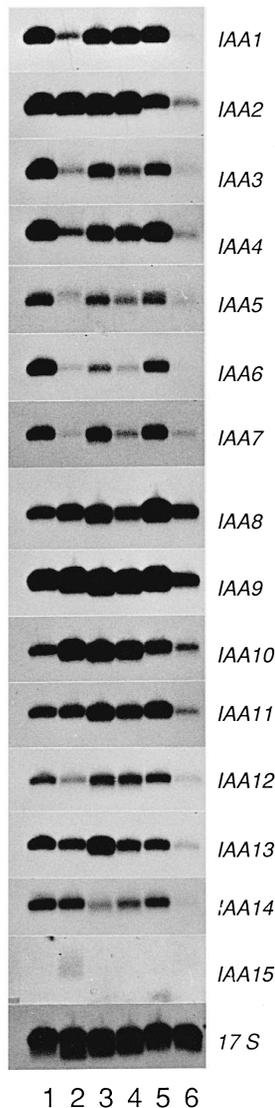


Figure 2. Expression of *IAA* genes in *Arabidopsis* plants. Poly(A)⁺ RNA (2.5 μg) isolated from untreated etiolated seedlings (5 days old) and from various organs of light-grown mature plants (25 days old) were separated in 1% agarose, transferred to a nylon membrane and hybridized with ³²P-labeled *PS-IAA4/5*-like DNA inserts or with a ³²P-labeled *17S* rDNA probe. The lanes are: 1, etiolated seedlings; 2, roots; 3, stems; 4, leaves; 5, flowers; and 6, siliques. Exposure times: six hours, *IAA7*, *IAA8*, *IAA9* and *IAA13*; 24 hours, *IAA1*, *IAA2*, *IAA4*, *IAA6*, *IAA10*, *IAA11*, *IAA12* and *IAA14*; 72 hours, *IAA3* and *IAA5*; 144 hours, *IAA15*.

i.e. in roots, stems, leaves, flowers or siliques (Figure 2, compare lane 1 with lanes 2 to 6). In general, *IAA1* to *IAA14* mRNAs are highly abundant in flowers but are significantly less abundant in developing siliques (compare lanes 5 and 6). Interestingly, a comparison of the expression pattern between the family members reveals differential preferences for the other three organs tested. For example, the mRNAs of *IAA1*, *IAA3* to *IAA7* and *IAA12* are in relatively low

abundance in roots. Low levels of mRNA are found for *IAA3* and *IAA5* to *IAA7* in leaves, and for *IAA14* in stems. Members of the *IAA* gene family fall into four groups according to their organ-preferential expression profile: (1) *IAA3*, *IAA5* to *IAA8* and *IAA11* are highly abundant in stems and flowers; (2) *IAA1*, *IAA4*, *IAA9* and *IAA12* are preferentially expressed in stems, leaves and flowers; (3) *IAA2* and *IAA10* are more abundant in vegetative than in reproductive organs; and (4) the expression patterns of *IAA13* and *IAA14* differ from the other *IAA* genes.

Response of the gene family to IAA and CHX

The effect of IAA and of the protein synthesis inhibitor CHX was examined on *IAA* gene expression (Figure 3). Treatment of intact etiolated *Arabidopsis* seedlings with 20 μM IAA for one hour (Figure 3A, lane 3) increases steady-state mRNA levels for all *IAA* genes tested, except for *IAA7* and *IAA8*, when compared with untreated (lane 1) or mock-treated (lane 2) seedlings. Accumulation of *IAA* mRNAs ranges from a two-fold increase for *IAA9* to a 25-fold increase for *IAA5*. Incubation with 50 μM CHX results in accumulation of high levels of *IAA* mRNA, again with the exception of *IAA7* and *IAA8* (compare lane 2 with lane 4). At this concentration, CHX effectively prevents protein biosynthesis (data not shown). In general, *IAA* mRNA accumulation is more profound in response to CHX than to IAA (compare lanes 3 and 4). CHX does not interfere with the effect of IAA (compare lane 3 with lane 5). Insensitivity of the auxin response to CHX is best revealed by an augmented response to both chemicals for some of the *IAA* genes, such as *IAA1*, *IAA2*, *IAA5* or *IAA6* (compare lane 4 with lane 5). These results qualify most of the *IAA* genes as primary response genes. Their expression is independent of *de novo* protein synthesis.

The *IAA* mRNA accumulation profiles in response to IAA and/or CHX treatment for one hour (Figure 3A) are essentially maintained when seedlings are treated for six hours (panel B). However, two significant variations are noticed. Firstly, mRNA levels of *IAA7* and *IAA8* increase (about threefold and 1.5-fold, respectively) in response to IAA (compare lane 2 with lane 3 in panel B). CHX alone has no effect on *IAA7* and *IAA8* (compare lane 2 with lane 4) but inhibits the response to auxin when administered together with the hormone (compare lane 3 with lane 5). *IAA7* and *IAA8* are late genes whose expression appears to be dependent on *de novo* protein synthesis. Secondly, IAA does not significantly increase *IAA3* mRNA or *IAA6* mRNA levels after a six hour treatment. Interestingly, the transcript levels are comparable with the mRNA concentrations of the mock control (compare lane 2 with lane 3 in both panels). This indicates transient response of gene expression to auxin.

Table 2. Characteristics of the *Arabidopsis* IAA cDNAs

| cDNA clone | GenBank accession number ^a | Insert size (bp) | Integrity of cDNA | RUUUR/DST motifs | Relative abundance (mRNA) ^b | Amino acids (no.) | Calculated molecular mass (kDa) |
|------------|---------------------------------------|------------------|----------------------|------------------|--|-------------------|---------------------------------|
| IAA1 | L15448 | 504 | N/A ^c | 1/0 ^d | + + | 168 | 19.1 |
| IAA2 | L15449 | 522 | N/A ^c | 2/0 ^d | + + | 174 | 19.9 |
| IAA3 | U18406 | 1062 | Poly(A) | 1/0 | + | 189 | 21.5 |
| IAA4 | L15450 | 558 | N/A ^c | 0/0 | + + | 186 | 21.0 |
| IAA5 | U18407 | 578 | Truncated | 0/0 | + | 174 | 19.6 |
| IAA6 | U18408 | 879 | No poly(A) | 2/0 | + | 189 | 21.0 |
| IAA7 | U18409 | 947 | No poly(A) | 0/0 | + + | 243 | 26.4 |
| IAA8 | U18410 | 1342 | Poly(A) | 2/0 | + + + | 321 | 35.1 |
| IAA9 | U18411 | 1450 | Poly(A) | 0/0 | + + + | 338 | 36.4 |
| IAA10 | U18412 | 1268 | Poly(A) | 1/0 | + + | 261 | 27.9 |
| IAA11 | U18413 | 1013 | Poly(A) | 1/0 | + + | 246 | 26.5 |
| IAA12 | U18414 | 1174 | Poly(A) | 3/1 | + | 239 | 26.3 |
| IAA13 | U18415 | 1341 | No poly(A) | 1/1 | + + | 246 | 26.6 |
| IAA14 | U18417 | 754 | 5'-Truncated/Poly(A) | 1/0 | + | N/A | N/A |

^a Additional Accession Numbers are: U18418 (genomic PCR fragment of *IAA15*); T04508, T20545, and Z47584 (novel *PS-IAA4/5*-like *Arabidopsis* EST's).

^b Based on Figure 2.

^c Cloning of coding region by PCR.

^d Based on inverse PCR product.

Specificity of the hormonal response

The specificity of the hormonal response is documented for the *IAA1* gene in Figure 4. Qualitatively the same pattern of expression is found for genes *IAA2* to *IAA14* (data not shown). *IAA15* mRNA was not detected under any conditions. The effect of various IAA analogs (20 μ M each) is shown for 2,4-D (lane 6), α -NAA (lane 7), PAA (lane 8) and L-tryptophan (lane 9). As compared with the response to 20 μ M IAA (compare lane 1 or lane 2 with lane 3; or lane 13 with lane 14), 2,4-D and α -NAA are highly effective in inducing accumulation of *IAA1* mRNAs. PAA has only a slight effect (compare lane 8 with lane 2), whereas the structural analog tryptophan is ineffective. Other plant hormones such as 20 μ M ABA (lane 15), 20 μ M GA (lane 16), 20 μ M BA (lane 17) or 10 ppm ethylene (compare lane 21 with lane 23) do not significantly increase *IAA1* mRNAs above control levels (lane 13). A combination of 20 μ M IAA with 20 μ M BA neither augments nor attenuates the response to auxin (compare lane 13 with lane 18).

The effect of several stress conditions on *IAA* gene expression was also studied. Wounding (lane 10), osmotic shock (lane 11), heat shock (lane 12), anaerobiosis (lane 22), and phosphate starvation (lane 25) fail to induce *IAA1* transcript accumulation. LiCl, which is known to interfere with phosphatidylinositol metabolism and signaling (Berridge *et al.*, 1989), has no effect, either when applied alone (lane 20) or in combination with 20 μ M IAA and 20 μ M benzyl adenine (lane 19). The only other inducer found is inhibition of protein synthesis by CHX (lane 3 and lane 4).

Differential kinetics of mRNA accumulation in response to IAA

We monitored the steady-state mRNA accumulation in intact etiolated seedlings treated with 20 μ M IAA for up to eight hours (Figure 5). All *IAA* genes except *IAA7* to *IAA10* respond to exogenous auxin within the first 30 minutes of treatment. *IAA9* and *IAA10* mRNA levels significantly increase between 30 and 60 minutes, whereas mRNAs of *IAA7* and *IAA8* start to increase only modestly between one and two hours of auxin exposure. The early responding genes, *IAA1* to *IAA6* and *IAA11* to *IAA14*, achieve maximal mRNA concentrations between 30 and 120 minutes of auxin treatment. These levels are approximately fourfold (see *IAA3*) to 25-fold (see *IAA5*) higher than the respective levels of the zero time control. Maximal mRNA transcripts are maintained for at least eight hours in the presence of auxin. *IAA3* and *IAA6* show a transient response to auxin. *IAA3* mRNA accumulates about fourfold within 30 minutes and subsequently decreases to the starting mRNA concentration after four hours of treatment. *IAA6* mRNA reaches a maximum within one hour (about fivefold increase), which drops to a new steady-state (twofold higher concentration than starting mRNA level) after four hours of auxin treatment. The transcripts of genes that respond relatively late to auxin, *IAA7* to *IAA10*, tend to accumulate steadily.

Figure 6 shows a short time-course experiment to study *IAA* mRNA accumulation between two and 30 minutes of auxin treatment. Interestingly, the group of early genes, *IAA1* to *IAA6* and *IAA11* to *IAA14*, does not respond to auxin in a uniform manner. Members respond in a spectrum between four minutes (see *IAA1* or *IAA5*) and 25 minutes (see *IAA4*) of hormone treatment. After induction, early

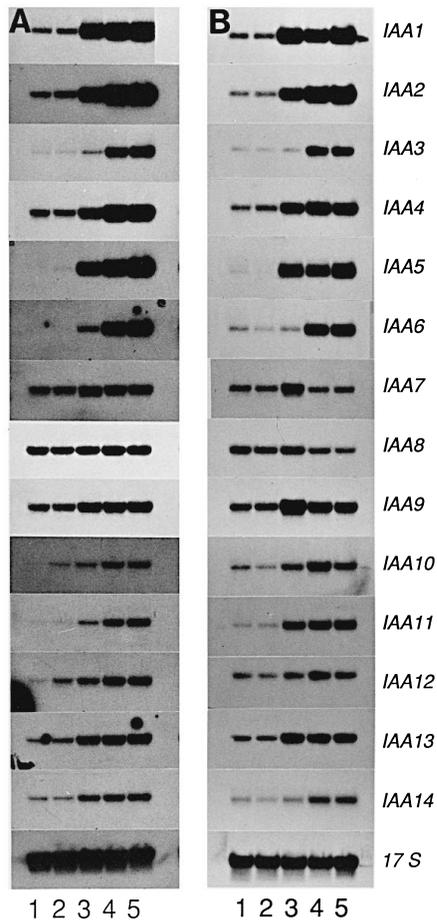


Figure 3. Response to IAA and CHX. Total RNA (25 μ g) isolated from intact etiolated seedlings (5 days old) was probed as in Figure 2. The seedlings were treated either for one hour (A) or for six hours (B). The lanes are: 1, untreated; 2, control-treated; 3, 20 μ M IAA; 4, 50 μ M CHX; 5, 20 μ M IAA and 50 μ M CHX after 30 minutes pretreatment with 50 μ M CHX only.

IAA mRNAs accumulate steadily during the remaining time of the experiment. However, the transiently expressed *IAA3* mRNAs achieve a nearly maximal level within the first ten minutes of auxin treatment, which remains essentially unchanged for up to 30 minutes.

Differential sensitivity of gene expression to IAA

Next, we studied the effect of different IAA concentrations on the expression of genes *IAA1* to *IAA14*. Since all expressed IAA genes respond to exogenous auxin within two hours of treatment (see Figure 5), a dose response curve ranging from 1×10^{-8} M to 5×10^{-4} M of IAA was obtained after a two hour auxin treatment of intact seedlings (Figure 7). The dose response of all IAA genes studied shows a characteristic bimodal shape. All genes respond maximally to exogenous IAA at a concentration of 1×10^{-5} M. Higher concentrations of IAA are suboptimal, and IAA at 5×10^{-4} M

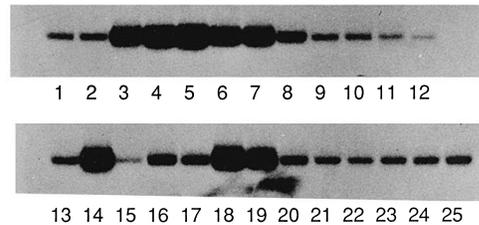


Figure 4. Specificity of the hormonal response. Total RNA (25 μ g) from six days old etiolated seedlings treated for one hour with various chemicals and conditions, if not otherwise indicated, were hybridized with a 32 P-labeled *IAA1*-specific probe. The lanes are: 1, untreated; 2 and 13, control-treated; 3 and 14, 20 μ M IAA; 4, 50 μ M CHX; 5, 20 μ M IAA and 50 μ M CHX after 30 minutes pretreatment with 50 μ M CHX only; 6, 20 μ M 2,4-D; 7, 20 μ M α -NAA; 8, 20 μ M PAA; 9, 20 μ M L-tryptophan; 10, wounding; 11, 0.5 M sorbitol; 12, heat treatment at 42°C for 15 minutes followed by 45 minutes recovery at room temperature; 15, 20 μ M ABA; 16, 20 μ M GA; 17, 20 μ M BA; 18, 20 μ M IAA and 20 μ M BA; 19, 20 μ M IAA/20 μ M BA and 50 mM LiCl; 20, 50 mM LiCl; 21, 10 ppm ethylene; 22, N₂; 23, air control; 24, control (six hours in 10 mM phosphate); 25, phosphate starvation (six hours, no phosphate present).

completely inhibits steady-state mRNA accumulation. However, at auxin concentrations below 1×10^{-5} M IAA, individual members of the IAA gene family respond in a differential fashion. For example, steady-state mRNA levels of *IAA1*, *IAA5*, *IAA6* or *IAA13* start to increase at an IAA concentration as low as 1×10^{-8} M and continue to accumulate over a concentration range of three orders of magnitude. The other IAA genes are gradually less sensitive in their response, and some of them begin to respond at IAA concentrations only as high as 5×10^{-6} M (see *IAA3*, *IAA7* and *IAA14*).

Expression in auxin-resistant mutants of Arabidopsis

Expression in *axr1-12* etiolated seedlings

Expression of IAA genes was studied in intact etiolated seedlings of the *Arabidopsis* mutant line *axr1-12* (Lincoln *et al.*, 1990). Wild-type seedlings and mutant seedlings were treated in the presence or absence of 20 μ M IAA for two hours. Relative to mock-treated, wild-type tissue, steady-state IAA mRNA levels are severely reduced in respectively treated *axr1-12* seedlings, between threefold for *IAA12* or *IAA14* and 15-fold for *IAA7* (Figure 8). Transcripts of *IAA5* and *IAA6* are not detectable by this analysis in control-treated mutant seedlings. Interestingly, when compared with the hormonal response in wild-type, auxin-inducibility of IAA mRNA accumulation in *axr1-12* appears not to be affected. Defective gene expression in *axr1-12* is most severe for *IAA5* and *IAA6* when based on a comparison between auxin-treated seedlings (Figure 8).

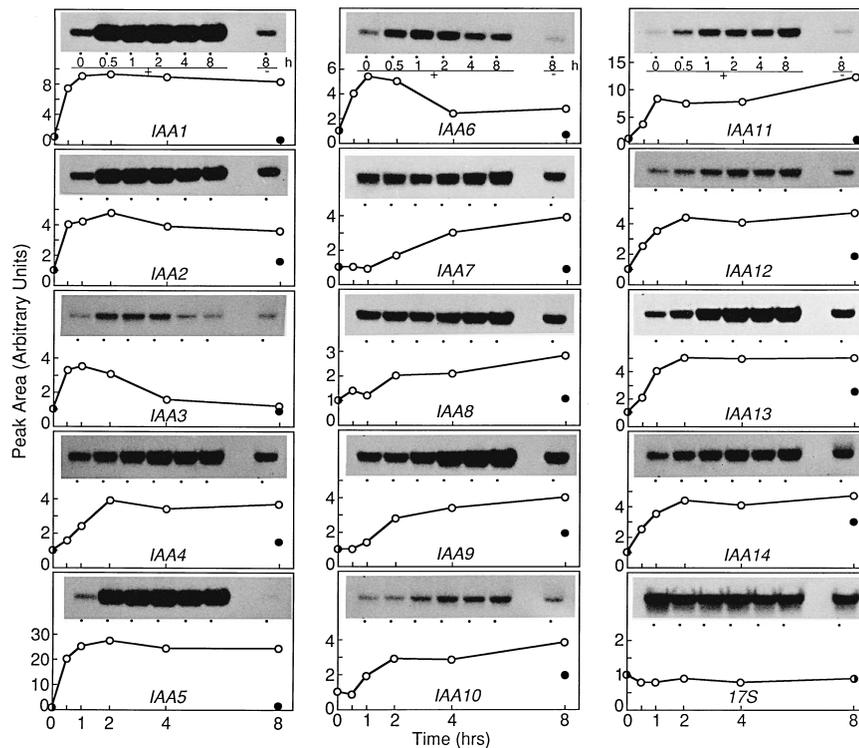


Figure 5. Long-term kinetics of mRNA accumulation in response to IAA. Intact etiolated *Arabidopsis* seedlings (five days old) were incubated in the presence (○) or absence (●) of 20 μM IAA. After the indicated period, total RNA (25 μg) was probed as for Figure 2. The results are shown graphically relative to the mRNA level of the zero time control (arbitrary value of 1). The original autoradiogram is included in each graph.

Expression in axr2-1 etiolated seedlings

An identical analysis was performed to study the expression of IAA genes in the mutant line *axr2-1* (Wilson *et al.*, 1990). Relative to wild-type, IAA mRNA levels in control-treated *axr2-1* seedlings are

severely reduced. The degree of IAA transcript reduction is similar to that observed in *axr1-12* seedlings, with the exception of IAA7, whose transcripts are barely detectable. However, the response of the IAA gene family to exogenous auxin is variable and more complex in the *axr2-1*

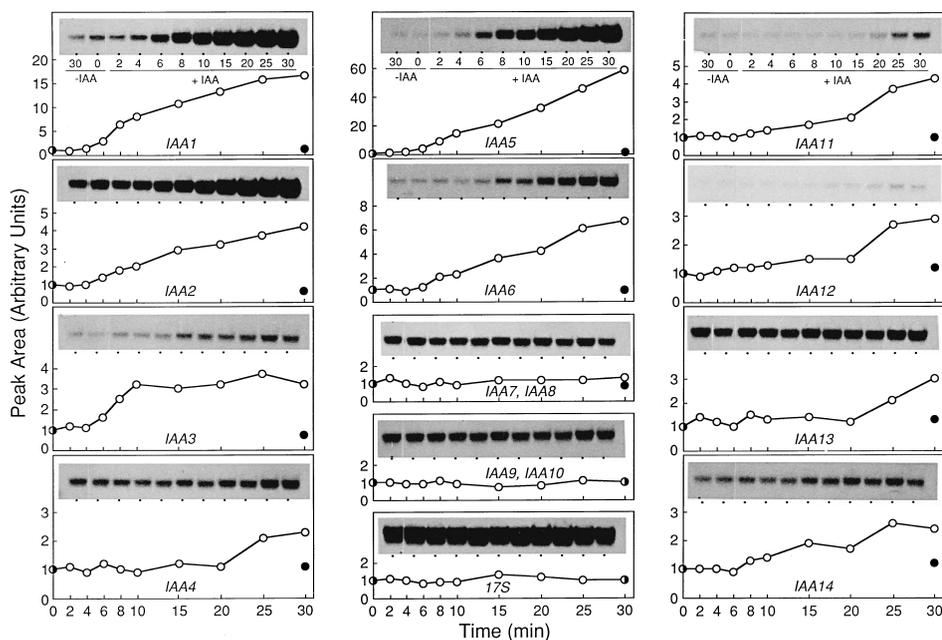


Figure 6. Short-term kinetics of mRNA accumulation in response to IAA. See the legend to Figure 5.

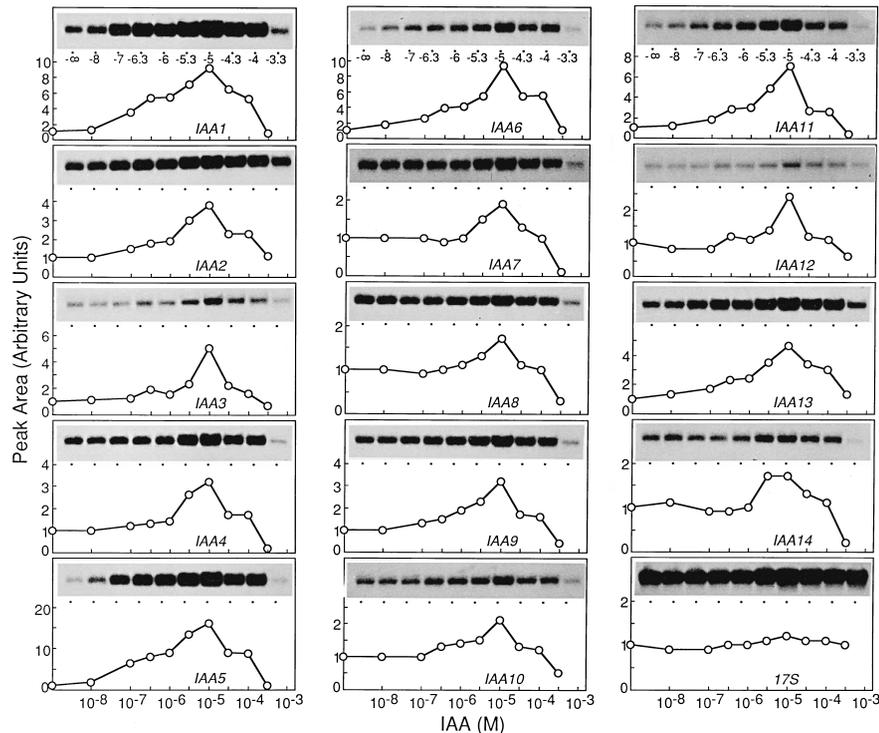


Figure 7. Dose response to IAA. Intact etiolated *Arabidopsis* seedlings (five days old) were treated with various concentrations of IAA for two hours. Total RNA (25 μ g) was probed as for Figure 2. The results are shown graphically relative to the mRNA level of the no auxin control (arbitrary value of 1). The original autoradiogram is included in each graph.

background. Auxin-inducibility of IAA mRNA accumulation is either unaffected (see IAA2, IAA9 to IAA11 and IAA13), reduced (see IAA1, IAA4 and IAA14) or abolished (see IAA3, IAA5 to IAA7, and IAA12). The most severely affected genes in the *axr2-1* mutant are IAA3 and IAA5 to IAA7 (Figure 8).

Expression in *aux1-7* seedlings

Etiolated seedlings. Unlike in the *axr1-12* and *axr2-1* mutant lines, IAA gene expression is less affected in *Arabidopsis aux1-7* seedlings (Figure 8). Absolute transcript levels are only moderately reduced, between 1.5-fold for IAA14 and fivefold for IAA6. Auxin-inducibility is retained for all IAA genes and is slightly enhanced for IAA1, IAA2 and IAA7.

Light-grown seedlings. Since only root morphology appears to be affected in *aux1-7* mutants (Pickett *et al.*, 1990), we extended our study to light-grown plants. Non-flowering wild-type and *aux1-7* plants were dissected into roots and shoots, which were subsequently incubated for two hours in the absence or presence of 20 μ M IAA. All IAA genes tested except IAA6 are detectable and auxin-responsive in wild-type roots and shoots (Figure 9). IAA6 mRNA is not detectable probably because of its low expression in vegetative organs (see Figure 2). Transcript levels and auxin-inducibility of most of the IAA genes are not affected in *aux1-7* roots, with the exception of IAA1, IAA5, IAA7 and IAA12. For

those genes, mRNA levels are about twofold lower in *aux1-7* than in wild-type. Auxin-inducibility appears to be reduced for IAA5 and IAA7. A slightly different expression profile is detected in *aux1-7* shoots. Auxin-inducibility appears to be unaffected for all IAA genes shown. However, relative to wild-type shoots, all IAA transcript levels are moderately reduced (about twofold), except for IAA5 (fourfold decrease).

Isolation of DNA sequences complementary to IAA1 to IAA14 mRNAs

The coding regions of IAA1 and IAA2 were reconstructed by PCR (see Materials and Methods). A sequence coding for the IAA4 protein was obtained by RT-PCR using amplimers synthesized to deduced non-translated regions of *AtAux2-11* (Conner *et al.*, 1990). A similar attempt to amplify sequences coding for IAA5, whose genomic PCR product corresponds to gene *AtAux2-27* (Conner *et al.*, 1990), repeatedly failed for unknown reasons.

All genomic PCR products except that corresponding to IAA15 allowed for the isolation of specifically corresponding cDNA clones. The characteristics of the isolated IAA cDNAs are described in Table 2. cDNA inserts containing putative full-length coding regions were isolated for IAA3, IAA6 and IAA8 to IAA13. The large ORF of most cDNAs is preceded by nonsense codons, however, the cDNAs of IAA3 and IAA11 start with an uninterrupted large ORF. In each

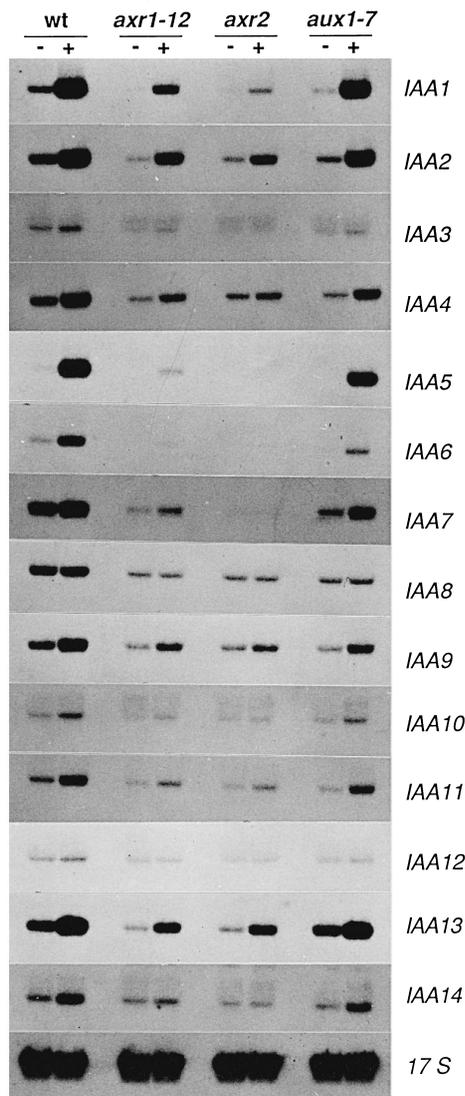


Figure 8. Expression of IAA genes in etiolated auxin-resistant seedlings. Intact etiolated *Arabidopsis* seedlings (five days old), wild-type or the mutant lines *axr1-12*, *axr2-1* or *aux1-7*, were incubated in the presence (+) or absence (-) of 20 μ M IAA for two hours. Total RNA (25 μ g) was probed as for Figure 2.

case, the first coding methionine residue of the large ORF is assigned as putative translational start site. Truncated coding sequences were recovered only for *IAA5* and *IAA14*. Upon library rescreening with the truncated *IAA14* cDNA, a weakly hybridizing clone was isolated coding for a novel IAA protein. The corresponding gene escaped the initial genomic PCR cloning and is designated *IAA7*.

Many IAA mRNAs contain the motif AUUUA and/or GUUUG in the deduced 3' non-translated region. DST-like sequences are present in the transcripts of *IAA12* and *IAA13*; **GGAN**₁₇**TTAGATATGN**₅**AATCCGTTTT** and **GAAN**₁₁**CTAGATTGAN**₅**TATCCAGTTTG**, 88 and 67 nucleotides downstream of the translational stop codon, respectively. Bold letters indicate nucleotides identical with the DST element of *SAUR-AC1*

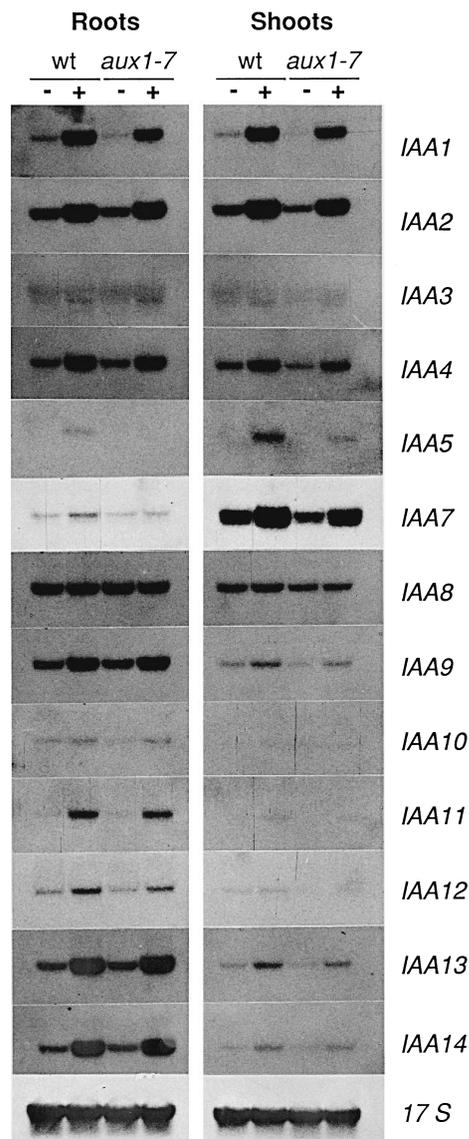


Figure 9. Expression of IAA genes in light-grown *aux1-7* seedlings. Light-grown wild-type and *aux1-7* seedlings (20 days old) were dissected into roots and shoots. The organs were incubated in the presence (+) or absence (-) of 20 μ M IAA for two hours. Total RNA (25 μ g) was probed as for Figure 2.

mRNA, 83 nucleotides downstream of the stop codon (Gil *et al.*, 1994).

Deduced amino acid sequences and phylogenetic analysis

The predicted primary structure of PS-*IAA4*-like proteins is shown in Figure 10. All polypeptides contain four conserved domains and seven invariant amino acids in the intervening sequences: a basic doublet, KR, between domain I and domain II, and five hydrophobic amino acid residues at conserved positions C-terminal to domain III (Ainley *et al.*, 1988; Conner *et al.*, 1990; Oeller *et al.*, 1993; Abel *et al.*, 1994). Noteworthy is the hypervariability of the

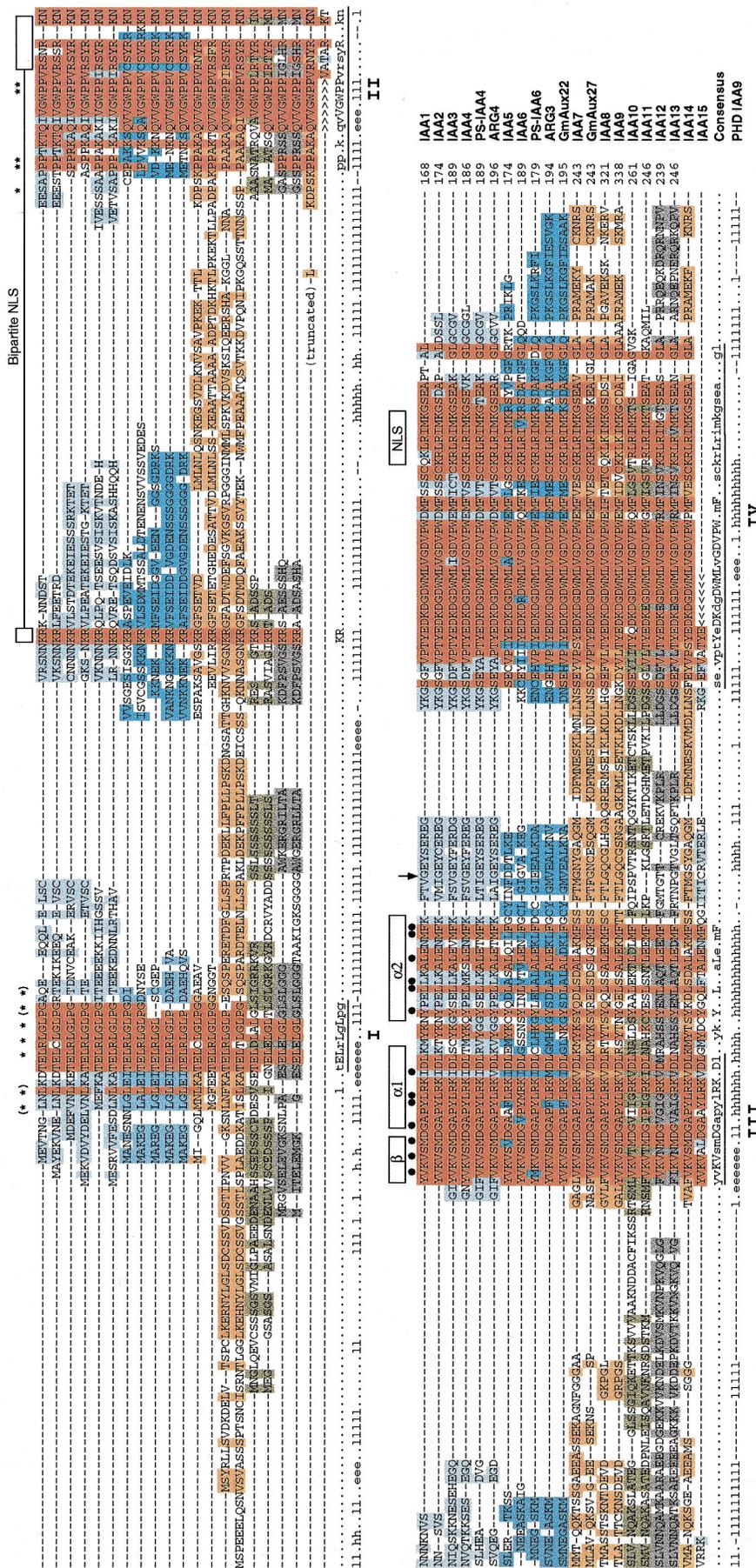


Figure 10. Sequence alignment and domain structure of primary auxin-responsive gene products. The multiple sequence alignment obtained by DARWIN (Gonnet et al., 1992) was manually improved. Identical and conserved amino acid residues (at least 11 of 21 matches) are shaded (orange) and appear in the consensus (capital and small letters, respectively). Conserved domains are underlined and indicated by Roman numerals. Similar amino acid residues conserved within distinct lineages are coloured (small, G, A; acidic, D, E; basic, H, R, K; generally hydrophobic, A, C, V, I, L, M, F, Y, W; polar, S, T; and N, Q). Amino acid residues corresponding to degenerate amplimers DC2 and DC4 are indicated in the IAA15 sequence (> > > and < < <, respectively). An arrow designates the conserved position of an intron in the PCR-generated gene fragments when present. Conserved basic residues that function as an NLS in PS-IAA4 and PS-IAA6 (Abel & Theologis, 1995) are indicated on top of the alignment. Stars indicate a pattern of conserved leucine residues in domain I and of conserved proline residues in domain II. Secondary structure predictions (PHD) for IAA9 were calculated according to Rost & Sander (1993) and are indicated by h (helix), e (extended sheet), l (loop); a period indicates no prediction. Amino acid residues that may form hydrophobic surfaces in the predicted conserved amphipathic $\beta\alpha\alpha$ motif are indicated by (●). Sources of the sequences are as follows: IAA1-IAA15 (Arabidopsis thaliana, this study); PS-IAA4, PS-IAA6 (pea (Pisum sativum), Oeller et al., 1993); ARG3 and ARG4 (mung bean (Vigna radiata), Yamamoto et al., 1992); GmAux22 and GmAux28 (soybean (Glycine max), Ainley et al., 1988).

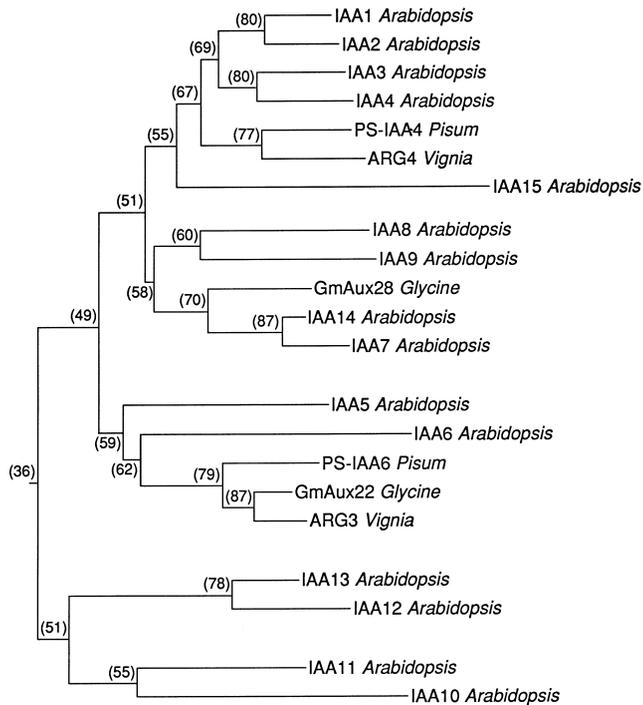


Figure 11. Dendrogram of auxin-regulated PS-IAA4-like proteins. The unrooted phylogenetic tree was constructed using DARWIN (Gonnet *et al.*, 1992) and is based on the estimated PAM (point accepted mutations) distances between each sequence pair. The horizontal distances are drawn to scale and the numbers in parentheses represent the approximate percentage identities between different members positioned to the right.

separating regions with respect to length. The calculated molecular mass of the IAA polypeptides ranges from 19 kDa for IAA1 to 36 kDa for IAA9. Homology searches against databases detect no significant similarity with any proteins other than with members of the PS-IAA4-like family. A search against the EST database indicated the existence of three additional PS-IAA4-like proteins in *Arabidopsis*, which extend the IAA gene family to 18 members (see Table 2).

Phylogenetic analysis of the 21 PS-IAA4-like amino acid sequences described thus far from various plant species yields the dendrogram shown in Figure 11. Overall sequence identity between individual polypeptides of the family is highly variable (36% to 87%), which is reflected by the four distinct branches of the unrooted phylogenetic tree. Interestingly, although pea, soybean and mungbean are closely related evolutionarily by being members of the Leguminosae family, PS-IAA4-like proteins of these species fall into two of the four main classes. In those lineages, the Leguminosae proteins are highly similar to the proteins from *Arabidopsis*, a member of the Cruciferae family. It has been proposed that the proteins of each class perform similar functions, which are distinct from the function of polypeptides in other lineages (Oeller *et al.*, 1995).

Discussion

Extent and differential expression of IAA gene families

We have described a family of PS-IAA4/5-like transcripts in *Arabidopsis thaliana* as a first step toward the elucidation of the biological function of their encoded proteins. To isolate a representative number of family members, we have used genomic DNA as the template in a PCR-based procedure. This approach has been facilitated by the occurrence of four conserved domains in PS-IAA4-like proteins (Conner *et al.*, 1990). Degenerate oligonucleotides corresponding to each of those domains were used in various combinations (data not shown). In view of the complexity and size variation of products obtained, we further characterized only the population amplified with degenerate primers DC2 and DC4, leading to the identification of 14 unique genomic fragments of the PS-IAA4/5-type. As expected from amplification biases of the degenerate primers used, the PCR selection was not exhaustive. IAA7 escaped the initial screen, and searching the *Arabidopsis* EST database revealed three additional novel cDNAs of the PS-IAA4/5-type, extending the *Arabidopsis* gene family to at least 18 members, 17 being expressed. IAA15 probably represents a pseudogene. Primers DC2 and DC4 amplify a large though similar population of fragments from genomic DNA of various plant species (see Figure 1, panel I). Likewise, antibodies against PS-IAA4 and PS-IAA6 from pea recognize seven to 12 distinct members of a similar group of polypeptides expressed in pea, soybean, *Arabidopsis* and maize (Oeller & Theologis, 1995). Therefore, PS-IAA4/5-like multigene families most likely occur ubiquitously in higher plants (Theologis, 1986).

Expression analysis in mature *Arabidopsis* plants reveals organ-preferential accumulation of IAA transcripts. More detailed information on cell and tissue-specific expression has been obtained for gene *AtAux2-11* (Wyatt *et al.*, 1993), whose transcript corresponds to the IAA4 cDNA. *AtAux2-11* is expressed in specific spatial and temporal patterns that correlate with sites and physiological processes known to be modulated by auxin (Wyatt *et al.*, 1993). A similar and comparative analysis has been performed in tobacco seedlings transgenic for promoter-GUS fusions of PS-IAA4/5 and PS-IAA6 from pea. The data support the observations reported by Wyatt *et al.* (1993) and reveal, in addition that both genes exhibit distinct and common patterns of restricted expression (L.-M. Wong and A.T., unpublished data). We anticipate, therefore, a complexity of specific and overlapping expression patterns for members of the IAA multigene family in *Arabidopsis*. This view is corroborated by a study in soybean demonstrating that two classes of early auxin-responsive transcripts, *GH3* and *SAUR*, display both unique and overlapping patterns of tissue-specific gene expression (Gee *et al.*, 1991).

Characteristics of the hormonal response

Basal mRNA levels of most *IAA* genes are readily detectable in untreated *Arabidopsis* seedlings, indicating that endogenous auxin concentrations are sufficient in at least some tissues to induce and maintain their expression. All genes expressed, *IAA1* to *IAA14*, respond specifically to natural and synthetic auxins as the only external inducers identified, besides CHX. This observation is in agreement with previous results for related genes (Theologis *et al.*, 1985; Hagen & Guilfoyle, 1985; Yamamoto *et al.*, 1992). The *SAUR* genes and *GH3* are the only other classes of early genes described to be specifically induced by auxin (Guilfoyle *et al.*, 1993).

Members of the *Arabidopsis* gene family react to exogenous IAA in a highly differential fashion. First detectable increases in steady-state IAA mRNAs occur between four and 120 minutes after auxin application. Some of the early genes, such as *IAA1* to *IAA3*, have mRNA accumulation kinetics very similar to the *SAUR* genes, the fastest responding genes known to be induced by auxin. The *SAUR* transcripts start to accumulate within two to five minutes and achieve maximal levels by 60 minutes of 2,4-D treatment (McClure & Guilfoyle, 1987). The dose response curves of IAA genes reveal differential sensitivities over a broad range of IAA concentrations, covering about three orders of magnitude from 10 nM to 5 μ M IAA. The differences in kinetics and sensitivity of transcript accumulation between individual IAA genes is likely due to a variety of factors. These may include unknown parameters such as tissue-specific auxin permeability and reception, cell-type dependent and differential regulation of free auxin concentrations, or different modes of auxin-dependent transcriptional activation and post-transcriptional regulation. However, all IAA genes respond maximally at about 10 μ M IAA. A similar saturable response has been described for *PS-IAA4/5* gene expression in pea epicotyl protoplasts (Ballas *et al.*, 1993), for the *PS-IAA4/5*-type gene *GH1* in soybean seedlings (Hagen & Guilfoyle, 1985), or for *SAUR* promoter-driven GUS expression in transgenic tobacco (Li *et al.*, 1991). Interestingly, the biphasic dose response to IAA of *PS-IAA4/5*-like mRNA accumulation is paralleled by similar bimodal characteristics of other auxin-elicited responses in plants, such as stem elongation, stomatal opening, or auxin-modulation of the inward-rectifying K⁺ channel, I_{k,in}, in stomatal guard cells (Blatt & Thiel, 1994). Intracellular pH and calcium concentrations have been proposed as opposing second messengers in auxin signaling, which reciprocal interaction evokes the bimodal control of the potassium transporter (Blatt & Thiel, 1994). It is tempting to extend this hypothesis to auxin-regulated gene expression.

Deficient expression in auxin-resistant mutants

Expression of IAA genes is severely inhibited in etiolated seedlings of the *Arabidopsis* auxin-resistant

mutants *axr1-12* and *axr2-1*. These mutants have a pleiotropic though auxin-related phenotype (Hobbie & Estelle, 1994). Most aspects of the *axr2-1* phenotype are due to a dramatic decrease in cell elongation rather than a reduction in cell number, whereas the opposite effect has been described for *axr1-12* mutant plants (Lincoln *et al.*, 1990; Timpote *et al.*, 1992). The alterations of *axr1-12* plants have been explained by a reduced sensitivity to auxin (Hobbie & Estelle, 1994). This interpretation is supported by dramatically reduced IAA mRNA levels in etiolated *axr1-12* seedlings, though auxin-inducibility appears to be maintained (Figure 8). The *AXR1* gene reveals significant similarity to the ubiquitin-activating enzyme E1, although *AXR1* is probably not a functional E1 homolog (Leyser *et al.*, 1993). Nonetheless, the possibility exists that ubiquitin-dependent proteolysis may play an important role in auxin responses, for instance, by controlling the level but not auxin sensitivity of the short-lived repressor postulated to regulate *PS-IAA4/5*-like gene expression (Ballas *et al.*, 1993). Expression of IAA genes in *axr2-1* seedlings is as severely inhibited as in *axr1-12* plants; however, auxin-inducibility is also impaired for most of the genes. A similar reduced expression in *axr2-1* seedlings has been described for the *SAUR-AC1* gene (Gil *et al.*, 1994; Timpote *et al.*, 1994). The *axr2-1* mutation has recently been characterized as a gain-of-function mutation and proposed to prevent auxin-mediated inactivation of a transcriptional repressor (Timpote *et al.*, 1994). Mutant *aux1-7* plants display a severe defect in root gravitropism but are otherwise wild-type in their aerial structures (Pickett *et al.*, 1990). Only a modest reduction of IAA gene expression is noticed, however, both in roots and in shoots of light-grown seedlings (Figure 9). This is in agreement with the suggested function of *AUX1* also in the aerial portions of the plant (Hobbie & Estelle, 1994). We have also studied the expression of an auxin-inducible ACC synthase gene and of the *SAUR-AC1* gene. Interestingly, in each of the three mutant lines tested both genes are inhibited to a similar extent as the IAA genes (unpublished results). Inhibition of three classes of genes with different auxin-responsive elements supports the previous notion that either mutation acts early in an auxin response pathway (Hobbie & Estelle, 1994).

Regulation of IAA gene expression

The expression of *PS-IAA4*-like proteins appears to be stringently controlled at the transcriptional and post-transcriptional level, which is a hallmark of regulatory proteins (Gottesman & Maurizi, 1992). *PS-IAA4/5*-like genes are rapidly transcriptionally activated by auxin (Theologis *et al.*, 1985; Hagen & Guilfoyle, 1985; Ainley *et al.*, 1988; T. Koshiba and A.T., unpublished results). The auxin-responsive *cis*-element of the *PS-IAA4/5* promoter has been functionally defined, and *in vivo* titration exper-

iments indicate binding to positively acting factors (Ballas *et al.*, 1993, 1995).

The transcripts of *PS-IAA4/5* and *PS-IAA6* are labile. Their mRNA half-lives, 60 minutes and 75 minutes, respectively (T. Koshiba and A.T., unpublished results), are comparable with those of many unstable mRNAs of mammalian early-response genes (Atwater *et al.*, 1990), or with the soybean *SAUR* transcripts, 40 to 50 minutes, (Franco *et al.*, 1990). The DST sequence element in the 3'-untranslated region of the *SAUR* genes has been shown to signal rapid mRNA decay (Newman *et al.*, 1993). Likewise, as in mammalian cells (Chen & Shyu, 1994), AU-rich elements can confer mRNA instability in plants (Ohme-Takagi *et al.*, 1993). Consistent with their expected instability, most of the *IAA* transcripts contain AU-rich elements and, *IAA12* and *IAA13*, a DST-like sequence element in the 3' untranslated region (see Table 2). A short half-life of *IAA* mRNAs is indicated by the transient accumulation of *IAA3* and *IAA6* transcripts in response to auxin (Figure 5).

The PS-*IAA4* and PS-*IAA6* proteins are of extremely low abundance *in vivo* (Oeller & Theologis, 1995). The metabolic half-lives of both proteins, between six and eight minutes (Abel *et al.*, 1994), are among the shortest known for eukaryotic proteins (Gottesman & Maurizi, 1992). Although the mechanism of PS-*IAA4* degradation is as yet unknown, we note that determinants for rapid protein turnover such as PEST sequences or other potential destruction signals (Gottesman & Maurizi, 1992) are not discernible in *IAA* polypeptides.

Effect of CHX

Protein synthesis inhibitors have been used as a tool to unmask regulatory mechanisms of early gene activation. Induction of early genes is often independent of *de novo* synthesized proteins, indicating a primary response to the stimulus *via* modification of pre-existing components (Herschman, 1991). In addition, inhibition of protein synthesis potentiates the strength and duration of many signal-induced transcriptional responses by elimination of attenuating factors that are often synthesized as part of the response (Hill & Treisman, 1995). Induction by auxin of most *IAA* genes is insensitive to CHX, qualifying them as primary auxin-responsive genes (Figure 3). CHX alone or in combination with auxin, superinduces *PS-IAA4/5*-like genes (Figure 3; Theologis *et al.*, 1985). This phenomenon is well described for many early genes and oncogenes in mammalia (Herschman, 1991). The effect of CHX on the expression of *PS-IAA4/5* has been investigated, implicating two targets of inhibitor action: mRNA stabilization and transcriptional activation presumably by preventing synthesis or activation of a labile repressor polypeptide (T. Koshiba and A.T., unpublished results). The induction of negatively acting factors as part of the auxin response is best revealed by the accumulation

kinetics of *IAA3* or *IAA6* mRNAs. Both genes are transiently stimulated by IAA, and transcript levels are adjusted to low basal levels after four hours in the presence of the hormone (Figure 5). However, in the presence of IAA and CHX, induced mRNA levels are maintained for at least six hours (Figure 3). This indicates *de novo* synthesis of a protein that directly or indirectly attenuates the transcriptional response. Such directly acting factors have been described and include the fos protein that *trans*-represses its own gene (Sassone-Corsi *et al.*, 1988). The effect of CHX on expression of the relatively late auxin genes *IAA7* and *IAA8* suggests dependence on *de novo* protein synthesis of auxin-induction. Auxin-inducibility of both genes is inhibited in the presence of CHX, and CHX alone has no effect, indicating that *IAA7* and *IAA8* are secondary response genes (Figure 3). These data suggest a hierarchical response to auxin of *IAA* genes.

Structural motifs of IAA proteins

All deduced PS-*IAA4*-like protein sequences described thus far contain the four conserved domains originally noticed by Ainley *et al.* (1988). More importantly, seven amino acids are absolutely conserved in hypervariable regions. These regions are highly polymorphic and responsible for the wide range of molecular mass calculated, 19 to 36 kDa. We have previously shown that PS-*IAA4*-like polypeptides are able to direct the GUS reporter protein into the cell nucleus (Abel *et al.*, 1994; Abel & Theologis, 1994). Functional NLSs have been identified in PS-*IAA4* and PS-*IAA6* (Abel & Theologis, 1995). The essential NLS is of bipartite structure, comprising the invariant basic doublet KR and basic amino acids in domain II. A second, SV40-type NLS is located in domain IV (Figure 10). Domain I is the core of a leucine-rich region. Most *IAA* proteins share the consensus motif LxLxxxxLxLxL or in *IAA10* to *IAA13*, LxLxLxLxL (overlapping residues are in bold-face type). This motif is reminiscent of the leucine-rich repeat unit that is proposed to mediate protein-protein interactions (Kobe & Deisenhofer, 1994). Domain II is relatively rich in proline residues, and the spacer of *IAA8* and *IAA9* contains short sequences, (PXX)_n, indicative of a polyproline II conformation. Proline-rich motifs have been implicated in protein-protein interactions (Williamson, 1994).

The calculated secondary structures are similar for all members of the *IAA* family, suggesting that these proteins have related tertiary folds. The predicted secondary structure for *IAA9* is shown in Figure 10. Most of the spacer sequences are likely to adopt a random conformation in all *IAA* proteins analyzed. Domain III and the adjacent five invariant hydrophobic amino acid residues at conserved positions are predicted to form an amphipathic $\beta\alpha\alpha$ -fold similar to the β -ribbon DNA recognition domain of prokaryotic repressor polypeptides, such as Arc and MetJ (Phillips, 1994). If experimentally

proven, IAA polypeptides may represent the first eukaryotic members of this class of DNA-binding proteins (Raumann *et al.*, 1994). Arc and MetJ are proteins with similar functions and tertiary structures but with no apparent sequence identities, which is probably a reason why additional members of this family have not been identified yet. Interestingly, a phylogenetic analysis shows that the $\beta\alpha\alpha$ -motif of PS-IAA4-like proteins is as distantly related to the prokaryotic motifs (Arc, Mnt, TraY and MetJ) as those are to each other (data not shown). The tertiary structures of Arc and MetJ have been resolved (Phillips, 1994). A three-dimensional (3D) compatibility analysis of the IAA $\beta\alpha\alpha$ -fold with the β -ribbon DNA recognition motif of Arc or MetJ as the tertiary template was performed using the 3D profile method of Bowie *et al.* (1991). In both profiles, the 3D-1D scores for Arc versus MetJ differ, since their $\beta\alpha\alpha$ -domains vary in length. However, the score for the IAA $\beta\alpha\alpha$ -motif is comparable with the scores obtained for the other prokaryotic $\beta\alpha\alpha$ -folds in each profile (data not shown). This indicates that the putative eukaryotic $\beta\alpha\alpha$ -motif is compatible with the prokaryotic β -ribbon DNA recognition motifs. We have proposed an analogous function of the putative $\beta\alpha\alpha$ -motif in PS-IAA4-like proteins (Abel *et al.*, 1994).

Functional implications

This study demonstrates that the plant growth hormone auxin induces in a highly differential and hierarchical fashion a large family of early genes that encode a structurally diverse class of short-lived nuclear proteins. These polypeptides contain a conserved putative DNA-binding motif, and their expression is stringently regulated at multiple levels. Therefore, it has been proposed that PS-IAA4-like proteins play a pivotal role in the regulation of subsequent auxin-induced events responsible for plant cell growth (Abel *et al.*, 1994). Thus, the mode of auxin action in plants appears to follow the paradigm of how, for instance, ecdysone (Andres & Thummel, 1992) or other growth factors and extracellular signals (Herschman, 1991; Hill & Treisman, 1995) regulate behavior and development of animal cells. Factors that promote long-term cellular responses such as proliferation and differentiation coordinate hierarchical changes in the pattern of gene expression. Initially, a set of various early response genes is rapidly and transiently expressed. Many of those early genes encode transcription factors that activate secondary genes in a tissue-specific manner to shape the morphological and functional properties of each target tissue (Herschman, 1991). For instance, members of a large and diverse family of POU-domain regulatory genes are expressed in a distinct temporal and spatial pattern during neuronal development (He *et al.*, 1989). However, co-expression of different POU-domain proteins in certain brain regions is evident and the combinatorial code is suggested to be critical for specifying mature cellular phenotypes in the

mammalian nervous system (He *et al.*, 1989). Likewise, the tissue coordination model predicts that ecdysone activates tissue-specific sets of early genes encoding ecdysone receptors and regulatory proteins. The combination of those leads to activation of distinct sets of late genes that define the unique properties of each ecdysone target tissue at each stage in its development (Karim & Thummel, 1991). The studies done by Gee *et al.* (1991), Wyatt *et al.* (1993) and our laboratory (unpublished results) demonstrate that expression of early auxin-responsive genes is restricted to a few tissue types at specific developmental stages. Furthermore, the existence of multiple auxin receptors has been inferred (Gee *et al.*, 1991). In view of the highly differential mode of IAA gene expression in *Arabidopsis*, with respect to timing, hormone concentration and spatially restricted expression patterns, the prospect arises that combinatorial interactions among co-expressed IAA proteins define tissue-specific responses to auxin during plant growth and development. Defining the expression profiles of the different IAA transcripts will provide clues regarding those potential combinatorial interactions. These studies should also provide insight into the molecular mechanisms that control the timing of sequential gene expression in response to auxin.

Materials and Methods

Arabidopsis strains, growth conditions and other plant material

Wild-type *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia and auxin-resistant mutant lines *axr1-3*, *axr1-12* (Lincoln *et al.* 1990), *aux1-7* (Pickett *et al.* 1990) and *axr2* (Wilson *et al.* 1990) were obtained from the Biological Resource Center at Ohio State University. Etiolated seedlings were grown as described (Liang *et al.*, 1992). Wild-type and *aux1-7* seedlings were grown in Petri dishes at 22°C for three weeks under illumination with fluorescent and incandescent light (16 hour photoperiod). Mature plants were grown in the greenhouse at 22°C to 25°C under continuous illumination. Etiolated seedlings of pea (*Pisum sativum* cv. Alaska), corn (*Zea mays* cv. Funk), rice (*Oryza sativa* L. cv. IR36) and tomato (*Lycopersicon esculentum* cv. Rutgers) were grown as described (Oeller & Theologis, 1995). Cauliflower was purchased from a local market.

Tissue treatment

Intact etiolated seedlings (five to six days old) were placed in 0.5 × MS salt solution (pH 5.6) and supplemented with the appropriate chemicals. The seedlings were incubated in the dark at room temperature with moderate shaking (50 to 100 rpm). Mock control incubations were supplemented with an equal amount of the solvent used to prepare the stock solution of the respective chemical. After the indicated time, aliquots (3 to 5 g fresh weight) of seedlings were removed, immediately frozen with liquid nitrogen and stored at -80°C. Light-grown wild-type and *aux1-7* seedlings were dissected into roots and shoots, and treated as described above. Different organs from 25-day-old light-grown plants were harvested and kept at -80°C before RNA extraction.

Isolation of nucleic acids

Genomic DNA of *Arabidopsis*, cauliflower, pea, tomato, rice, and corn was isolated from etiolated seedlings (Dellaporta *et al.*, 1983). The DNA preparations were further purified by equilibrium sedimentation in cesium chloride gradients (Davis *et al.*, 1980). Total nucleic acids were prepared from frozen *Arabidopsis* tissues (Liang *et al.*, 1992). Poly(A)⁺ RNAs were isolated by affinity chromatography using oligo(dT)-cellulose (Theologis *et al.*, 1985).

PCR

Two degenerate oligonucleotide primers were synthesized, DC2 (512-fold degeneracy) and DC4 (64-fold degeneracy), which correspond to motifs I/VVGWPP in domain II and DKDGDWM in domain IV of PS-IAA4-like proteins, respectively (Figure 10). The sequence and orientation of both primers is as follows: DC2 5'-GCC GAA TTC RTN GTN GGN TGG CCN CC-3'; DC4 5'-GCC AGG CTT CAT CCA RTA NCC RTC YTT RTC-3'; N is A, C, G or T; R is A or G; Y is C or T. The *Eco*RI site in DC2 and the *Hind*III site in DC4 are underlined. The degenerate PCR (Compton, 1990) reaction mixture contained 100 ng of *Arabidopsis* genomic DNA, 1 μM of each primer, 0.5 unit of Taq polymerase (Perkin-Elmer-Cetus) and was incubated in a thermocycler (Perkin-Elmer-Cetus) for 30 cycles, each cycle consisting of one minute at 94°C, one minute at 43°C and one minute at 72°C. The PCR products were fractionated in 3% NuSieve (FMC BioProducts)/1% agarose and recovered by adsorption to Schleicher & Schuell NA-45 paper. Amplified genomic DNA fragments were directionally subcloned into pUC19(*Eco*RI/*Hind*III) and subsequently characterized by cross-hybridization and DNA sequencing of both strands.

Poly(A)⁺ RNA from IAA/CHX-treated *Arabidopsis* seedlings was used to synthesize circularized cDNA (Huang *et al.*, 1990). To reconstruct the coding region of *IAA1* and *IAA2*, inverse PCR amplimers were designed using the sequence information of the DC2-DC4 PCR products that correspond to *IAA1* and *IAA2*, respectively. Sequence and orientation of these primers is as follows: *IAA1*, 12/1A 5'-TTCACAGTAGGTGAATATTC-3' (residues 111 to 117) and 12/1B 5'-ACATAACTCAGTTTTTGTG-3' (residues 77 to 70); *IAA2*, 8/15A 5'-AATATGTTCAAAGTCATGATT-3' (residues 109 to 116) and 8/15B 5'-TTTCACGTACTCACTGTT-3' (residues 81 to 75). Using these primer sets and circular cDNA, inverse PCR products corresponding to the coding region of *IAA1* and *IAA2* were generated. The respective DNA fragments were purified, subcloned into the *Eco*RV site of pIC20R and sequenced.

The complete deduced coding region of *IAA1* and *IAA2* was generated by reverse transcription of poly(A)⁺ RNAs followed by PCR (Jones, 1993). Based on sequences of the respective inverse PCR products, the following amplimers complementary to non-translated regions of *IAA1* cDNA and *IAA2* cDNA were synthesized (the *Eco*RI and *Hind*III sites are underlined): *IAA1*, CD1-1 5'-GCCGAATTCGAGAGAATATGGAAGTCACC-3' and CD1-2 5'-GCCAAGCTTCAAAGGTGTTTTGAGACAAT-3'; *IAA2*, CD2-1 5'-GCCGAATTCACAAAGATTGATATTGATCC-3' and CD2-2 5'-GCCAAGCTTCAAAGATTCTCAGCTTCTC-3'. The coding region of *IAA4* cDNA was obtained by the same method using amplimers complementary to deduced non-translated sequences of the corresponding gene *AtAux2-11* (Conner *et al.*, 1990): CD4-1 5'-GCCGAATTCACAAAGAGGTTCTTTCTTTG-3' and CD4-2 5'-GCCAAGCTTTATCTCTCAATTAGGTTT-3'. The amplified cD-

NAs were directionally subcloned into pUC19(*Eco*RI/*Hind*III) and both strands were sequenced.

Hybridization analysis and cDNA library screening

PCR products obtained with the degenerate amplimers DC2 and DC4 were electrophoresed on 2% (w/v) agarose gels and transferred to Nytran nylon membrane (Schleicher & Schuell). The degenerate oligonucleotide DC3 (5'-AAR GTN AGY RTN GAY GG-3'; N is A, C, G or T; R is A or G; Y is C or T), corresponding to the motif KVSM/V/I/DG in domain III of PS-IAA4-like auxin-regulated polypeptides (see Figure 10), was used as hybridization probe according to Sambrook *et al.* (1989).

Appropriate enzyme digests of plasmid DNAs or total *Arabidopsis* genomic DNA were electrophoresed on 2% or 0.8% agarose gels, respectively. Southern transfer and hybridization was performed according to Oeller *et al.* (1993).

Northern analysis was performed according to Ecker & Davis (1987). The autoradiograms were quantified using an LKB ultrascan laser densitometer (Bromma, Sweden). After exposure, the probe was removed by rinsing the filters in 0.1% (w/v) SDS at 95 to 100°C for 15 to 30 minutes.

An *Arabidopsis* cDNA library (Elledge *et al.*, 1991) was screened with individual PS-IAA4/5-like DNA fragments. *IAA* cDNA inserts were recovered as described (Elledge *et al.*, 1991). Both strands of the cDNAs were sequenced.

Other molecular techniques

Standard molecular techniques and colony hybridizations were performed according to Sambrook *et al.* (1989). Plasmid DNA sequencing was carried out with the chain termination method (Sanger *et al.*, 1977) using [α -³⁵S]dATP and the modified phage T7 DNA polymerase Sequenase (U.S. Biochemicals).

Protein structure computer analysis

Homology searches were performed in January 1995 against protein data banks (SwissProt, GenPept, PIR, PROSITE) and nucleotide data banks (EMBL, GenBank, dBEST) using BLAST programmes (Altschul *et al.*, 1990) through NCBI (The National Center for Biotechnology Information; blast@ncbi.nlm.nih.gov). Secondary structure predictions were obtained using a neural network algorithm (Rost & Sander, 1993) at the EMBL Heidelberg (PredictProtein@EMBL-Heidelberg.de). The phylogenetic tree of *IAA* protein sequences was constructed using DARWIN (Gonnet *et al.*, 1992) at the public server cbrg@inf.ethz.ch.

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References

- Abel, S. & Theologis, A. (1994). Transient transformation in *Arabidopsis* leaf protoplasts: a versatile experimental system to study gene expression. *Plant J.* **5**, 421–427.
- Abel, S. & Theologis, A. (1995). A polymorphic bipartite motif signals nuclear targeting of early auxin-inducible proteins related to PS-IAA4 from pea (*Pisum sativum*) *Plant J.* **8**, 101–110.
- Abel, S., Oeller P. W. & Theologis, A. (1994). Early auxin-induced genes encode short-lived nuclear proteins. *Proc. Natl Acad. Sci. USA*, **91**, 326–330.
- Ainley, W. M., Walker, J. C., Nagao, R. & Key, J. L. (1988). Sequence and characterization of two auxin-regulated genes from soybean. *J. Biol. Chem.* **263**, 10658–10666.
- Altschul, S. F., Gish, W., Miller, W. Myers, E. & Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.
- Andres, A. J. & Thummel, C. S. (1992). Hormones, puffs and flies: the molecular control of metamorphosis by ecdysone. *Trends Genet.* **8**, 132–138.
- Assmann, S. M. (1993). Signal transduction in guard cells. *Annu. Rev. Cell Biol.* **9**, 345–375.
- Atwater, J. A., Wisdom, R. & Verma, I. M. (1990). Regulated mRNA stability. *Annu. Rev. Genet.* **24**, 519–541.
- Ballas, N., Wong, L.-M. & Theologis, A. (1993). Identification of the auxin-responsive element, *AuxRE*, in the primary indoleacetic acid-inducible gene, *PS-IAA4/5*, of pea (*Pisum sativum*). *J. Mol. Biol.* **233**, 580–596.
- Ballas, N., Wong, L. M., Ke, M. & Theologis, A. (1995). Two auxin responsive domains interact positively to induce expression of the early indoleacetic acid-inducible gene, *PS-IAA4/5*. *Proc. Natl Acad. Sci. USA*, **92**, 3483–3487.
- Berridge, M. J., Downes, C. P. & Hanley, M. R. (1989). Neural and developmental actions of lithium: a unifying hypothesis. *Cell*, **59**, 411–419.
- Blatt, M. R. & Thiel, G. (1993). Hormonal control of ion channel gating. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 543–567.
- Blatt, M. R. & Thiel, G. (1994). K⁺ channels of stomatal guard cells: bimodal control of the K⁺ inward-rectifier evoked by auxin. *Plant J.* **5**, 55–68.
- Bowie, J. U., Lüthy, R. & Eisenberg, D. (1991). A method to identify protein sequences that fold into a known three-dimensional structure. *Nature*, **253**, 164–170.
- Chen, C.-Y. A. & Shyu, A.-B. (1994). Selective degradation of early-response gene mRNAs: functional analyses of sequence features of the AU-rich element. *Mol. Cell. Biol.* **14**, 8471–8482.
- Compton, T. (1990). Degenerate primers for DNA amplification. In: *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J., eds.), pp. 39–45, Academic Press, New York.
- Conner, T. W., Goekjian, V. H., LaFayette, P. R. & Key, J. L. (1990). Structure and expression of two auxin-inducible genes from *Arabidopsis*. *Plant Mol. Biol.* **9**, 2487–2492.
- Davis, R. W., Botstein, D. & Roth, J. R. (1980). *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983). A plant DNA miniprep: version II. *Plant Mol. Biol. Rep.* **1**, 19–21.
- Ecker, J. R. & Davis, R. W. (1987). Plant defense genes are regulated by ethylene. *Proc. Natl Acad. Sci. USA*, **84**, 5202–5206.
- Elledge, S. J., Mulligan, J. T., Ramer, S. W., Spottswood, M. & Davis R. W. (1991). λYES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. *Proc. Natl Acad. Sci. USA*, **88**, 1731–1735.
- Estelle, M. (1992). The plant hormone auxin: insight in sight. *Bioessays*, **14**, 439–443.
- Franco, A. R., Gee, M. A. & Guilfoyle, T. J. (1990). Induction and superinduction of auxin-responsive mRNAs with auxin and protein synthesis inhibitors. *J. Biol. Chem.* **265**, 15845–15849.
- Gee, M., Hagen, G. & Guilfoyle, T. J. (1991). Tissue-specific and organ-specific expression of soybean auxin-responsive transcripts GH3 and SAURs. *Plant Cell*, **3**, 419–430.
- Gil, P., Liu, Y., Orbovic, V., Verkamp, E., Poff, K. L. & Green, P. (1994). Characterization of the auxin-inducible *SAUR-AC1* gene for use as a genetic tool in *Arabidopsis*. *Plant Physiol.* **104**, 777–784.
- Gonnet, G. M., Cohen, M. A. & Benner, S. A. (1992). Exhaustive matching of the entire protein sequence database. *Science*, **256**, 1443–1445.
- Gottesman, S. & Maurizi M. R. (1992). Regulation of proteolysis: Energy-dependent proteases and their targets. *Microbiol. Rev.* **56**, 592–621.
- Gray, J., Pictor, S., Shabbeer, J., Schuch, W. & Grierson, D. (1992). Molecular biology of fruit ripening and its manipulation with antisense genes. *Plant Mol. Biol.* **19**, 69–87.
- Guilfoyle, T. J. (1986). Auxin-regulated gene expression in higher plants. *CRC Crit. Rev. Plant Sci.* **4**, 247–276.
- Guilfoyle, T. J., Hagen, G., Li, Y., Ulmasov, T., Lui, Z., Strabala, T. & Gee, M. (1993). Auxin-regulated transcription. *Aust. J. Plant Physiol.* **20**, 489–502.
- Hagen, G. & Guilfoyle, T. J. (1985). Rapid induction of selective transcription by auxins. *Mol. Cell. Biol.* **5**, 1197–1203.
- He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. W. & Rosenfeld, M. G. (1989). Expression of a large family of POU-domain regulatory genes in mammalian brain development. *Nature*, **340**, 35–42.
- Herschman, H. R. (1991). Primary response genes induced by growth factors and tumor promoters. *Annu. Rev. Biochem.* **60**, 281–319.
- Hill, C. S. & Treisman, R. (1995). Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell*, **80**, 199–211.
- Hobbie, L. & Estelle, M. (1994). Genetic approaches to auxin action. *Plant Cell Environ.* **17**, 525–540.
- Huang, S. H., Hu, Y. Y., Wu, C. H. & Holcenberg, J. (1990). A simple method for direct cloning cDNA sequence that flanks a region of known sequence from total RNA by applying the inverse polymerase chain reaction. *Nucl. Acids Res.* **18**, 1992.
- Jones, M. D. (1993). Reverse transcription of mRNA by *Thermus aquaticus* DNA polymerase followed by polymerase chain reaction amplification. *Methods Enzymol.* **218**, 413–419.
- Karim, F. D. & Thummel, C. S. (1991). Ecdysone coordinates the timing and amounts of *E74A* and *E74B* transcription in *Drosophila*. *Genes Dev.* **5**, 1067–1079.
- Kobe, B. & Deisenhofer, J. (1994). The leucine-rich repeat: a versatile binding motif. *Trends Biochem. Sci.* **19**, 415–421.
- Leyser, H. M. O., Lincoln, C., Timpte, C., Lammer, D., Turner, J. & Estelle, M. (1993). The auxin-resistance gene *AXR1* of *Arabidopsis* encodes a protein related to ubiquitin-activating enzyme E1. *Nature*, **364**, 161–164.

- Li, Y., Hagen, G. & Guilfoyle, T. J. (1991). An auxin-responsive promoter is differentially induced by auxin gradients during tropisms. *Plant Cell*, **3**, 1167–1175.
- Liang, X., Abel, S., Keller, J. A., Shen, N. F. & Theologis, A. (1992). The 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **89**, 11046–11050.
- Lincoln, C., Britton, J. & Estelle, M. (1990). Growth and development of the *axr1* mutants of Arabidopsis. *Plant Cell*, **2**, 1071–1080.
- McClure, B. A. & Guilfoyle, T. J. (1987). Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. *Plant Mol. Biol.* **9**, 611–623.
- Meyerowitz, E. M. (1989). *Arabidopsis*, a useful weed. *Cell*, **56**, 263–269.
- Napoli, C., Lemieux, C. & Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. *Plant Cell*, **2**, 279–289.
- Newman, T. C., Ohme-Takagi, M., Taylor, C. B. & Green P. J. (1993). DST sequences, highly conserved among plant SAUR genes, target reporter transcripts for rapid decay in tobacco. *Plant Cell*, **5**, 701–714.
- Oeller, P. W. & Theologis, A. (1995). Induction kinetics of the nuclear proteins encoded by the early indoleacetic acid-inducible genes, *PS-IAA4/5* and *PS-IAA6*, in pea (*Pisum sativum* L.). *Plant J.* **7**, 37–48.
- Oeller, P. W., Keller, J. A., Parks J. E., Silbert, J. E. & Theologis, A. (1993). Structural characterization of the early indoleacetic acid-inducible genes, *PS-IAA4/5* and *PS-IAA6*, of pea (*Pisum sativum* L.). *J. Mol. Biol.* **233**, 789–798.
- Ohme-Takagi, M., Taylor, C. B., Newman, T. C. & Green P. J. (1993). The effect of sequences with high AU content on mRNA stability in tobacco. *Proc. Natl Acad. Sci. USA*, **90**, 11811–11815.
- Osborne, B. I. & Baker, B. (1995). The maize transposons are moving and mutagenizing in other genomes. *Curr. Opin. Cell Biol.* In the press.
- Phillips, S. E. V. (1994). The β -ribbon DNA recognition motif. *Annu. Rev. Biophys. Biomol. Struct.* **23**, 671–701.
- Pickett, F. B., Wilson, A. K. & Estelle, M. (1990). The *aux1* mutation of *Arabidopsis* confers both auxin and ethylene resistance. *Plant Physiol.* **94**, 1462–1466.
- Raumann, B. E., Brown, B. M. & Sauer, R. T. (1994). Major groove DNA recognition by β -sheets: the ribbon-helix-helix family of gene regulatory proteins. *Curr. Op. Struct. Biol.* **4**, 36–43.
- Rost, B. & Sander, C. (1993). Improved prediction of protein secondary structure by use of sequence profiles and neural networks. *Proc. Natl Acad. Sci. USA*, **90**, 7558–7562.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. 2nd edit., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanger, F., Nicken, S. & Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
- Sassone-Corsi, P., Sisson, J. C. & Verma, I. M. (1988). Transcriptional autoregulation of the proto-oncogene fos. *Nature*, **334**, 314–319.
- Scherer, S. & Davis, R. W. (1979). Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. *Proc. Natl Acad. Sci. USA*, **76**, 4951–4955.
- Theologis, A. (1986). Rapid gene regulation by auxin. *Annu. Rev. Plant Physiol.* **37**, 407–438.
- Theologis, A., Huynh, T. V. & Davis R. W. (1985). Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. *J. Mol. Biol.* **183**, 53–68.
- Timpte, C., Wilson, A. K. & Estelle, M. (1992). Effects of the *axr2* mutation of *Arabidopsis* on cell shape in hypocotyl and inflorescence. *Planta*, **188**, 271–278.
- Timpte, C., Wilson, A. K. & Estelle, M. (1994). The *axr2-1* mutation of *Arabidopsis thaliana* is a gain-of-function mutation that disrupts an early step in auxin response. *Genetics*, **138**, 1239–1249.
- Went, F. W. & Thimann, K. V. (1937). *Phytohormones*, Macmillan, New York.
- Williamson, M. P. (1994). The structure and function of proline-rich regions in proteins. *Biochem. J.* **297**, 249–260.
- Wilson, A. K., Pickett, F. B., Turner, J. & Estelle, M. (1990). A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. *Mol. Gen. Genet.* **222**, 377–383.
- Wyatt, R. E., Ainley, W. M. Ainley, Nagao, R. T., Conner, T. W. & Key J. L. (1993). Expression of the *Arabidopsis AtAux2-11* auxin-responsive gene in transgenic plants. *Plant Mol. Biol.* **22**, 731–749.
- Yamamoto, K. T., Mori, H. & Imaseki, H. (1992). cDNA cloning of indole-3acetic acid-regulated genes: Aux22 and SAUR from mung bean (*Vigna radiata*) hypocotyl tissue. *Plant Cell Physiol.* **33**, 93–97.

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