

Early auxin-induced genes encode short-lived nuclear proteins

(plant hormone action/plant cell growth/protein stability/ $\beta\alpha\alpha$ DNA binding motif/nuclear localization)

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ABSTRACT The plant growth hormone indoleacetic acid (IAA) transcriptionally activates gene expression in plants. Some of the genes whose expression is induced by IAA encode a family of proteins in pea (PS-IAA4 and PS-IAA6) and *Arabidopsis* (IAA1 and IAA2) that contain putative nuclear localization signals that direct a β -glucuronidase reporter protein into the nucleus. Pulse-chase and immunoprecipitation experiments have defined the $t_{1/2}$ of the PS-IAA4 and PS-IAA6 proteins to be 8 and 6 min, respectively. Their most prominent feature is the presence of a $\beta\alpha\alpha$ motif similar to the β -sheet DNA-binding domain found in prokaryotic repressors of the Arc family. Based on these data, we suggest that plant tissues express short-lived nuclear proteins as a primary response to IAA. We propose that these proteins act as activators or repressors of genes responsible for mediating the various auxin responses.

The plant hormone auxin, typified by indoleacetic acid (IAA), regulates various aspects of plant growth and development (e.g., cell division, differentiation, morphogenesis, oncogenesis, and tropisms) and is generally considered to be responsible for regulating plant cell growth (1, 2). The mechanism responsible for auxin-induced cell growth is unknown. Auxin-mediated cell elongation is associated with rapid changes in the expression of a select set of primary genes (3, 4). Although several auxin-responsive genes have been isolated and structurally characterized, the function of the proteins they encode is unknown (5–12).

Expression of two genes in pea [*Pisum sativum* (PS)], PS-IAA4/5 and PS-IAA6, and two members of a family of similar genes in *Arabidopsis*, IAA1 and IAA2,‡ are rapidly induced by IAA (within 4–8 min) and by protein synthesis inhibitors (refs. 5 and 12; S.A. and A.T., unpublished data). The encoded proteins of the PS-IAA4/5-like genes share similar physical properties and extensive amino acid sequence identity with six other proteins from other plant species [Aux22 and Aux28, soybean (9); ARG3 and ARG4, mungbean (10); AtAux2-11 and AtAux2-27, *Arabidopsis* (11)]. Data base searches do not find any significant sequence similarity with any other protein (12, 13). Our initial attempts to immunolocalize the pea proteins, PS-IAA4 and PS-IAA6, were unsuccessful (13) probably because these proteins are short-lived and thus of low abundance. We show that these proteins are localized to the nucleus and contain a putative $\beta\alpha\alpha$ motif reminiscent of the β -ribbon DNA-binding domain of prokaryotic repressor polypeptides (14).

MATERIALS AND METHODS

Plant Material. Abraded third internode segments from 7-day-old etiolated pea seedlings (*Pisum sativum* cv. Alaska) were treated with 20 μ M IAA as described (5).

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Antibody Production for the PS-IAA4/5 and PS-IAA6 Proteins. Rabbit polyclonal antibodies to PS-IAA4/5 and PS-IAA6 proteins were raised by expressing the pIAA4/5 and pIAA6 cDNAs in *Escherichia coli* (5, 12, 13) and immunizing a New Zealand White rabbit three times at 3-week intervals with 1 mg of recombinant protein.

$t_{1/2}$ Determination by *in Vivo* Labeling and Immunoprecipitation. Tissue samples [40 segments; 0.8 g (fresh weight) per sample] were depleted for 2 hr and pulse-labeled for an additional 2 hr with or without 20 μ M IAA in the presence of 1 mCi of [³⁵S]methionine per sample (5) (1 Ci = 37 GBq). The chase was carried out in buffer containing a 1000-fold excess of unlabeled methionine. The incubation medium was changed every 5 min during the chase period. Labeled tissue was frozen in liquid N₂ and lyophilized. Protein was extracted using boiling extraction buffer (100 mM sodium phosphate, pH 7.0/140 mM 2-mercaptoethanol/1% SDS) to inactivate endogenous proteases (15). Immunoprecipitations with affinity-purified antisera were performed as described by Giudice *et al.* (16) except the trichloroacetic acid precipitation was omitted and the wash buffer was adjusted to 0.4 M LiCl for the first two of the five wash steps. The immunoprecipitates were eluted into boiling Laemmli loading buffer and resolved on a SDS/12% polyacrylamide gel (17). The $t_{1/2}$ was calculated using the formula, $(0.693 \times t)/\ln(N_0/N)$, where t is the chase time and N is the fraction remaining of the initially labeled protein N_0 (18).

Protein Structure Analysis. The data base maintained by Genentech (containing the GenBank, EMBL, and other data bases) was used for sequence searches (June 1992). Secondary-structure predictions were obtained using Chou–Fasman (19) and Garnier–Osguthorpe–Robson (20) parameters with the Genetics Computer Group suite of programs on the Stanford University VAX computer and a neural network algorithm (PHD) at the European Molecular Biology Laboratory (Heidelberg) (21).

Nuclear Localization. Translational fusions between β -glucuronidase (GUS) and the IAA-inducible proteins were constructed as follows: full-length coding sequences for PS-IAA4 and PS-IAA6 (12), AtAux2-11 (11), and IAA1 and IAA2 (S.A. and A.T., unpublished data) proteins were synthesized by PCR (22). The PCR products were subcloned into pRTL2-GUS/Nla Δ Bam (23, 24) after removing the Nla Δ Bam fragment by *Bgl* II/*Xba* I or *Bgl* II/*Bam*HI digestion, respectively. The *Bgl* II site provides the C-terminal translational fusion site to GUS, extending the GUS protein by Arg-Ser. The following amplimers (5' → 3') were used (with the *Bgl* II, *Xba* I, or *Bam*HI linkers underlined and boldface type is the start or termination codon, respectively): N-4, **GCCGGATC-**

Abbreviations: IAA, indoleacetic acid; GUS, β -glucuronidase; NLS, nuclear localization signal; PS, *Pisum sativum*.

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. for pIAA1, L15448; for pIAA2, L15449; and for pIAA4, L15450).

CATGGAATTCAAGGCCAAGTGGAGC (PS-IAA4, aa 1-7); C-4, GCCGGATCCTCATACACCACAACCCAATCC (PS-IAA4, aa 189-183); N-6, GCCAGATCTATGGCAA-GAGAAGTTTATAGGAC (PS-IAA6, aa 1-7); C-6, GCCTCTAGACTATATGAATCTCTTCAAAGATCC (PS-IAA6, aa 179-173); N-1, GCCGGATCCATGGAAGTCAC-CATGGGCTTAACC (IAA1, aa 1-8); C-1, GCCTCTA-GATCATAAGGCAGTAGGAGCTTCAGATCCTTTC (IAA1, aa 167-158); N-2, GCCGGATCCATGGCCTAC-GAGAAAGTCAACGAG (IAA2, aa 1-8); C-2, GCCTCTA-GATCATAAGGAAGAGTCTGGAGCAGGAGCGTCT-GATCCCTTC (IAA2, aa 174-163); N-7, GCCGGATC-CATGGAAAAAGTTGATGTTTATGATGAGC (AtAux2-11, aa 1-9); C-7, GCCTCTAGATTAAA GACCAC-CACAACCTAACCTTAAAC (AtAux2-11, aa 186-178). The cDNA inserts of plasmids pP037 (PS-IAA4), pP057 (PS-IAA6) (12), pIAA1, pIAA2, and pIAA4 (AtAux2-11) were used as PCR templates. pIAA4 was obtained by reverse transcription of RNA (22) prepared from auxin-treated *Arabidopsis* seedlings followed by PCR using amplimers complementary to nontranslated sequences of the deduced AtAux2-11 cDNA (11) and subcloning the PCR product into pUC19 (23). All plasmid constructs were verified by dideoxynucleotide sequencing. Preparation, transfection, and histochemical staining of GUS activity in tobacco protoplasts were performed as described (25). In addition, Hoechst 33342 (Sigma) at 20 μg/ml was added to the GUS assay buffer for staining the DNA. Protoplasts and cell nuclei were visualized using differential interference contrast and epifluorescence optics (Zeiss Axiophot microscope), respectively.

RESULTS AND DISCUSSION

The Pea Proteins PS-IAA4 and PS-IAA6 Are Short Lived. Two rapidly auxin-inducible genes, *PS-IAA4* and *PS-IAA6* from pea, encode proteins of 21 and 20.3 kDa, respectively, that are 46% identical (12). They also share a significant degree of identity (42-80%) with other proteins encoded by early auxin-regulated genes in soybean, mungbean, and *Arabidopsis* (9-11). All these proteins contain four conserved domains ranging in size from 8 to 33 aa (Fig. 1 and ref. 12). The pea proteins, PS-IAA4 and PS-IAA6, were expressed in

E. coli, and polyclonal antibodies were raised that detect by Western blot analysis <1 ng of protein made in bacteria. The proteins cannot be detected on immunoblots of pea extracts from IAA-treated tissue, nor can they be subcellularly localized by immunofluorescence (13). However, antibodies to PS-IAA4 protein specifically precipitate IAA-inducible proteins of 25 (PS-IAA4) and 26 (PS-IAA5) kDa from *in vitro* translation products and *in vivo* [³⁵S]methionine-labeled proteins. Similarly, antibodies to PS-IAA6 protein (20 kDa) precipitate a 21-kDa IAA-inducible protein made in the same ways (13). These data demonstrate that (i) the proteins are not extensively modified posttranslationally, and (ii) our failure to detect them on immunoblots of pea extracts or on thin sections of pea epicotyls by immunofluorescence is probably due to their low abundance.

This raises the prospect that the PS-IAA4 and PS-IAA6 proteins are short lived and do not accumulate at high levels even though their corresponding mRNAs are induced 30- to 100-fold in the presence of IAA (5). Fig. 2 shows pulse-chase and immunoprecipitation experiments for determining the t_{1/2} of the IAA-regulated proteins. The results show that both PS-IAA4/5 and PS-IAA6 proteins have a remarkably short t_{1/2} (Fig. 2 a and b, respectively), whereas β-tubulin is stable over the 1-hr chase period (Fig. 2c). The 25/26-kDa doublet of PS-IAA4/5 is degraded with t_{1/2} of ≈8 min and the 21-kDa PS-IAA6 protein is degraded with t_{1/2} of ≈6 min (Fig. 2 a and b). The effect of IAA on the stability of the proteins is negligible. The results in Fig. 2 a and b indicate that the degradation of the proteins does not obey first-order kinetics. Therefore, the calculated t_{1/2} refers to the first 15 min of the chase period. This apparent decrease in the degradation rate has been previously observed in other systems and is not fully understood (27-29). It may reflect a pool of protein that is more stable either because of its interaction with other cellular components or because of its intracellular localization.

The t_{1/2} values of PS-IAA4 and PS-IAA6 are among the shortest known for eukaryotic proteins and are characteristic of regulatory molecules (27-30). Rapid degradation of specific proteins is a component of many biological regulatory mechanisms, including aspects of growth control, metabolic regulation, embryonic development, and cell-cycle progres-

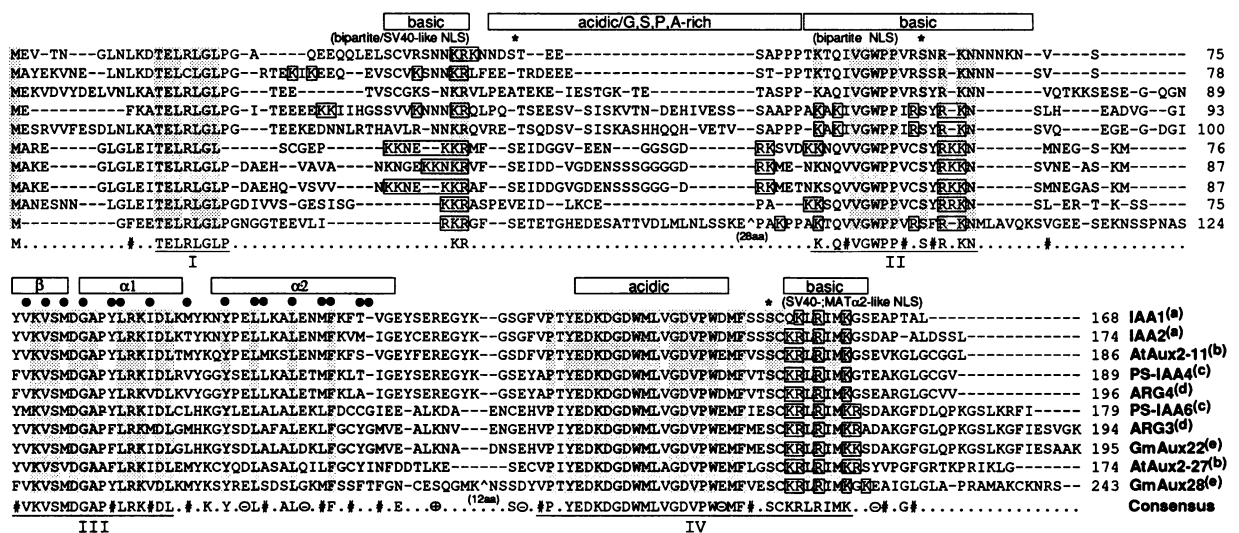


FIG. 1. Sequence alignment and domain structure of primary auxin-responsive gene products. Identical (shaded) and generally conserved amino acid residues appear in the consensus [at least 8 of 10 matches [⊖, acidic (D, E); ⊕, basic (R, K); #, hydrophobic (A, C, V, I, L, M, F, Y, W)]]. Conserved domains are underlined and indicated by Roman numerals. Basic residues that may contribute to putative nuclear localization signals (NLS) are boxed (26). Conserved phosphorylation sites proximal to putative NLS are indicated by stars on top of the alignment (casein kinase II protein kinase, S/TXK/E/D; protein kinase C, S/TXR/K). Amino acids that may form hydrophobic surfaces in the predicted conserved amphipathic βαα motif are indicated by ●. Sources of the sequences are as follows: a and b, *Arabidopsis thaliana* (S.A. and A.T., unpublished data and ref. 11); c, pea (*Pisum sativum*) (12); d, mung bean (*Vigna radiata*) (10); e, soybean (*Glycine max*) (9).

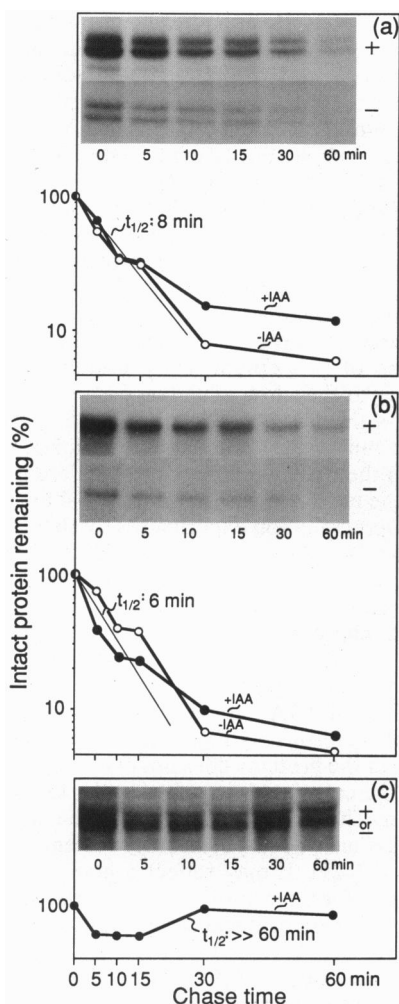


FIG. 2. $t_{1/2}$ of PS-IAA4 and PS-IAA6 proteins. Etiolated pea epicotyl tissue was pulse-labeled *in vivo* with [35 S]methionine in the presence (+) or absence (-) of IAA for 2 hr, chased in the presence of excess unlabeled methionine for the times indicated, and processed for immunoprecipitation using affinity-purified PS-IAA4/5 (a), PS-IAA6 (b), and β -tubulin (c) antibodies. Portions of the fluorograms are shown. The results were quantified with a scanning densitometer and presented graphically.

sion (28, 29). The *Drosophila ftz* and yeast MAT α 2 transcription factors have a $t_{1/2}$ of 5–10 min (30) and the protooncogene products Myb, Myc, and Fos are also known to be rapidly turned over with a $t_{1/2}$ of 25–60 min (27, 31, 32). Although we have no information on the mechanism of PS-IAA4/5 and PS-IAA6 degradation, we note that neither protein contains a typical "PEST" sequence associated with rapid turnover (33).

Mutations altering the $t_{1/2}$ of these types of proteins have profound effects on the development of an organism (28, 29). The importance of selective protein turnover is dramatically illustrated by mutations in the *Drosophila ftz* gene. Mutations in this gene (*Ual* alleles) cause homeotic transformations of the anterior first abdominal segment (A1) to the third (A3) (28, 29). Each of the alleles are missense mutations that increase the Ftz protein $t_{1/2}$ from 5 to \approx 40 min but do not appear to alter the function of the protein. Transformation is likely due to the higher steady-state levels of Ftz protein but could also reflect an aberrant temporal gradient of expression resulting from the increased $t_{1/2}$ (28, 29). The short $t_{1/2}$ of these auxin-regulated proteins is intriguing considering recent results regarding the auxin-resistant mutant *axr1* of *Arabidopsis* (34). The *AXR1* gene encodes a protein related to the

ubiquitin-activating enzyme E₁ (34). The possibility exists that the *AXR1* gene product may affect the $t_{1/2}$ of the *Arabidopsis* PS-IAA4/5- and PS-IAA6-like proteins (see Fig. 5). Alternatively, it may regulate the level of the short-lived repressor molecule postulated to be responsible for the regulation of the *PS-IAA4/5* and *PS-IAA6* gene expression (refs. 4 and 35 and see Fig. 5).

Nuclear Localization of the PS-IAA4-Like Proteins. The amino acid alignment of this family of early auxin-inducible proteins shown in Fig. 1 reveals the presence of three generally conserved clusters of basic amino acid residues. Interestingly, the composition of these short basic regions satisfy sequence requirements of three classes of NLS (26, 36). A putative simian virus 40-like NLS (PKKKRKV) is found at the end of conserved domain IV [smallest consensus, KR/KXR/K (26)]. This region also appears similar to the MAT α 2-like NLS (KIPIK), which is usually composed of short hydrophobic strings with few interspersed basic residues (26). Putative simian virus 40-like NLS and bipartite NLS are also found in domain II and near the N terminus (Fig. 1).

The presence of a putative NLS raises the prospect that these proteins reside in the nucleus. To test this possibility, the coding sequences of five genes from two plant species, pea [PS-IAA4 and PS-IAA6 (5, 12)] and *Arabidopsis* [IAA1 and IAA2 (S.A. and A.T., unpublished data) and AtAux2-11 (11)], were fused to the 3' end of the *GUS* gene. These five proteins represent the structural diversity of the protein family shown in Fig. 1. The chimeric gene constructs were introduced into tobacco protoplasts and the intracellular localization of the transiently expressed chimeric *GUS* proteins was determined histochemically. The authentic 68-kDa *GUS* protein, which has been shown to be located in the cytoplasm unless it is tagged with an NLS (37), does not accumulate in tobacco nuclei (Fig. 3 A and G). However, a translational fusion of *GUS* with the nuclear protein VirD2 (25) is directed to the nucleus (Fig. 3 B and H). Similar nuclear accumulation of the *GUS* product is evident in tobacco protoplasts transiently expressing fusions of *GUS* with all the auxin-regulated polypeptides tested (Fig. 3 C–F and I–L; data for AtAux2-11 are not shown). In addition, transient expression of *GUS::PS-IAA4* and *GUS::PS-IAA6* in pea protoplasts confirmed the results in a homologous transformation system (data not shown). The uniformity of the subcellular localization and the presence of putative NLS in all polypeptides of this family indicate that the nucleus is the cellular compartment where they function.

Perhaps small proteins (<60 kDa) such as the auxin-regulated polypeptides (18–27 kDa) simply diffuse into the nucleus and are retained. However, growing experimental evidence indicates that nuclear proteins are selectively recognized and actively transported into the nucleus regardless of their size (26, 36). In fact, no physiologically relevant macromolecule has been demonstrated to enter the nucleus by diffusion, and indeed, many small cytoplasmic proteins are excluded from the nucleus (38–40). Conversely, certain small nuclear proteins, such as the yeast histone H2B (15 kDa), maize Opaque-2 protein (47 kDa), and calf thymus histone H1 (21 kDa), either contain NLS or are actively transported into the nucleus (38–40). Further studies will be required to confirm the function of the putative NLS in the auxin-regulated polypeptides. In addition, conserved phosphorylation sites in the vicinity of putative NLS (Fig. 1) may play a role in the regulation of their nuclear transport, as was demonstrated for several mammalian transcription factors (36).

The β aa Motif. We speculate that the anticipated nuclear function of the early auxin-inducible gene products is linked to a conspicuous structural feature centered around domain III of their primary structure (Fig. 1). Secondary-structure

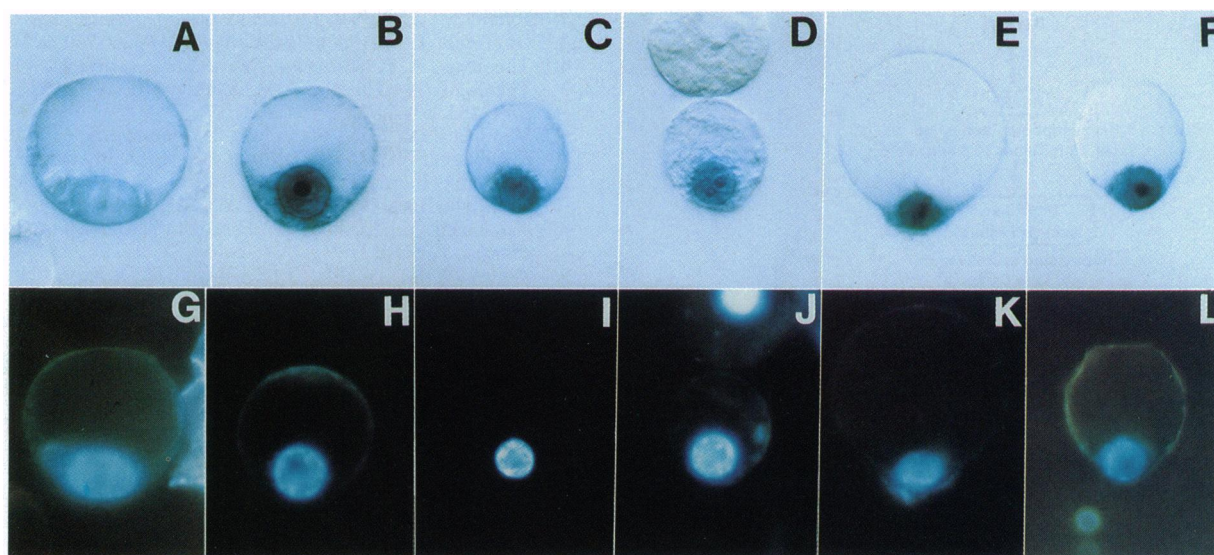


FIG. 3. Nuclear localization of the auxin-regulated polypeptides. Tobacco (*Nicotiana tabacum*) protoplasts were purified, transfected with plasmid DNA (containing *GUS*-auxin gene fusions), incubated in culture medium for 12–16 hr, and assayed for GUS activity (A–F) and stained for nuclei (G–L). (A and G) Authentic GUS. (B and H) GUS::VirD2. (C and I) GUS::PS-IAA4. (D and J) GUS::PS-IAA6. (E and K) GUS::IAA1. (F and L) GUS::IAA2.

analyses predict [≈50% prediction accuracy for the Chou–Fasman algorithm (19) and ≈70% prediction accuracy for a neural network algorithm (21)] that this region, including the five invariant hydrophobic amino acid residues at conserved positions C-terminal to the third domain, forms an amphipathic $\beta\alpha\alpha$ structure (Figs. 1 and 4). The helix–turn–helix motif therein is unlike the motif found in prokaryotic (e.g., λ repressor) and eukaryotic (e.g., MAT α 2 homeodomain) regulatory proteins (14). However, we note a possibly significant similarity between the predicted $\beta\alpha\alpha$ motif in auxin-regulated proteins and the $\beta\alpha\alpha$ DNA-binding domain of prokaryotic repressor proteins such as Arc (14). This similarity is based on common features of their secondary structures and not on their primary structures, which do not exhibit a high degree of identity. Interestingly, the prokaryotic $\beta\alpha\alpha$ motif itself shows limited sequence conservation (Fig. 4A), and the relationship between Arc and MetJ was only clearly established after their structures were elucidated (14). Searches for

additional members of the prokaryotic family identified the TraY proteins as probable relatives, when based on hydrophobicity patterns in Arc and Mnt (14). Most noteworthy is the close match between the pattern of hydrophobic amino acids in the putative plant $\beta\alpha\alpha$ motif and the hydrophobicity pattern in the prokaryotic $\beta\alpha\alpha$ domain, including a conserved acidic residue in the second helix (Fig. 4A). The periodicity of hydrophobic and hydrophilic amino acid residues is reflected in the amphipathic nature of the respective secondary structure elements (Fig. 4B). The conservation of the putative plant $\beta\alpha\alpha$ motif, which is present in at least 14 expressed members of the auxin-regulated *Arabidopsis* gene family (S.A. and A.T., unpublished data), the relatively short sequences of the proteins (167–243 aa, Fig. 1), and their likely function in the plant cell nucleus (Fig. 3) increase the probability that the matches found between the two protein families are significant (Fig. 4A). However, the structure of the plant $\beta\alpha\alpha$ motif remains to be verified.

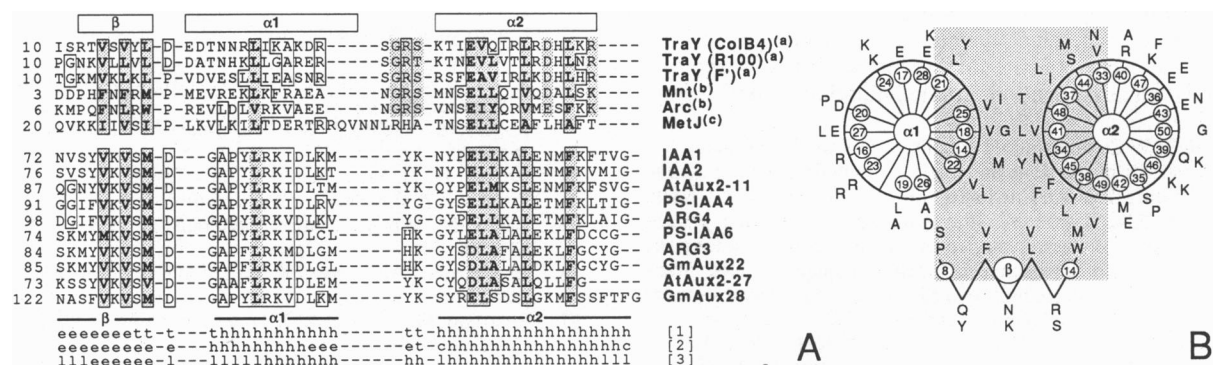


FIG. 4. $\beta\alpha\alpha$ motif in prokaryotic repressor proteins and auxin-inducibile gene products. (A) Sequence alignment. Similar amino acid residues occurring frequently at a given position in both protein families are boxed (hydrophobic, A, C, V, I, L, M, F, Y, and W; acidic, D and E; basic, K, R, and H; polar, S, T, N, and Q). Positions that are generally conserved in the prokaryotic sequences are shaded (similar residues in at least five out of six sequences). Similar amino acids in positions that are generally conserved in both protein families are shaded and shown in boldface type. The secondary structure known for MetJ from crystal structure and for Arc from NMR analysis (14) is indicated above the alignment. The predicted secondary structure of the plant $\beta\alpha\alpha$ motif is that of IAA1 and is given below the sequences. Methods: 1, Chou–Fasman (19); 2, Garnier–Osguthorpe–Robson (20); 3, profile network (21). Predictions of helical (h), extended (e), turn (t), coil (c), and loop (l) conformations are also indicated. Sources of the sequences (in parentheses): a, *E. coli* (14); b, *Salmonella* phage P22 (14); c, *E. coli* (14). (B) Planar projection of the three secondary structure elements. Positions of amino acids refer to the Arc repressor (Arc residues closer to wheel peripheries and β -ribbon). Corresponding amino acids in the motif of IAA1 are placed next to those of Arc. Apolar sides of the secondary structure elements are shaded.

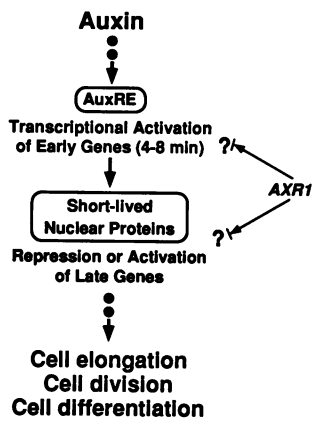


Fig. 5. Model for early auxin events.

Based on these structural similarities, an analogous function of the predicted plant $\beta\alpha\alpha$ domain is proposed. The best-studied members of the β -ribbon repressor family are MetJ and Arc (14). These proteins regulate in part methionine biosynthesis in *E. coli* and lysogeny in the *Salmonella* phage P22, respectively, and bind to DNA as tetramers (14). The antiparallel β -ribbon and additional N-terminal residues make specific operator contacts in an extended conformation of each dimer. The second helix of the $\beta\alpha\alpha$ domain is involved in dimerization, whereas the first helix mediates cooperative binding of dimer units (14). In this context, we note a cluster of basic amino acid residues on the N-terminal side of the β -ribbon in the predicted plant $\beta\alpha\alpha$ structure and a less-conserved nature of the second helix among the auxin-regulated polypeptides (Fig. 1). These regions may convey specificity for DNA binding and heterodimerization.

CONCLUSION

The results presented demonstrate that elongating plant cells produce short-lived nuclear proteins with structural features of prokaryotic transcription factors as a primary response to auxin. We suggest that these auxin-regulated proteins play a central role in the regulation of subsequent auxin-induced events responsible for plant cell growth (Fig. 5), similar to the ecdysone-mediated cascade of gene expression during insect development (41). The ability of auxin to act through a biochemical machinery whose components are short-lived is a prerequisite for execution of rapid responses to auxin, such as elongation, gravistimulation, and tropisms.

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