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Expression of the *Arabidopsis AtAux2-11* auxin-responsive gene in transgenic plants

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Abstract

Five constructions containing deletions of the promoter from an auxin-inducible gene of *Arabidopsis thaliana*, *AtAux2-11*, were fused to the coding region of the reporter gene LacZ, which encodes β-galactosidase, and a polyadenylation 3′-untranslated nopaline synthase sequence from *Agrobacterium*. These chimeric genes were introduced into *Arabidopsis* by *Agrobacterium tumefaciens*-mediated transformation, and expression of the gene was examined by spectrophotometric and histochemical analyses. A 600 bp fragment from the *AtAux2-11* promoter conferred histochemical patterns of staining similar to the longest 5′ promoter tested, a 3.0 kb fragment. Localization of *AtAux2-11*/LacZ activity in the transgenic plants revealed spatial and temporal expression patterns that correlated with tissues and cells undergoing physiological processes modulated by auxin. LacZ activity was expressed in the elongating region of roots, etiolated hypocotyls, and anther filaments. Expression was detected in the vascular cylinder of the root and the vascular tissue, epidermis, and cortex of the hypocotyl, and filament. The *AtAux2-11*/LacZ gene was preferentially expressed in cells on the elongating side of hypocotyls undergoing gravitropic curvature. Expression of the chimeric gene in the hypocotyls of light-grown seedlings was less than that in etiolated seedling hypcotyls. The *AtAux2-11*/LacZ gene was active in the root cap, and expression in the root stele increased at sites of lateral root initiation. Staining was evident in cell types that develop lignified cell walls, e.g. trichomes, anther endothecial cells, and especially developing xylem. The chimeric gene was not expressed in primary meristems. While the magnitude of expression increased after application of exogenous auxin (2,4-D), the histochemical localization of *AtAux2-11*/LacZ remained unchanged.

Transgenic plants with a 600 bp promoter construct (−0.6 kb *AtAux2-11*/LacZ) had higher levels of basal and auxin-inducible expression than plants with a 3.0 kb promoter construct. Transgenic plants with a −500 bp promoter had levels of expression similar to the −3.0 kb construct. The −0.6 kb *AtAux2-11*/LacZ gene responded maximally to a concentration of 5×10^{-6} to 5×10^{-5} M 2,4-D and was responsive to as little as 5×10^{-8} M. The evidence presented here suggests that this gene may play a role in several auxin-mediated developmental and physiological processes.

Introduction

The plant growth regulator auxin is responsible for a number of physiological effects and developmental responses in growing plants. Auxin is associated with tropisms, cell division and elongation, initiation and development of vascular tissue, branch root initiation, trichome development, apical dominance, and leaf abscission [18]. Many of these auxin-induced processes are thought to involve changes in gene expression, and a number of mRNAs have been identified that increase or decrease in abundance in response to auxin treatment [2, 4, 11, 12, 19, 21, 24, 26, 28]. The isolation and characterization of two auxin-inducible genes (*GmAux22* and *GmAux28*) expressed in elongating etiolated soybean hypocotyls have been previously reported [1, 29]. Genes homologous to *GmAux22* and *GmAux28* were isolated from *Arabidopsis thaliana* and characterized [6]. These *Arabidopsis* genes (designated *AtAux2-11* and *AtAux2-27*) belong to a small gene family and are similar to the soybean Aux gene family with regard to the amino acid sequence of the predicted proteins, intron/exon locations, and the auxin-enhanced expression of their transcripts.

To date, no function has been determined for any of these auxin-induced gene products, and only a limited number of correlations between auxin-induced changes in gene expression and the various auxin-mediated developmental responses have been described. Analyses of one auxin-regulated gene, the *dbp* gene of *Arabidopsis*, suggests that it is a DNA-binding protein, and *in situ* localization studies show that *dbp* transcripts are present at higher concentrations in regions undergoing cell division [2]. Two different classes of auxin-inducible transcripts isolated from soybean hypocotyl have been localized using *in situ* hybridization. One class includes the pGH3 transcript [11]. This transcript is found in the vascular tissues of 4-day old soybean seedling roots and etiolated hypocotyls. Expression of pGH3 transcripts occurred in additional tissue after exogenous addition of auxin [9]. A second class of auxin-responsive mRNAs, the small auxin up-regulated mRNAs (SAURs), were localized to the xylem in sections taken from the apical region of soybean seedlings and the epidermis and cortical region of the elongating portion of the hypocotyl or epicotyl [9]. A SAUR promoter fused to the β-glucuronidase reporter gene (GUS) directed expression of GUS in a manner that correlated with the expected concentration of endogenous auxin in transgenic tobacco plants [17].

As part of a continuing effort to elucidate the function of the gene products and mechanism of activation of auxin-regulated genes, the promoter region of the *AtAux2-11* gene was fused to the reporter gene, β-galactosidase (LacZ), and analyses were performed on transgenic *Arabidopsis* seedlings and plants containing this chimeric gene. Both quantitative and histochemical assays were performed using five different 5′-deletion constructs to determine the degree and location of expression of the reporter gene and to localize important *cis*-regulatory elements. The results demonstrate that the 617 bp fragment of the *AtAux2-11* promoter region 5′ to the transcriptional start site confers tissue-specific patterns of expression and auxin inducibility similar to those of much longer promoter constructs. Activity observed by histochemical staining in transgenic plants was greatest in lignified tissues, including xylem, leaf trichomes, and endothecilal cells within the anther. LacZ activity was also observed in the elongating region of etiolated seedlings and, asymmetrically, on the elongating side of seedlings undergoing gravitropic curvature. Expression from the *AtAux2-11* promoter was enhanced by as little as 0.01 μM exogenous auxin, and the maximum response was observed when 5 to 50 μM auxin was used. These results are consistent with the hypothesis that the *AtAux2-11* gene is involved in many of the physiological and developmental growth processes modulated by auxin.

Materials and methods

Plasmid and promoter constructions

The LacZ reporter gene fragment was isolated by *Bam* HI digestion of pHTT27 [25] and ligated

into Bluescript KS (–) (Stratagene, La Jolla, CA) making pARG105. pARG105 was linearized by *Spe* I digestion; the NOS 3′ polyadenylation containing sequence was isolated by *Eco* RI/*Bam* HI digestion of pMA404 [1]. Both fragments were Klenow filled and ligated to form pLacZBam. The LacZNOS insert was isolated from pLacZBam as a *Xba* I/Asp fragment, Klenow-filled and ligated into *Bgl* II digested, Klenow-filled T-DNA vector pGG102 making pARG110. pGG102 was derived from pGA470 [3] by *Eco* RI digestion and addition of *Bgl* II linkers. The promoter deletion constructs of *AtAux2-11* [6] were blunt-end ligated into the Klenow-filled *Hind* III site of pARG110 from the restriction fragments as follows: −3 kb (*Hinc* II), −1.5 kb (*Bam* HI), −0.6 kb (*Spe* I), −0.5 kb (*Eco* RV) and −0.4 kb (*Nde* I).

Arabidopsis *transformations*

Arabidopsis thaliana (ecotype Norway) was transformed by the root explant procedure essentially as described by Valvekens *et al.* [27]. The *AtAux2-11* promoter deletion/LacZ constructs were transferred into the *Agrobacterium tumefaciens* strain EHA101 [13] using the triparental mating procedure of Ditta *et al.* [7] with pRK2013 [8] providing the genes for mobilization of the binary vector into *Agrobacterium*. Transformants were selected on media containing 50 μg/ml kanamycin. Kanamycin-resistant S2 seeds were used in all quantitative analyses.

Auxin application

Seeds from independent transgenic *Arabidopsis* plants were germinated and grown on MS salts media with 50 μg/ml kanamycin at 22 °C with a day length of 18 h. For experiments assaying promoter deletions at later stages of development, seedlings were transferred to pots containing Peatlite for the indicated time. For experiments with auxin application, plants were sprayed until run-off with a 50 μM solution of 2,4-D.

β-galactosidase assay in solution

Plant tissue, whole or partial *Arabidopsis* seedlings, were first fixed with 1% glutaraldehyde in Z′ buffer [25] for 6 h at room temperature. Plant tissue was homogenized in Z′ buffer supplemented with 100 mM (final concentration) 2-mercaptoethanol. The extract was cleared by centrifugation (10 min at 14000 rpm, 4 °C in a microcentrifuge) and assayed spectrophotometrically by a modification of the method of Miller [20]. A blank reaction with no extract was used as a spectrophotometric control for each assay. Protein content was determined by the dye-binding method (Bio-Rad). One unit of β-galactosidase is defined as the amount of enzymatic activity hydrolyzing 1 nmol of ONPG (o-nitro-phenyl-β-*D*-galactoside) per minute at 25 °C [20].

β-galactosidase assay in gel

Extracts were prepared as described for solution assays and normalized for protein content. The extracts were mixed with cold loading buffer and applied in an 8% polyacrylamide gel [16]. After electrophoresis, the gel was washed for 15 min in cold Z′ buffer and allowed to equilibrate in Z′ buffer for 15 min at room temperature. The fluorogenic β-galactosidase substrate 4-methyl, umberlliferyl β-*D*-galactoside was dissolved at 20 mg/ml in dimethylsulfoxide and diluted 1000-fold in Z′ buffer. The gel was incubated with the substrate for 10 min at room temperature and photographed under UV illumination.

Histochemical β-galactosidase assay

Plant tissue was first fixed in glutaraldehyde as described above. The fixative was then removed and replaced with the staining solution (880 μl Z′ buffer, 50 μl 100 mM $K_3[Fe(CN)_6]$, 50 μl 100 mM $K_4[Fe(CN)_6]$, 20 μl 8% 5-bromo-4-chloro-3-indolyl-β-*D*-galactoside in N,N-dimethylformamide). The tissue was incubated at 28 °C

until blue staining was visible, usually overnight. The material was rinsed with Z′ buffer and soaked in 95% ethanol to remove chlorophylls. After rinsing with absolute ethanol, the tissue was embedded in Immunobed (Jansen, Life Sciences) and cut into 2–4 μm sections on a Sorvall (Newtown CT) MT-2 microtome using glass knives. Micrographs were taken on a Zeiss (Vienna, Austria) dissecting microscope (whole plants) or a Zeiss light microscope (sectioned material) using Kodacolor 100 film (Kodak).

Results

Construction of the LacZ fusions and Arabidopsis *transformation*

The *AtAux2-11* promoter/LacZ constructs used in these experiments are detailed in Fig. 1. Each construct has an identical fusion of the promoter fragment to the LacZ protein coding sequence followed by a portion of the nopaline synthase gene that contains a polyadenylation sequence. Promoter fragments beginning at approximately −3.0, −1.5, −0.6, −0.5 and −0.4 kb (Fig. 1A) were analyzed for their ability to confer LacZ expression in transgenic *Arabidopsis* plants.

LacZ was chosen as a reporter gene because of its reported superiority to β-glucuronidase for histochemical analysis of gene promoter activity [25]. Any endogenous plant β-galactosidase activity can be eliminated by fixation with glutaraldehyde. Evidence that penetration of the fixative and X-gal (the histochemical substrate of LacZ) is not limiting in young *Arabidopsis* plants is derived from observations that the innermost cells of the root, cotyledon and primary leaves are stained in transgenic plants, while endogenous activity is completely eliminated by fixation in control plants. Fig. 1B shows the effect of fixation on the activities of the endogenous and LacZ β-galactosidases. In extracts from transformed unfixed tissues (lane 1), both enzymes are present and active, while in extracts from glutaraldehyde fixed material (lane 2), only the LacZ (upper band) β-galactosidase remains active. The upper

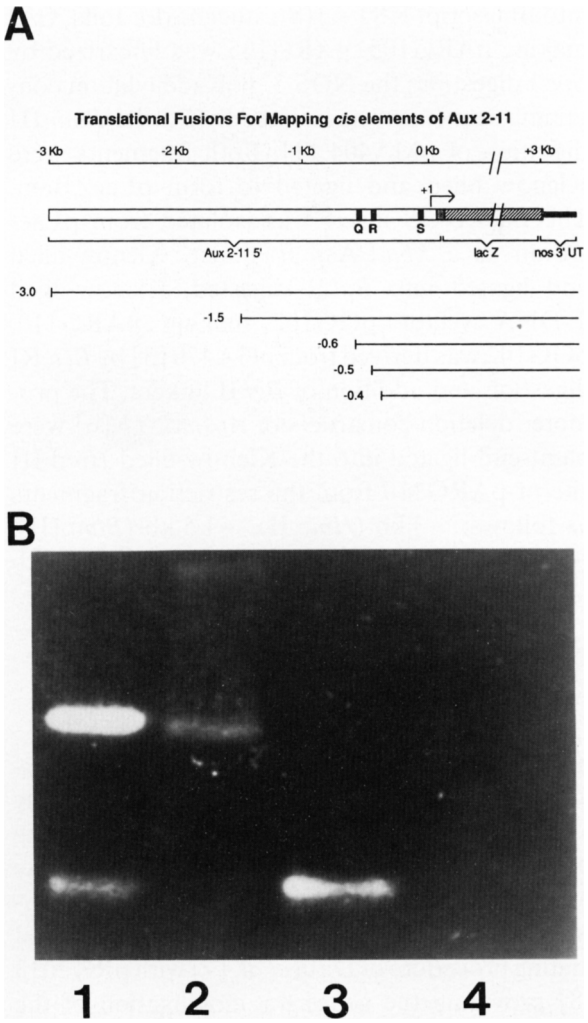


Fig. 1. Structure of 5′ promoter deletion constructs and effect of glutaraldehyde fixation on β-galactosidases in transgenic *Arabidopsis* plants. A. Schematic representation of the *AtAux2-11* promoter-LacZ constructs. Promoter fragments were isolated as described in Materials and methods. Approximate promoter length is indicated with respect to the transcription start site. Q, R, and S are regions of sequence which show homology with the *Arabidopsis* auxin-inducible gene *AtAux2-27* [6]. B. Protein gel analyses showing the effect of 1% glutaraldehyde fixation on β-galactosidase activity in *Arabidopsis* seedlings. Extracts from fixed and unfixed transgenic (−0.6 kb promoter) and control (untransformed) plants were standardized with respect to protein concentration and loaded on a SDS polyacrylamide gel. β-galactosidase activity was analyzed using the fluorogenic substrate 4-methyl umbelliferyl-β-*D*-galactoside. The gel was photographed under a UV fluorescent light. Lane 1, extract from unfixed transgenic seedlings; lane 2, extract from glutaraldehyde-fixed transgenic seedlings; lane 3, extract from unfixed control seedlings; lane 4, extract from glutaraldehyde-fixed control seedlings.

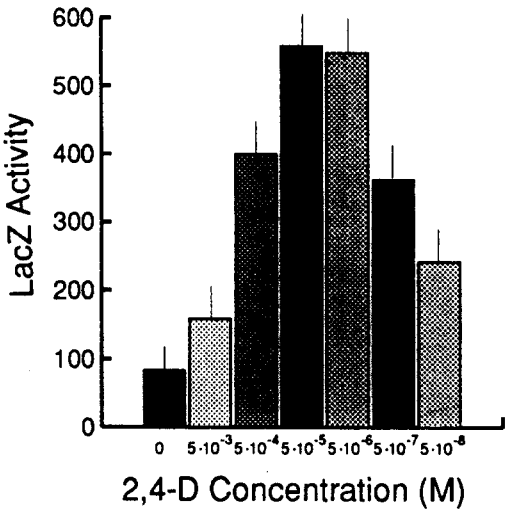


Fig. 2. Effect of auxin concentration on *AtAux2-11*/LacZ expression assayed from transgenic *Arabidopsis* plants. Extracts from eight 14-day old independent transformants, each containing the −0.6 kb promoter *AtAux2-11*/LacZ gene, were analyzed for LacZ activity after treatment with various concentrations of auxin (2,4-D) for 3 h. Each bar represents the mean LacZ activity, the small line indicates standard error from triplicate assays for the eight transformants.

band appears to consist of several, apparently higher molecular weight species of β-galactosidase. This is probably due to the glutaraldehyde crosslinking of LacZ to other plant proteins. This technique allows for direct quantitative and histochemical assay of the various *AtAux2-11*/LacZ fusion products in transgenic plants without background endogenous β-galactosidase activity. Unfixed and untransformed plant materials were routinely used as control tissues for all experiments.

Auxin concentration response

The auxin dose response for the *AtAux2-11* −0.6 kb promoter-driven LacZ expression is shown in Fig. 2. Ten-day old *Arabidopsis* transgenic seedlings were sprayed until run off with various concentrations of 2,4-D, fixed after three hours, and assayed for LacZ expression to determine the range of auxin concentrations that increase expression. The promoter responded to as little as 0.05 μM exogenous 2,4-D, the lowest concentration tested, while the maximum increase in LacZ activity occurred after treatment with 5 to 50 μM 2,4-D.

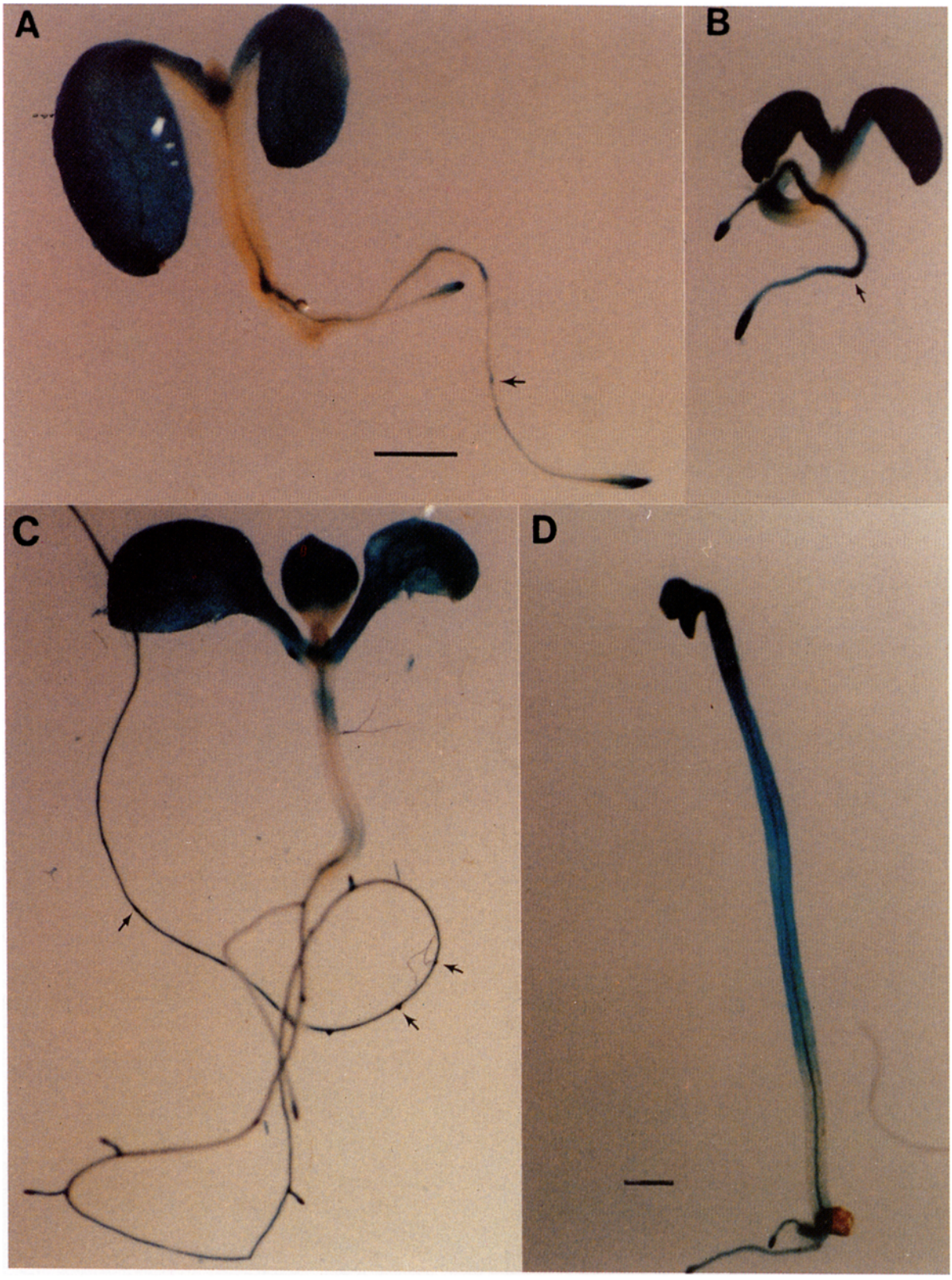
Expression and auxin inducibility of the AtAux2-11 *promoter 5′ deletion fragments/LacZ fusions in transgenic* Arabidopsis *seedlings*

The auxin inducibility of the *AtAux2-11* gene was previously reported, using RNA gel blot and S1 nuclease fragment protection analyses. The *AtAux2-11* gene encodes transcripts that increase in abundance after auxin application [6]. An

Table 1. LacZ enzymatic activities in auxin-treated independent transgenic plants containing different 5′ upstream deletion constructs of the *AtAux2-11* promoter[1].

Construct	0–100	100–250	250–400	400–550	550–700	>700	Mean auxin inducibility
−3.0 kb	2	4	1				2.3 ± 0.5
−1.5 kb	2	4					2.7 ± 0.5
−0.6 kb	1		4	3	1	4	5.5 ± 1.1
−0.5 kb	6	5	2			1	2.1 ± 1.0
−0.4 kb	5	1	1	1			1.4 ± 0.6

[1] In the first column the promoter deletion constructs are given (see Fig. 1). In columns 2 to 7 the number of independent transgenic plants for which the enzymatic assay of LacZ activity was in the range above the column is shown. Activity is given as nmol ONPG hydrolyzed per minute per mg protein. In column 8 the mean auxin inducibility is given as the fold auxin enhancement of LacZ enzymatic activity of auxin-treated activity divided by untreated control activity. The auxin-induced activity is an increase in reponse to auxin treatment above a significant total seedling activity which was present at the time of auxin treatment.


A
B
C
D

AtAux2-11 gene probe detected two auxin-inducible transcripts (1000 and 1200 nucleotides) in *Arabidopsis* seedlings and cells grown in culture, that increased rapidly (within 15 to 20 min) with kinetics similar to the corresponding genes in etiolated soybean hypocotyl sections [29].

In an initial attempt to identify regions of the 5′ sequence that may be involved in auxin-modulated induction of *AtAux2-11*, LacZ was extracted and assayed from control and 50 μM auxin-treated transgenic *Arabidopsis* seedlings using a modification of the hydrolysis assay [25]. Table 1 summarizes the results from quantitative assays for LacZ activity of auxin-treated seedlings with the various promoter constructs. Auxin-induced increases in activity varied from 5.5 × (−0.6 kb promoter) to 1.4 × (−0.4 kb promoter) two hours after treatment. The −3.0, −1.5, and −0.5 kb promoters produced similar levels of control or basal LacZ activity in control seedlings (presumably as a result of endogenous auxin mediated expression), and showed about 2-fold induction after auxin treatment over the same time period. Transgenic seedlings containing the −0.6 kb promoter showed the greatest levels of control and auxin-induced increases in expression. This indicates that sequences in the −1.5 to −0.6 kb region of the promoter may encompass a *cis*-acting regulatory sequence(s) which down regulates auxin-inducible expression. A significant drop in expression and auxin-inducibility was observed when the promoter was deleted to −0.5 kb, suggesting the presence of an enhancer-like *cis*-regulatory element in this region. Control and auxin-induced activity was further reduced in plants containing the −0.4 kb promoter construct, and these plants had higher variation in expression levels among individual, independent transformants. Transformants containing the −0.6 kb/LacZ promoter construct showed no differences in histochemical staining; therefore, it appears that regions upstream of −0.6 kb do not contain sequences controlling tissue specificity.

Histochemical expression of AtAux2-11/*LacZ in whole* Arabidopsis *seedlings*

The soybean *GmAux22* gene has been shown to be preferentially expressed in the elongating region of etiolated soybean hypocotyl. A similar analysis of *AtAux2-11* transcripts would be technically difficult, given the small size of *Arabidopsis* seedlings. Histochemical staining of plants transformed with the *AtAux2-11* promoter fused to LacZ provides this information regarding the tissue specificity of *AtAux2-11* gene. Analyses of the various promoter deletion constructs may further delineate sequence regions that confer tissue specificity.

Transgenic plants with the −3.0 kb, −1.5 kb, and −0.6 kb constructs showed identical histochemical patterns of expression after staining. The consensus pattern of *AtAux2-11*/LacZ staining in transgenic *Arabidopsis* seedlings is shown in Fig. 3. In every transgenic plant carrying the *AtAux2-11*/LacZ construct, the vascular tissue in the root was stained. The root cap was also darkly stained, but the root epidermis and cortex were not (Figs. 3A, 3B and 3C). Expression in the stele of roots was especially intense at the sites of branch root initiation (arrows in Fig. 3A and 3B). Plants with the −0.5 and −0.4 kb promoters showed reduced levels of expression in roots relative to plants with the longer constructs, and the pattern of histochemical staining was not as consistent between plants in the −0.4 kb group (data not shown). The shoot systems of both light-grown and etiolated plants expressed the *AtAux2-11* gene promoter but in different tissues. The vascular tissue in the hypocotyl of light grown seedlings did not stain, and staining was only occasionally observed in any tissues of the region of elongation (Fig. 3C). In contrast, most of the hypocotyl of etiolated seedlings was stained, and the staining was most intense in the region of greatest elongation and in the vascular cylinder (Fig. 3D).

Fig. 3. Histochemical localization of LacZ expression in representative transgenic *Arabidopsis* plants with the −0.6 kb promoter. A, B, and C. Light-grown 7- to 10-day old *Arabidopsis* seedlings; arrows show sites of lateral root initiation. D. Etiolated 4- to 5-day old *Arabidopsis* seedling. Bar = 1 mm in A, 0.5 mm in D.

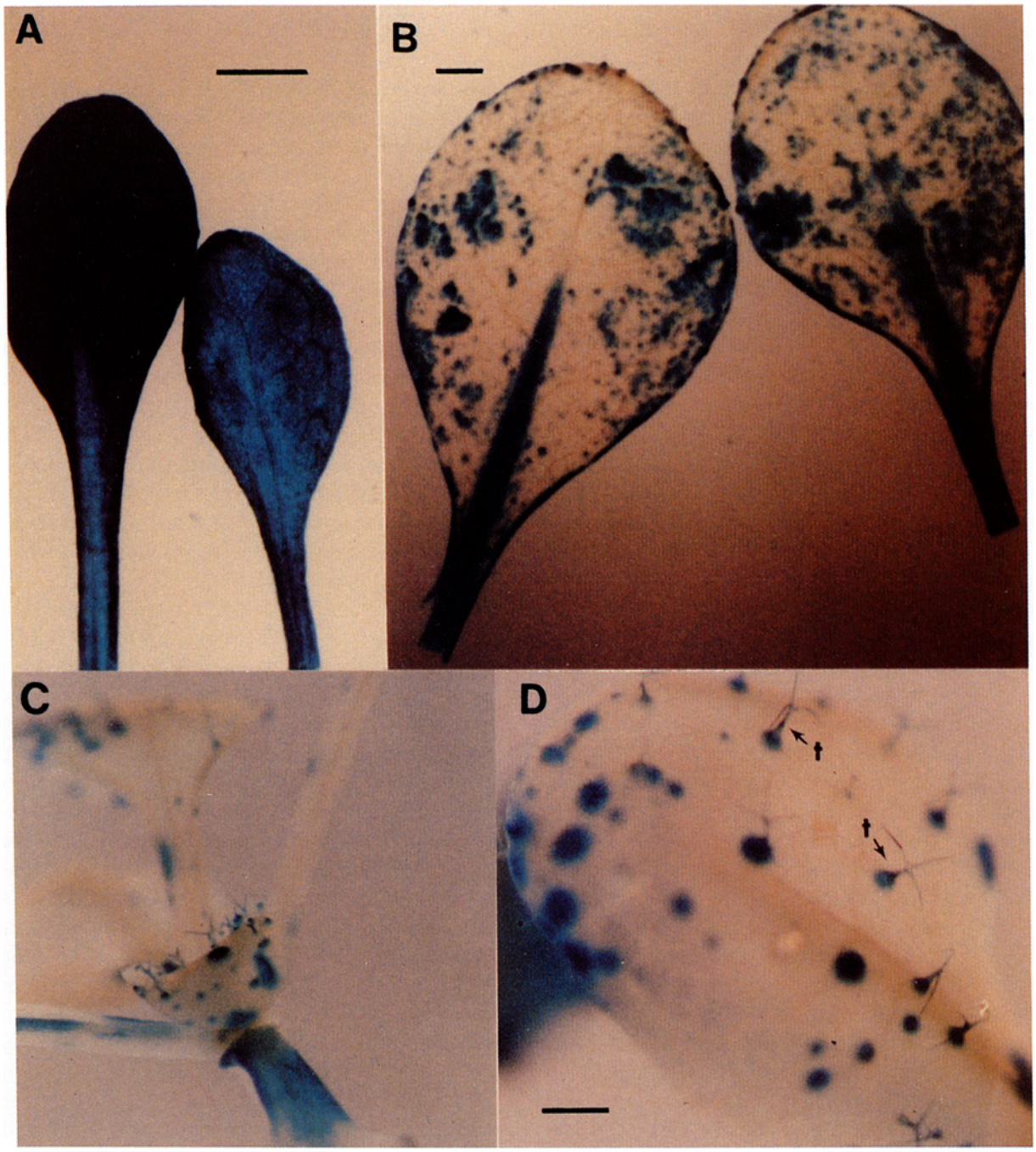


Fig. 4. Histochemical localization of −0.6 kb *AtAux2-11*/LacZ expression in cotyledons and leaves of a 14-day old transgenic *Arabidopsis*. A. Paired cotyledons from a transgenic *Arabidopsis* seedling. B. Paired set of first leaves from the same seedling. C. Transgenic *Arabidopsis* seedling showing LacZ staining of immature true leaves. D. Magnification of unexpanded true leaf showing staining of epidermis and trichome. Bar = 1 mm in A and B, 0.2 mm in D. Abbreviation: t, trichome.

Fig. 4 shows the localization of *AtAux2-11*/LacZ in cotyledons and the first true leaves of approximately 14-day old transgenic *Arabidopsis* plants. Mature cotyledons stained throughout

(Fig. 4A), while mature leaves showed mottled staining patterns (Fig. 4B). The timing of fixation and staining with respect to leaf age affected the number and size of the stained areas. On fully expanded leaves (> 12 days after leaf initiation) the spots appear larger and more diffuse; on less developed leaves, staining appeared to be confined to single epidermal cells. Observation of true

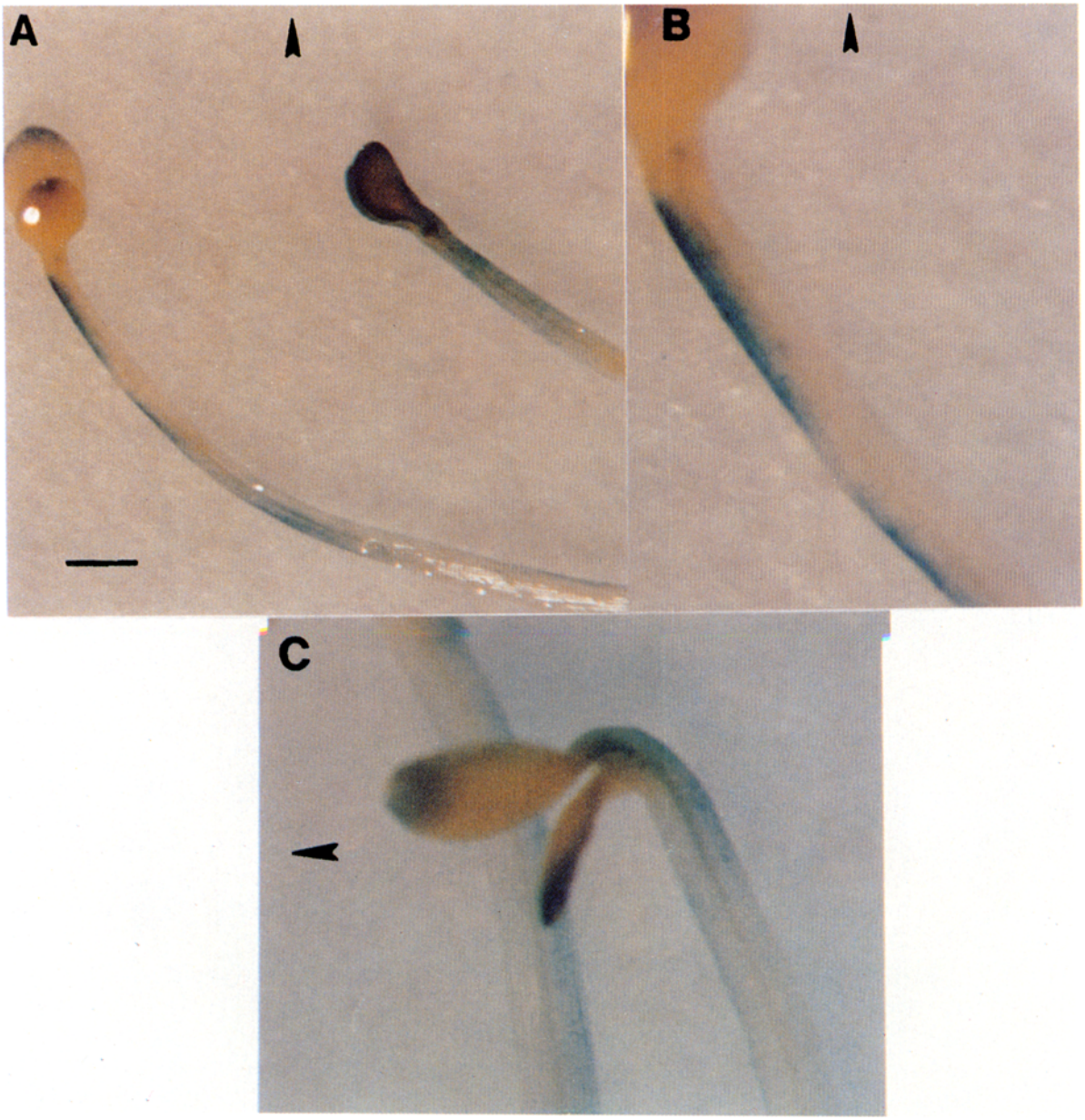
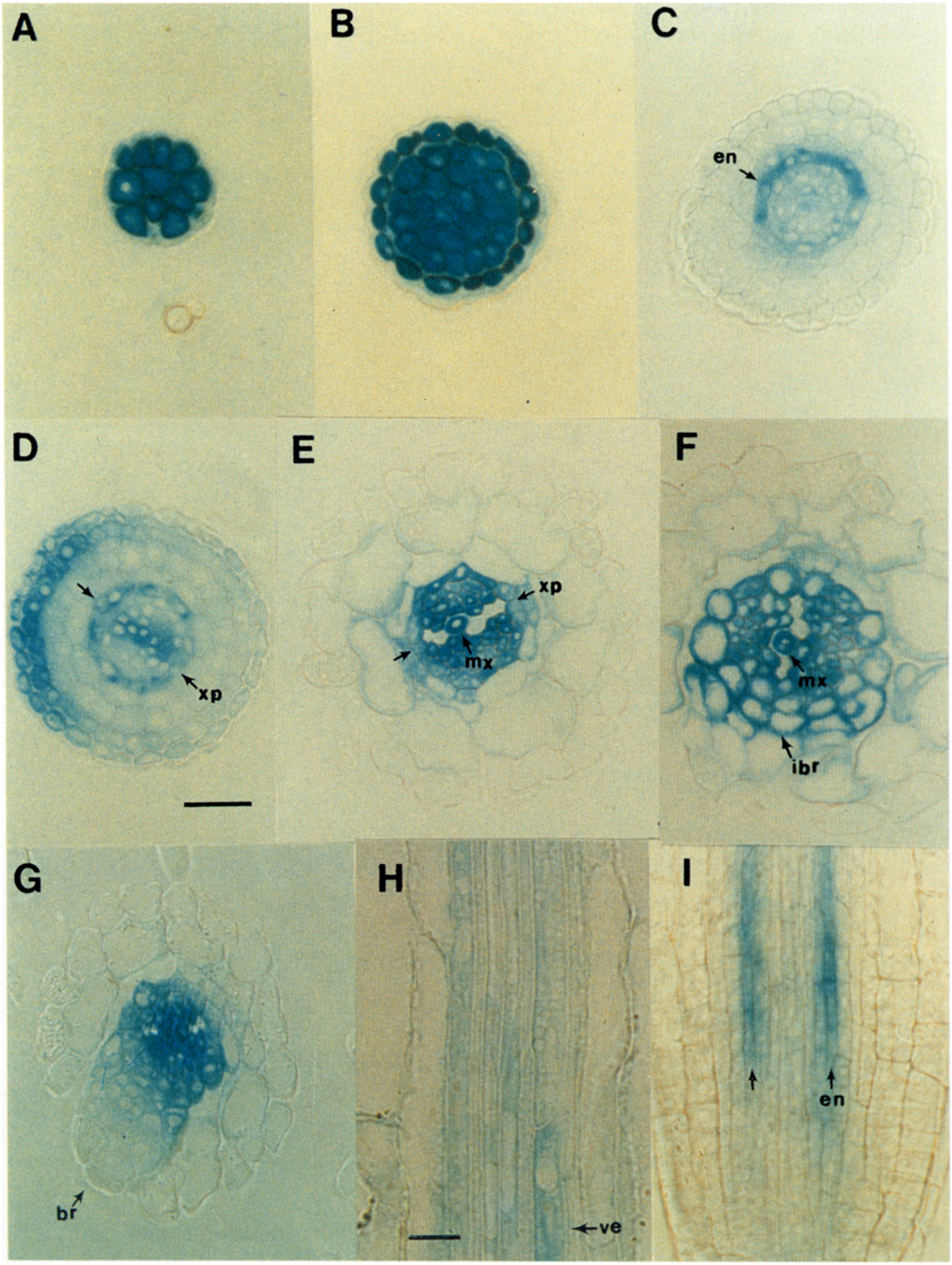


Fig. 5. Histochemical localization of *AtAux2-11*/LacZ in transgenic *Arabidopsis* seedlings undergoing gravitropic curvature. Five-day old etiolated transgenic *Arabidopsis* seedlings were turned horizontally (reoriented) and grown for 10 h; after which time they were fixed and stained for LacZ activity. A. Reoriented *Arabidopsis* seedlings; LacZ activity is on the elongating (lower) side of hypocotyls exhibiting curvature. B. Higher magnification of seedling in A. C. Reoriented *Arabidopsis* seedlings, staining varied with gravitropic response. Seedlings with little curvature had less staining. Arrows point up in all cases. Bar = 1 mm.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leaves under magnification revealed the stained spots to be single or small clusters of cells surrounding epidermal cells with trichomes (Figs. 4C and 4D). Staining was also visible in the trichomes prior to their complete maturation (data not shown). Trichomes are not normally found on the cotyledons of *Arabidopsis* but are present on the true leaves and stems of seedlings.

When transgenic etiolated seedlings were turned and grown horizontally for 10 hours, the hypocotyls of the seedlings underwent an upward gravitropic curvature. Figure 5 shows the asymmetry of the LacZ staining observed in these seedlings. Staining was found primarily on the lower, more rapidly elongating side of the hypocotyl (Figs. 5A, 5B, and 5C) correlating *AtAux2-11* expression with the asymmetric increase in auxin concentration to the faster elongating underside of the hypocotyl during gravitropic curvature.

Cellular localization of AtAux2-11/*LacZ*

In seedlings of the ages studied here, 7–10 days old, auxin would regulate many aspects of development, including development of vascular tissue in shoots and leaves, lateral root initiation, expansion of cotyledons and leaves, root elongation, and elongation of the hypocotyl of etiolated plants. IAA, the natural auxin, is made primarily in leaf primordia, apical meristems, and developing seeds. One set of independent transformants containing the −0.6 kb promoter construct was analyzed by sectioning stained plastic-embedded plants to examine, at the cellular level, *AtAux2-11*/LacZ expression during these auxin-mediated processes.

Expression in roots

The *AtAux2-11*/LacZ histochemical staining pattern in roots, as shown in Fig. 6, was characterized by intense staining of the root cap, staining of the stele along most the root, and more intense staining of the vascular tissue adjacent to sites of incipient or emerging lateral roots. In the most intensely stained region of the plant, the root cap, all cells were intensely blue (Figs. 6A and 6B). Just above the root cap, staining was primarily visible in the developing endodermis (Fig. 6C). Along most of the length of the root, the stele was consistently stained with cells of the vascular tissue showing a developmental pattern of staining. The vascular tissue in *Arabidopsis* has a diarch arrangement (or two poles); in younger parts of the roots of transgenic seedlings the protoxylem can be distinguished, prior to other visible signs of development, by darker staining than the adjacent protophloem and other procambium tissues. This darker blue region corresponds to cells of the future protoxylem and marks the two xylem poles (Figs. 6D and 6E). Cells of the xylem stained most intensely during mid to late development while they were elongating, and intense staining of xylem elements was observed even as the secondary cell wall began to form and the central tube developed. Mature metaxylem vessels were devoid of staining, consistent with the lack of functional cytoplasm in this cell type (Fig. 6E). In regions of the root that are more mature, phloem and xylem tissues (excluding mature xylem) were about equally stained (Fig. 6F). Interestingly, endodermal cells were stained darkly in regions close to the apex and also in areas of the root where branch root formation was initiated. In regions where branch root for-

Fig. 6. Cellular localization of *AtAux2-11*/LacZ in roots of 10-day old transgenic seedlings. Stained transgenic *Arabidopsis* plants were embedded in plastic resin and sectioned. The cross-sections shown proceed up the root from the tip (A) to the mature portion of the root (G). A and B. Cross-sections through the root cap. C and D. Cross-sections through the elongating region of the root. Staining is evident in the endodermis and the future sites of the xylem poles. E and F. Cross-sections through the maturing portion of the root. Most cells within the vascular cylinder express *AtAux2-11*/LacZ. G. Cross-section through an emerging branch root. H. Longitudinal section through the stele in the mature portion of the root. I. Longitudinal section through a maturing branch root. Staining in the endodermis marks the developing vascular cylinder. Bar = 200 μm in D, 50 μm in H. Abbreviations: en, endodermis; xp, xylem poles; mx, metaxylem; ibr, incipient branch root; br, branch root; ve, vessel element.

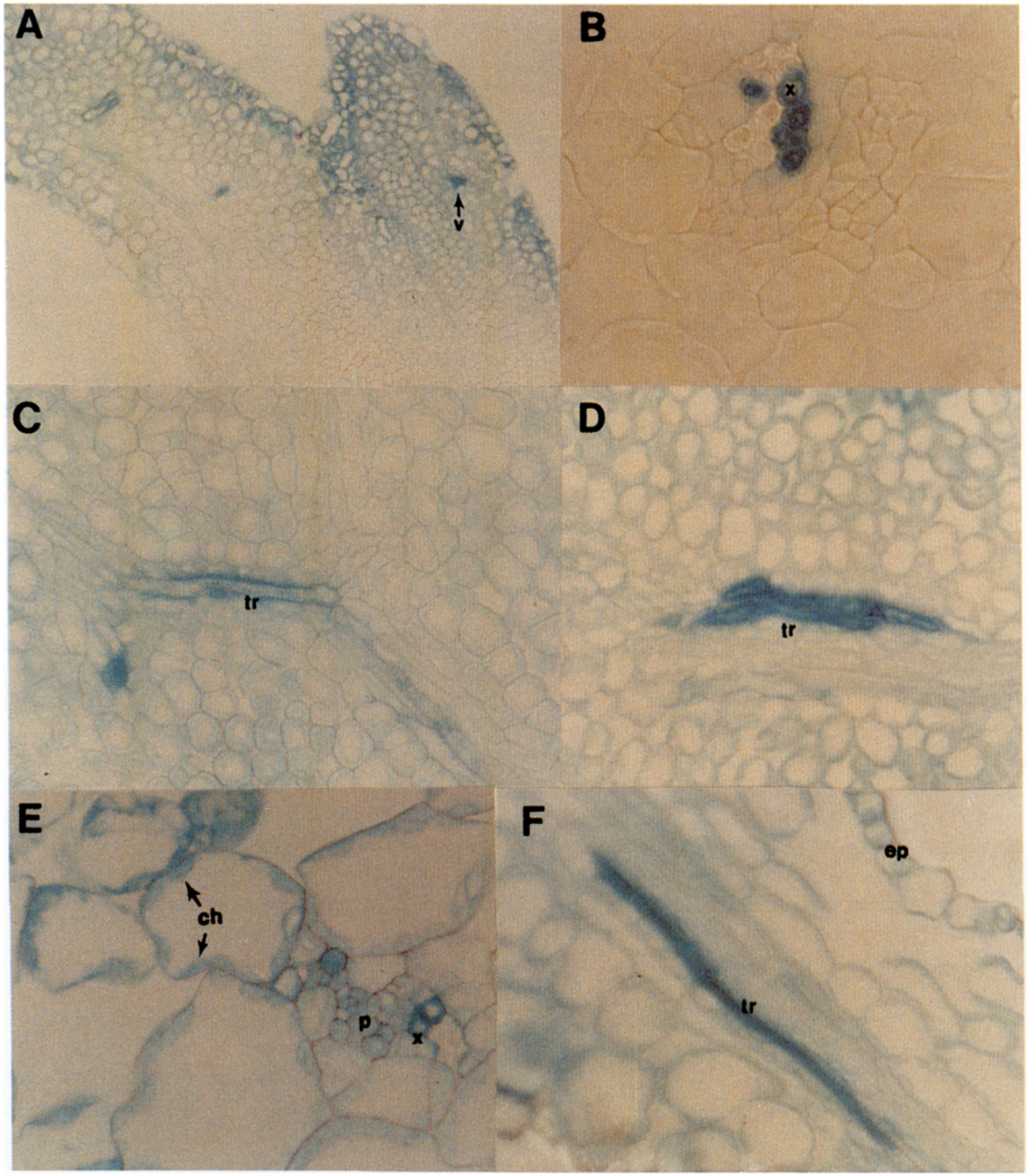


Fig. 7. Cellular localization of *AtAux2-11*/LacZ in the cotyledons of light-grown transgenic *Arabidopsis* seedlings. A. Oblique section through the cotyledon. B. Cross-section through a cotyledon vascular bundle. Only the tracheids stain. C and D. Longitudinal sections through the veins of *Arabidopsis* cotyledons. Cells in the xylem (tracheids) stain during development of secondary cell walls. E. Section through distal portion of a cotyledon. Mesophyll cells containing chloroplasts stain slightly. F. Section through pair of tracheids. Staining is greatest during initiation of secondary cell wall thickenings. Bar = 400 μm. Abbreviations: v, vein; tr, tracheid; ch, chloroplast; p, phloem; x, xylem; ep, epidermis.

mation occurred, staining in most of the stele also was more intense (Figs. 6F and 6G). In longitudinal sections, xylem elements containing helical or annular secondary walls were observed to have near maximum staining just after development of secondary wall structures (Fig. 6H). This suggests that the gene is active during a stage when elongation of the tracheary elements is occurring. Apparent expression ends rather abruptly when the xylem element matures (and cytoplasmic volume

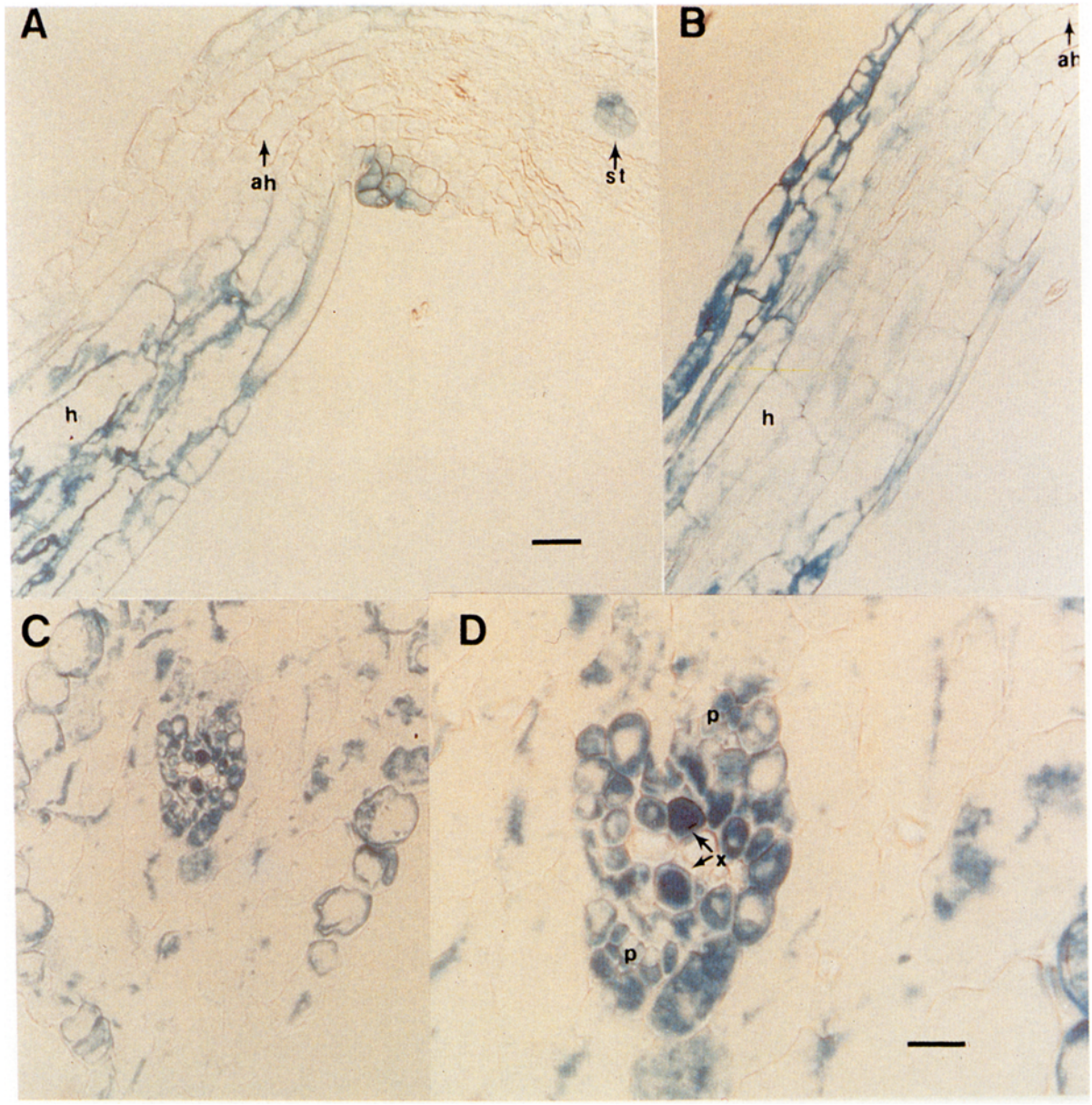
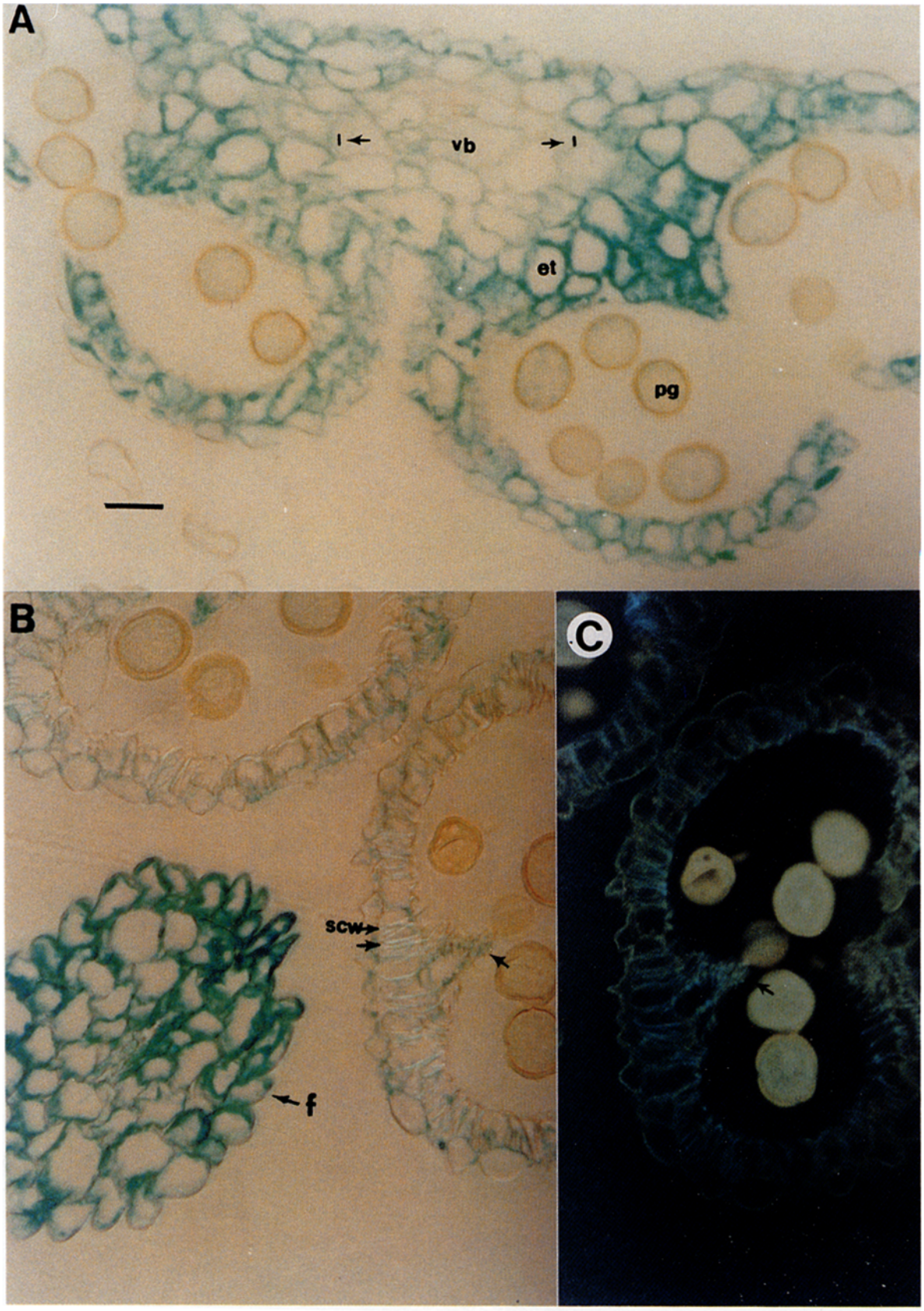


Fig. 8. Cellular localization of *AtAux2-11*/LacZ in etiolated transgenic *Arabidopsis* seedling hypocotyls. A and B. Longitudinal section through the apical and elongating regions of etiolated transgenic seedlings. C. Cross-section through the mature region of the hypocotyl. D. Higher magnification of C showing vascular cylinder in hypocotyl. Bar = 400 μm in A, 200 μm in D. Abbreviations: ah, apical hook; h, elongating region of hypocotyl; st, stipule; p, phloem; x, xylem.


A
vb
et
pg
B
SCW
f
C

diminishes). Longitudinal sections also illustrate the developmental expression in the endodermis near the root tip, defining the future vascular cylinder (Fig. 6I).

Expression in cotyledons

Expression of the *AtAux2-11*/LacZ fusion product in the cotyledons of *Arabidopsis* seedlings is shown in Fig. 7. In general, staining of the distal portions of the cotyledons was greater than staining in the basal portion of the organ (Fig. 7A). In contrast to the roots and hypocotyls from etiolated seedlings in which the entire vascular bundle stains, the xylem elements (tracheids) in cotyledons were darkly stained while the phloem stained lightly or was not stained at all (Fig. 7B). In addition, the xylem elements with the highest activity have secondary cell walls (as evident by annular thickenings (Figs. 7C, 7D, 7E and 7F). The *AtAux2-11* gene appears not to be expressed in the most mature phloem tissue in cotyledon vascular bundles. Mesophyll cells, containing chloroplasts, were lightly stained especially near the distal tips of the cotyledons (Figs. 7D and 7E).

Expression in hypocotyls

The hypocotyls of light grown plants showed large variations in staining patterns, ranging from no staining to light staining. The areas of light staining had variable locations in the hypocotyl, and if staining was present it was often associated with curved or elongating regions of the hypocotyl (data not shown). Figure 8 shows the cellular staining patterns of hypocotyls of etiolated seedlings. Sections prepared from the hypocotyls of etiolated seedlings showed staining intensities and distributions that varied dependent on the position of the section in the hypocotyl (Figs. 8A and 8B). In the regions showing the most staining such as the elongating region, essentially all cell types in a cross section were stained (Fig. 8C). The apparent differences in staining intensity observed between adjacent cells in the hypocotyl could be due to differences in cytoplasmic volume of the different cell types. Like the root, but unlike the hypocotyl of the light-grown plants, the vascular tissue of the hypocotyl was stained (Fig. 8D) with most cell types within the vascular tissue showing some *AtAux2-11*/LacZ activity.

Expression in flowers

Within *Arabidopsis* flowers, *AtAux2-11*/LacZ activity was detected in sepals and stigmas but not in petals (data not shown). Expression within the flower was greatest in the stamen. Figure 9 details the localization of expression in the anther and filament. Very little staining was seen in the tapetum or in pollen grains, but dark staining was observed in cells of the anther endothecium (Fig. 9A). The intensity of the staining diminished as the cells matured, when secondary cell walls were formed (Fig. 9B). These secondary wall thickenings appeared to contain lignin, as indicated by the characteristic blue-light emissions observed under UV fluorescence (Fig. 9C). Strong expression (dark staining) was also seen in all cells of the elongating region of the filament (Fig. 9B). The histochemical pattern of expression in the elongating filament was similar to that of elongating hypocotyls in that most of the cells in the organ, such as vascular and cortical cells, were stained in those regions undergoing elongation.

Fig. 9. Cellular localization of *AtAux2-11*/LacZ in stamen of transgenic *Arabidopsis* plants. A. Cross-section through the anther. Staining in primarily in the endothecium. Endothecial cells have no apparent secondary cell walls. B. Cross-section through the anther and filament of an older flower. The elongating section of the filament stains, while the endothecium no longer has activity. C. Above anther viewed under UV excitation. Bluish bands on endothecial cells indicate the presence of lignified secondary cell wall thickenings. Bar = 25 μm. Abbreviations: vb, vascular bundle; et, endothecium; f, filament; pg, pollen grain; scw (arrows), lignified secondary cell wall thickenings.

Discussion

AtAux2-11 *promoter analysis*

The results reported here identify regions of the *AtAux2-11* promoter that contain putative *cis*-acting sequences responsible for modulating auxin inducibility and cellular expression patterns. The 5′ regulatory region was analyzed for the ability to control auxin-mediated increases in expression using serial sequence deletion constructs. Independent transformants each containing the identical −0.6 kb promoter construct displayed some quantitative variability in levels of expression (Table 1), possibly due to the position of gene insertion; however, all transformants that had measurable basal expression showed similar increases in LacZ expression when treated with auxin. These changes in the levels of *AtAux2-11*/LacZ control expression and auxin-induced increases in *AtAux2-11*/LacZ expression reflect a response to changes from endogenous auxin levels (control expression) to auxin enhanced levels (auxin-induced expression). The apparent lack of high fold auxin inducibility of LacZ (2- to 6-fold vs. 10- to 100-fold depending on clone based run-off transcription [10] or approximately 25-fold based on mRNA levels following auxin depletion of pJCW2 in incubated, elongating soybean hypocotyl sections [15]) is due to the significant amount of auxin-regulated basal activity arising from normal endogenous auxin levels. Secondly the LacZ reporter assay is based on a relatively stable protein whose activity is difficult to quantitate kinetically to auxin-induced mRNA levels. Thus, auxin-induced expression in this case refers to expression in auxin-treated tissue over the control level and probably substantially underestimates true auxin responsiveness of the promoter. In most of the transgenic plants, auxin-inducible increases of *AtAux2-11*/LacZ expression in transformants containing the −0.6 kb promoter (5- to 6-fold increases) were greater than increases found using the 'full-length' promoter of ca. 3 kb (Table 1). This suggests that either a negatively acting *cis*-regulatory sequence or a portion of sequences necessary for auxin-modulated expression are upstream of −0.6 kb. Basal activity of transgenic plants with the −0.6 kb promoter was also generally greater than that with the full-length promoter. Auxin-induced increases in expression were not due to changes in tissue specificity since the observed histochemical staining patterns of the −3.0 and −0.6 kb promoter constructs were identical. The −0.5 kb transformants were auxin-inducible, but both basal and induced levels of expression were approximately one-third of those of seedlings with the −0.6 kb constructs indicating that the −0.6 to −0.5 kb region also plays some role in auxin-modulated expression. Previous sequence analyses of the promoter region of the two homologous auxin-inducible genes of *Arabidopsis*, *AtAux2-11* and *AtAux2-27*, revealed three short segments of sequence homology in the 5′ flanking region (designated Q, R, and S [6], Fig. 1). One of these, Q, is found at position −548 GACTATGAATATGTT −532 within the deleted region in the −0.5 kb promoter construct. DNase I protection experiments demonstrates DNA protein-binding encompassing but not limited to this sequence (Goekjian, Nagao, and Key unpublished data). This sequence may merely enhance general expression of the *AtAux2-11*/LacZ fusion gene (independent of auxin concentration) or it may be a part of sequences necessary for complete auxin-modulated expression. Additional experiments will be required to distinguish these possibilities. Transgenic plants containing the shorter deletion construct (−0.4 kb promoters) showed weak inducibility and variable basal expression and histochemical localization. Current work is focused on analyzing nested deletion and fragment replacement analyses of the region 600 bp 5′ of the initiation of transcription in order to more precisely identify sequences that control auxin inducibility.

Histochemical localization

Histochemical localization of *AtAux2-11*/LacZ activity in transgenic plants revealed spatial and temporal expression patterns correlated with tissues, cell types, and developmental processes

which are accepted to be modulated by auxin. Expression of the *AtAux2-11*/LacZ fusion was detected in the elongation zones of both etiolated hypocotyls and roots (Fig. 3). In other regions of the shoot, activity was greatest in developing xylem and leaf epidermal cells with trichomes (Fig. 4). In the cotyledons, expression occurred throughout most mesophyll and epidermal cells (Figs. 4 and 7). Staining of most epidermal and mesophyll cells was of similar intensity with a slightly darker staining in the distal portion of the cotyledon. The vascular tissue stained very intensely, and guard cells were occasionally darker than the surrounding epidermal cells. In unexpanded true leaves epidermal cells with trichomes stained distinctly. The cotyledons of *Arabidopsis* do not have trichomes; therefore, differences in staining patterns between cotyledons and leaves may be linked to the presence or absence of trichomes and could reflect a developmental difference between the epidermal tissues of these organs. The *AtAux2-11*/LacZ constructs were active in the root cap and stele of the root, and expression increased in the stele at points where branch root initials occurred (Figs. 3 and 6). Control of branch root formation and differentiation of vascular tissues by auxin is well documented. Auxin is the primary, if not exclusive, plant hormone that regulates the differentiation and maturation of both phloem and xylem tissue. Vascular tissue formation, specifically xylem, can be induced from undifferentiated callus by the application of auxin and sucrose [14]. Vascular strands of xylem also can be induced to form around wounds or given axial polarity within organs by the application of auxin [22, 23]. The *AtAux2-11*/LacZ fusion was expressed in xylem of all tissues and in all cell types of the xylem. The expression of *AtAux2-11*/LacZ occurred throughout xylem development, with greatest activity occuring just prior to and during the formation of the secondary cell walls that surround tracheids and vessel elements.

Another major site of *AtAux2-11*/LacZ expression was within the vascular tissue of roots at points of lateral root initiation. Lateral root formation requires anticlinal and periclinal divisions of the pericycle and can involve anticlinal cell divisions in the endodermis, which in some plants also serves as a temporary root cap. The vascular strands of the lateral root develop from cells arising from the pericycle. The initiation of extra laterals in *Raphanus* can be stimulated by auxin with the largest increase in lateral root formation occurring after application of 5 to 50 μM IAA [5]. *AtAux2-11*/LacZ staining increased at the site of lateral root formation, even in mature regions of the root where expression was not usually present. At these sites, cells within the endodermis expressed *AtAux2-11*/LacZ, thus marking the point of branch root formation prior to any observed anatomical changes in the stele.

The *AtAux2-11*/LacZ gene was also expressed in tissues undergoing rapid cell elongation or expansion, including regions in the root, cotyledons, leaves, and hypocotyl of etiolated seedlings. Cell elongation is another process in which auxin is known to be involved. An interesting difference was observed between the expression patterns observed in the hypocotyls of light-grown and etiolated seedlings. The etiolated seedlings expressed the LacZ gene at a much higher level than the hypcotyls of light-grown plants (Figs. 3 and 8). Possible explanations for this are: (1) the rate of auxin-induced cell elongation determines expression of the *AtAux2-11* gene; (2) there is a tissue-dependent threshold concentration of auxin concentrations that modulates expression of the gene with much lower levels occurring in green hypocotyls; (3) the half-life of LacZ is much lower in hypocotyls of light-grown plants. However the correlation remains that in the hypocotyl, and other tissues, *AtAux2-11*/LacZ was expressed most intensively in regions undergoing the greatest elongation.

Although auxin is known to affect cell division, the *AtAux2-11*/LacZ gene was not expressed at observable levels in primary meristems or in very young leaves even though these tissues are undergoing rapid mitotic divisions. However, the root cap and sites of branch root formation, two other tissues which are also undergoing rapid mitosis, showed high levels of *AtAux2-11* expression. The root cap shares cell lineage and some

initials with the root epidermis and is often associated with the gravitropic response of the root. However, most epidermal cells of the root do not express the *AtAux2-11*/LacZ construct. This may suggest some developmental change in these closely related cell types, which is also reflected by differences in *AtAux2-11* gene expression.

Like the stele in the etiolated hypocotyl, most cells in the root vascular cylinder stained. Anatomically, the vascular cylinder of an etiolated hypocotyl may resemble a root more than a true stem, especially near the base. It is organized as a true cylinder and the xylem and phloem tissues are not organized into discrete bundles surrounding a pith as in stems. The similarity of *AtAux2-11* expression patterns in etiolated hypocotyl and root vascular tissue may reflect this anatomical resemblance.

The *AtAux2-11*/LacZ gene is expressed in most of the mature region as well as the elongating region of *Arabidopsis* seedling roots. Auxin is known to affect cell growth in the root at a much lower optimum concentration than the shoot. Furthermore, the gradient of auxin concentration increases basipetally in roots. This increase in auxin concentration in the more mature regions of the root could explain the observed expression of *AtAux2-11*/LacZ in the mature region of the root stele. Alternatively, auxin concentrations or cellular sensitivity to auxin concentration may be different in roots than in hypocotyls, and the expression pattern of the *AtAux2-11*/LacZ construct may simply reflect this.

In flowers of *Arabidopsis*, the *AtAux2-11*/LacZ gene was expressed primarily in the stamen. Expression within the filament resembled that of etiolated hypocotyls, with expression in most or all cells of the structure, both vascular and cortical, with the darkest staining occurring in the region of greatest elongation. Again, this may reflect some similarity in the development of these organs, especially with respect to cell elongation. The localization of *AtAux2-11*/LacZ in anthers correlated with the development of lignified secondary cell walls around endothecial cells (Fig. 9). The *AtAux2-11* fusion gene was found to be strongly expressed in three cell types that have lignified secondary cell walls: xylem, trichomes, and anther endothecial cells.

Overall, a generalized description of the localization of the *AtAux2-11*/LacZ fusion gene could be put as follows: it is primarily expressed in tissues undergoing elongation and/or in cells with lignified secondary cell walls. Both of these processes are at least partially modulated by auxin. Thus, an auxin-responsive gene is expressed in a wide variety of cell types which are involved in auxin-mediated processes. These observations may not be surprising; however, in order to determine the function of hormonally-regulated genes of plants and to discern what exact role they play in physiological processes, it is necessary to examine when and where these genes are expressed during the development of the plant.

A comparison of *AtAux2-11* localization patterns with those of other auxin-inducible genes reveals both similarities and differences. The SAUR genes of soybean are primarily expressed in the outer tissues of the hypocotyl, including the epidermis, cortex, and starch sheath. The SAURs are only weakly expressed in vascular tissue [9]. In transgenic tobacco plants, a SAUR promoter/GUS fusion gene showed auxin dose sensitivity nearly identical to that of *AtAux2-11*/LacZ and had a similar asymmetrical position during gravitropically-induced curvature [17]. The usual staining pattern of etiolated hypocotyls, that of near complete staining along the length of the hypocotyl, was not observed (Fig. 5). Even older regions of the hypocotyl which had not undergone gravitropic bending were not stained. This may indicate that the half-life of the β-galactoside protein is shorter in light-grown *Arabidopsis* and/or that expression of the *AtAux2-11*/LacZ gene is quickly reduced upon reorientation of the seedlings. Alternatively, differential degradation of LacZ in non-elongating tissues could occur during the gravitropic response.

Another auxin-inducible gene, GH3, is expressed in xylem and trichomes similar to *AtAux2-11*. However, unlike *AtAux2-11*, GH3 transcripts also were detected in seeds, pollen, ovary, and pistil. Also, in contrast to *AtAux2-11*, GH3 spatial expression is dramatically altered by

exogenous auxin application [17]. Expression of the GH3 gene extends to other tissues and cells after auxin application while *AtAux2-11* and the SAUR gene families do not. The relationships between and precise roles of any and all of these genes in development still remains unclear.

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