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Differential activation of the primary auxin response genes, *PS-IAA4/5* and *PS-IAA6*, during early plant development

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Summary

The plant growth hormone auxin typified by indoleacetic acid (IAA) transcriptionally activates early genes in pea, *PS-IAA4/5* and *PS-IAA6*, that are members of a multigene family encoding short-lived nuclear proteins. To gain first insight into the biological role of *PS-IAA4/5* and *PS-IAA6*, promoter–β-glucuronidase (GUS) gene fusions were constructed and their expression during early development of transgenic tobacco seedlings was examined. The comparative analysis reveals spatial and temporal expression patterns of both genes that correlate with cells, tissues, and developmental processes known to be affected by auxin. GUS activity in seedlings of both transgenic lines is located in the root meristem, sites of lateral root initiation and in hypocotyls undergoing rapid elongation. In addition, mutually exclusive cell-specific expression is evident. For instance, *PS-IAA4/5–GUS* but not *PS-IAA6–GUS* is expressed in root vascular tissue and in guard cells, whereas only *PS-IAA6–GUS* activity is detectable in glandular trichomes and redistributes to the elongating side of the hypocotyl upon gravitropic stimulation. Expression of *PS-IAA4/5* and *PS-IAA6* in elongating, dividing, and differentiating cell types indicates multiple functions during development. The common and yet distinct activity patterns of both genes suggest a combinatorial code of spatio-temporal co-expression of the various *PS-IAA4/5*-like gene family members in plant development that may mediate cell-specific responses to auxin.

Introduction

The plant hormone auxin typified by the naturally prevalent indoleacetic acid (IAA), is a major regulator of plant growth and development. The hormone profoundly affects turgor, elongation, division, and differentiation of cells, and thereby morphogenetic processes such as development of lateral roots and vascular tissues, formation of adventitious roots and trichomes, branching of shoots, and tropic responses (Went and Thimann, 1937). The molecular mechanisms of auxin action in plant cell growth and development are unknown. Developmental processes governed by auxin in concert with other plant growth regulators are the target of current genetic analyses (Aeschbacher *et al.*, 1994; Celenza *et al.*, 1995; Marks, 1994; Masson, 1995; Meinke, 1995) and most likely involve sequential changes in the pattern of gene expression. It is well established that auxin modulates gene expression in a wide variety of plant tissues and cell types over a broad period of time (Guilfoyle, 1986; Theologis, 1986). However, a causal relationship between auxin-mediated gene expression and a particular growth process has not yet been demonstrated.

Early genes induced as a primary response to auxin and prior to the initiation of cell growth, are likely candidates to encode proteins that mediate the growth-stimulating effects of the hormone. In animal cells, products of early-response genes serve mainly three functions: stress response, intercellular communication, and transcriptional regulation of downstream genes to establish long-term biological consequences (Herschman, 1991). Auxin-mediated cell elongation is one of the fastest and phenotypically simplest hormonal responses known in plants, with a lag period of 15–25 min (Evans, 1975). The process is associated with rapid changes in the expression of a select set of early genes prior to or concomitant with the onset of cell expansion (Guilfoyle, 1986; Theologis, 1986).

In a continuing effort to understand how auxin promotes plant cell growth, we have focused on the functional analysis of two early auxin-inducible genes from pea, *PS-IAA4/5* and *PS-IAA6*, using a combination of biochemical, molecular and reverse genetic approaches. The induction of both genes by IAA qualifies as a primary response (Koshiba *et al.*, 1995; Theologis *et al.*, 1985). *PS-IAA4/5* and *PS-IAA6* have been structurally characterized (Oeller *et al.*, 1993), and the auxin-responsive domains of *PS-IAA4/5* have been identified (Ballas *et al.*, 1993, 1995). Both genes are members of a multigene family in pea, and the proteins encoded are related to products of early auxin-inducible genes from various plant species (Abel *et al.*, 1995; Oeller and Theologis, 1995; Oeller *et al.*, 1993). PS-IAA4 and PS-IAA6 are short-lived nuclear proteins that contain functional targeting signals and a putative βαα DNA-binding domain (Abel and Theologis, 1995; Abel *et al.*, 1994). Expression of both proteins is post-transcriptionally regulated at multiple

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levels (Abel *et al.*, 1994; Koshiba *et al.*, 1995; Oeller and Theologis, 1995). Stringent control of expression is a hallmark of genes encoding regulatory molecules. Thus, from a molecular point of view, the prospect arises that PS-IAA4-like polypeptides mediate growth-stimulating effects of auxin.

The biological significance of any class of early auxin-regulated genes is elusive. Only a limited number of correlations between auxin-induced changes in early gene expression and the various auxin-mediated developmental responses have been described (Gee *et al.*, 1991; Li *et al.*, 1991; Wyatt *et al.*, 1993). As for *PS-IAA4/5* and *PS-IAA6*, little is known of their spatial and temporal activities in developing plants. Previous studies have shown that both genes are preferentially expressed in the elongation zone of etiolated pea seedlings (Ballas *et al.*, 1993; Theologis *et al.*, 1985). Here, we present a detailed histochemical characterization of *PS-IAA4/5* and *PS-IAA6* expression during early plant development, using transgenic tobacco seedlings with promoter–β-glucuronidase (GUS) gene fusions. The data obtained suggest differential developmental control of both genes. Implications of overlapping expression patterns of *PS-IAA4/5*-like gene products are discussed. Furthermore, knowledge of the precise spatio-temporal expression pattern will guide characterization of mutant plants defective in the expression of *PS-IAA4/5*-like genes.

Results

Analysis of PS-IAA4/5–GUS *and* PS-IAA6–GUS *expression in transgenic tobacco*

To study the developmental expression patterns of *PS-IAA4/5* and *PS-IAA6*, we have fused each primary auxin-responsive promoter upstream of the *Escherichia coli* gene *uidA* encoding β-glucuronidase (Jefferson *et al.*, 1987). Since we lack an efficient method to stably transform pea, transgenic tobacco plants (*Nicotiana tabacum* cv. Samsun) have been generated expressing either *PS-IAA4/5–GUS* or *PS-IAA6–GUS*. Previous studies indicated preferential expression of *PS-IAA4/5* and *PS-IAA6* in elongating pea epicotyl (Ballas *et al.*, 1993; Theologis *et al.*, 1985). Therefore, we focused our histochemical analysis on patterns of spatio-temporal GUS expression in early development of etiolated and light-grown seedlings.

Figure 1 shows an overview of consensus GUS expression in untreated seedlings during the first week of growth. Seeds transgenic for *PS-IAA4/5–GUS* (panels I and IIa) or *PS-IAA6–GUS* (panels IIb and III) were germinated either in a light dark cycle (panel I, a–d, and panel III, a–d) or in complete darkness (panels II, Ie and IIIe). *PS-IAA4/5–GUS* seedlings developing in light, express GUS in the inner curve of the apical hook, the hypocotyl, the root–shoot junction, the root vascular tissues, and in the root tip (panel I, a–d). Light-grown *PS-IAA6* seedlings show GUS expression in the root tip, the vascular tissue of the hypocotyl, and in the basal portion of cotyledons (panel III, a–d). In etiolated seedlings transgenic for either chimeric gene, GUS expression is highly confined to the elongation zone of the hypocotyl (panel II). In addition, preferential GUS staining is detectable in the inner curve of the apical hook, the outer curve of the collet which is the root-hypocotyl junction, and in the root tip (panels II, Ie and IIIe). Control plants transgenic for the promoterless *uidA* gene, do not show any detectable GUS expression (data not shown).

Expression in early skotomorphogenesis

Next, we studied in more detail the expression pattern and auxin-responsiveness of both promoters in germinating etiolated seedlings. Figure 2 shows dark-grown tobacco seedlings transgenic for *PS-IAA4/5–GUS* (panel I) or *PS-IAA6–GUS* (panel II). Expression of the reporter gene is visualized histochemically at days 1, 3, 4, and 5 after incubation of the seedlings for 6 hs in the absence (a–d) or presence (e–h) of 10 μM IAA. Tobacco seeds do not germinate synchronously, and in our conditions seedlings begin to emerge between late in day 3 to early in day 4 after imbibition.

To study day 1 embryos, imbibed mature seeds were dissected and embryos separated from the seed coat and endosperm. Intact and dissected seeds transgenic for either chimeric gene, express GUS activity at the radicle pole of the endosperm (a and e in both panels). Embryos of both transgenic lines stain for GUS at the radicle. In addition, *PS-IAA4/5–GUS* embryos express reporter activity in the hypocotyl, and *PS-IAA6–GUS* embryos stain at the outer edge of the cotyledons, in particular where they fuse (Figure 2, panel IIa; see also Figure 3, panel IIa; and Figure 1, panel IIIa). Endosperm and embryos from seeds of both transformants imbibed for 1 or 2 days (data not shown for day 2), do not show differences in GUS expression when incubated in the absence or presence of auxin (Figure 2, compare a with e in both panels).

About 3 days after imbibition, the radicle penetrates the seed coat. GUS expression is detectable in the root tip and in the hypocotyl (Figure 2, panel Ib and panel IIb). At this stage of development both transgenic transformants gain responsiveness to exogenous auxin which results in GUS staining of the entire hypocotyl and root (compare b and f in both panels).

At day 4, fully emerged *PS-IAA4/5–GUS* seedlings express reporter activity in the inner curve of the apical hook, the elongating hypocotyl, the root vascular tissue, the root elongation zone, and in the root tip (Figure 2, panel Ic). Auxin treatment greatly enhances GUS staining

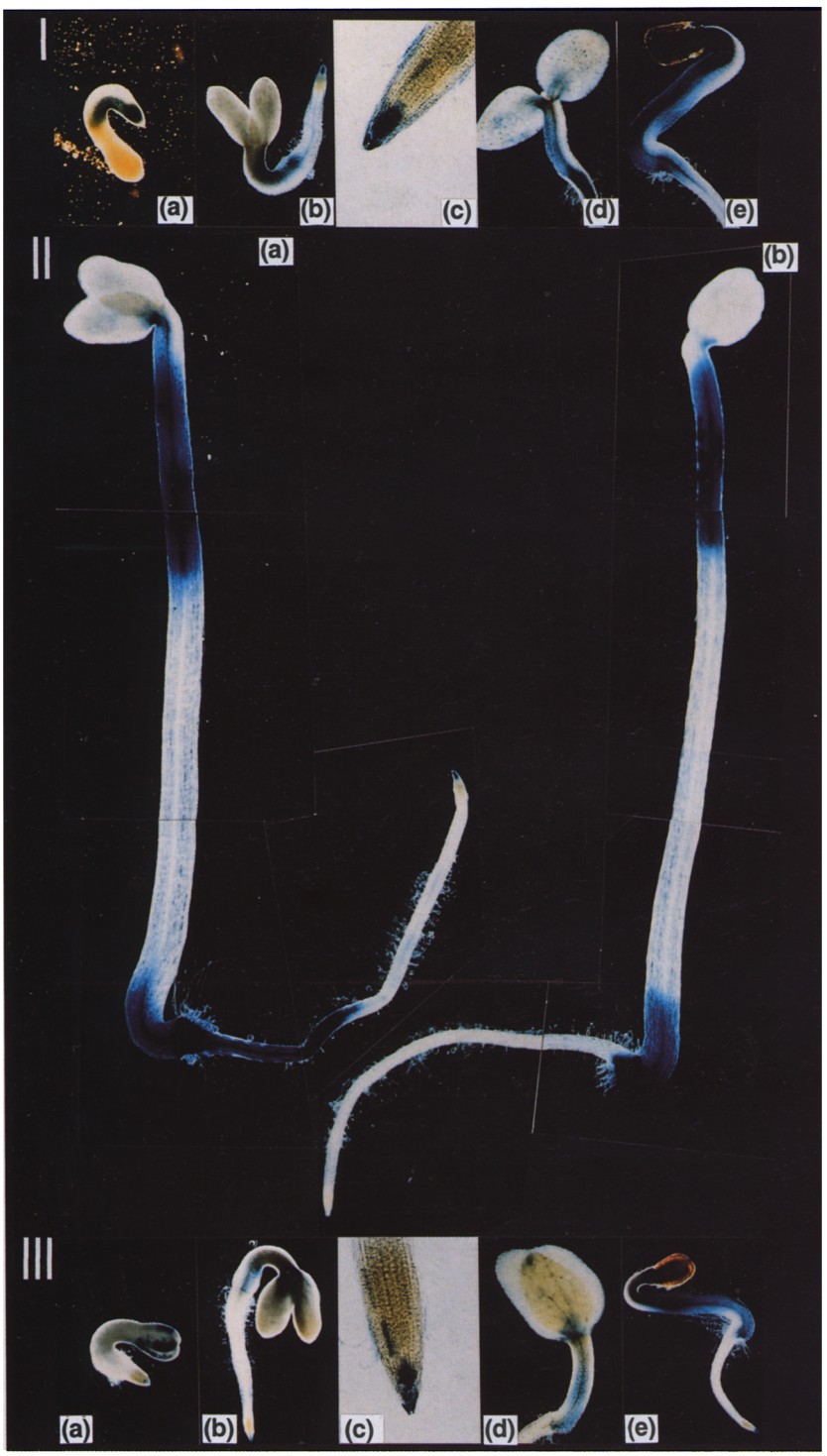


Figure 1. Promoter activity of *PS-IAA4/5* and *PS-IAA6* in early development of transgenic tobacco.
PS-IAA4/5–GUS seedlings (panels I and IIa) and *PS-IAA6–GUS* seedlings (panels IIb and III) were grown for the indicated time and stained for GUS activity without mock-control or auxin pre-treatment. Seeds were germinated either in a light–dark cycle (panel I, a–d, and panel III, a–d) or in the dark (panels Ie, IIIe, and II). The following stages of early seedling development are shown (days after imbibition): day 3 (panels Ia and IIIa); day 5 (panel I, b–c and e; and panel III, b–c, and e); day 6 (panels Id and IIId); and day 7 (panel II).

of the apical hook, hypocotyl, and root vascular tissues (panel Ig). In *PS-IAA6–GUS* seedlings, GUS is preferentially expressed at the edges of cotyledons and in the root tip. Low and inconsistent GUS staining is detectable in the hypocotyl and vascular cylinder (panel IIc). However, auxin treatment causes high *PS-IAA6–GUS* expression in the cotyledons, hypocotyl, and additionally, in the root vascular tissue, collet, and root elongation zone (Figure 2, compare c and g in panel II).

Beyond day 4, etiolated seedlings undergo rapid elongation. After 5 days of germination, *PS-IAA4/5–GUS* and *PS-IAA6–GUS* seedlings show reporter activity expression

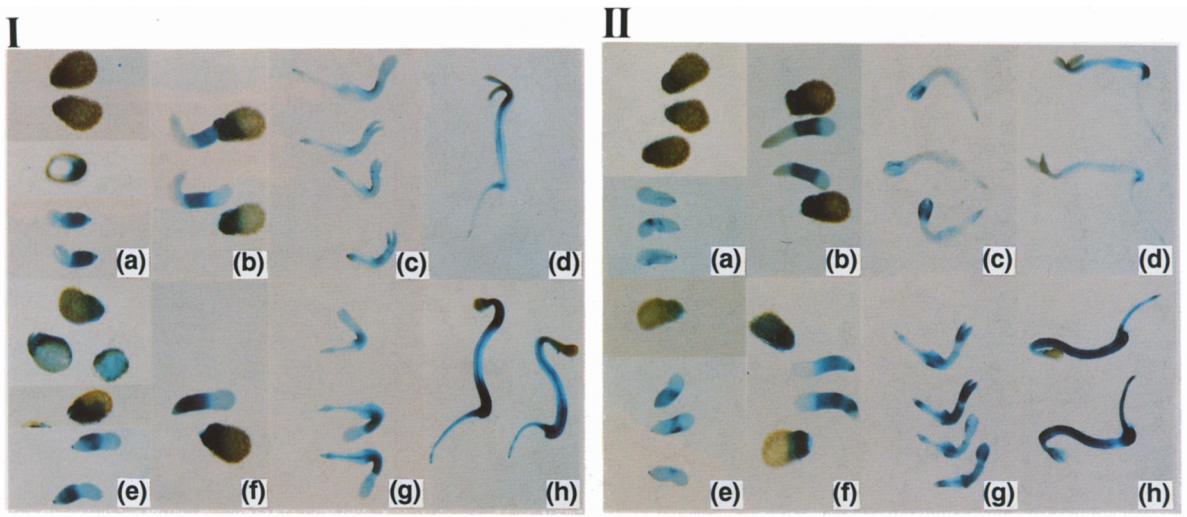


Figure 2. Promoter activity and responsiveness to exogenous auxin of *PS-IAA4/5* and *PS-IAA6* in developing etiolated seedlings.
Tobacco seeds transgenic for *PS-IAA4/5–GUS* (panel I) or *PS-IAA6-GUS* (panel II) were germinated in the dark for the indicated time, then incubated for 6 h in the presence (a–d) or absence (e–h) of 10 μM IAA, and subsequently stained for GUS activity. The following stages of early development are shown (days after imbibition): seeds and dissected embryos at day 1 (a and e); seedlings at day 3 (b and f), day 4 (c and g), and day 5 (d and h).

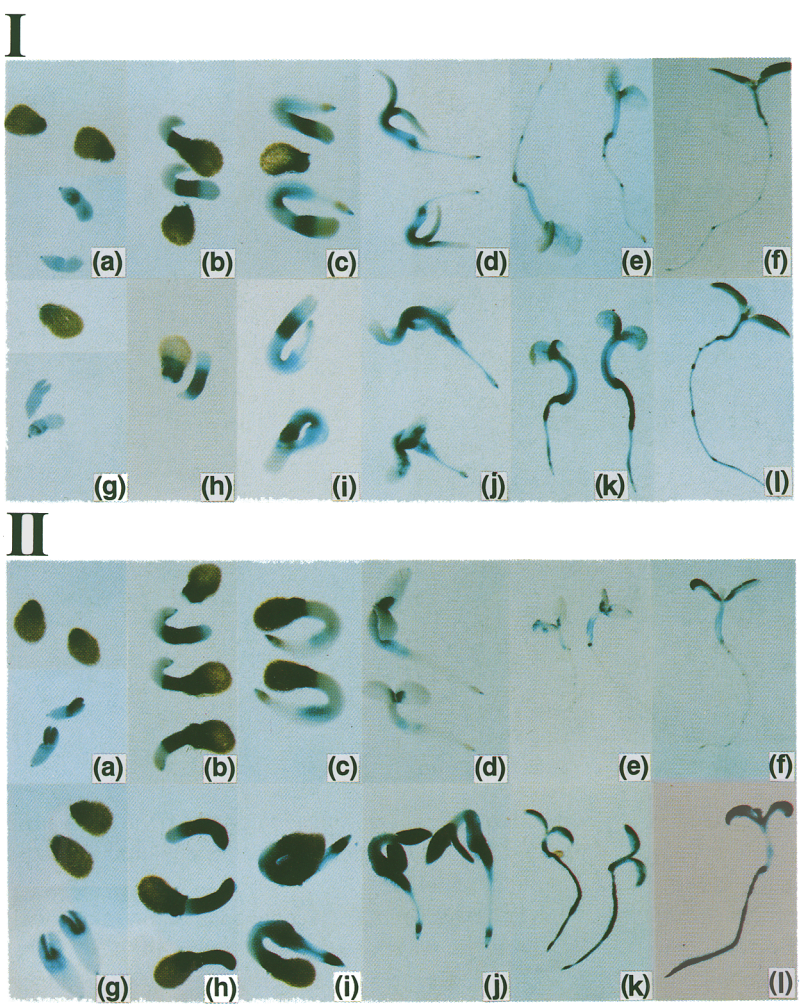


Figure 3. Promoter activity and responsiveness to exogenous auxin of *PS-IAA4/5* and *PS-IAA6* in developing seedlings grown in light.
Tobacco seeds transgenic for *PS-IAA4/5–GUS* (panel I) or *PS-IAA6-GUS* (panel II) were germinated for the indicated time, then incubated for 6 h in the presence (a–f) or absence (g–l) of 10 μM IAA, and subsequently stained for GUS activity. The following stages of early development are shown (days after imbibition): seeds and dissected embryos at day 1 (a and g); seedlings at day 3 (b and h), day 4 (c and i), day 5 (d and j), day 7 (e and k), and day 11 (f and l).

in the hypocotyl (Figure 2, d in both panels). Again, seedlings of both transgenic lines express GUS preferentially in the inner curve of the apical hook. Similarly, GUS expression around the collet is localized to the outer curve. Incubation of *PS-IAA4/5–GUS* and *PS-IAA6–GUS* seedlings with auxin increases GUS activity in all organs (Figure 2, compare d and h in both panels).

Expression in early photomorphogenesis

Figure 3 shows seedlings transgenic for *PS-IAA4/5–GUS* (panel I) or *PS-IAA6-GUS* (panel II) grown in a light-dark cycle for up to 11 days. Seedlings were stained for GUS activity after an incubation for 6 h in the absence (a–f) or presence (g–l) of 10 μM IAA. Patterns of *PS-IAA4/5–GUS* and *PS-IAA6–GUS* expression in light-grown seedlings are similar to those of etiolated seedlings germinated for up to 3 days (compare Figure 2 with Figure 3, a and b in both panels).

For light-grown *PS-IAA4/5–GUS* plants, the pattern of GUS expression and the responsiveness to exogenous auxin at day 4 and day 5 in development is similar to that in etiolated seedlings, except for additional strong GUS staining at the basal portion of the expanding cotyledons (Figure 3, panel I, c and d). At this age, GUS activity is preferentially localized to the root tip, vascular tissues, inner surface of the curled seedling, and shoot apex. *PS-IAA4/5–GUS* seedlings seem to preferentially express GUS in one half of the hypocotyl for up to day 7 (panel I, c–f). The preferential staining is not a result of the mock-control treatment since untreated seedlings show an identical GUS expression pattern (Figure 1, panel I, b and d). Treatment with auxin results in enhanced vascular GUS expression (Figure 3, panel I, compare c and d with i and j). At day 7 and day 11 after imbibition, in addition to *PS-IAA4/5–GUS* expression in the vascular tissues, hypocotyl and root tip, GUS activity is detectable in lateral root primordia and stomata (panel I, e and f; see below for detailed analysis). Auxin treatment increases GUS expression in all organs of 7-day-old seedlings but only in roots of 11-day-old plants (panel I, compare e and f with k and l).

For *PS-IAA6–GUS* seedlings, a similar pattern of GUS expression and responsiveness to auxin is observed in etiolated as well as light-grown plants at day 4 and day 5 after imbibition (Figure 3, panel II, c and d). Unlike in *PS-IAA4/5–GUS* seedlings, GUS activity is equally expressed in the hypocotyl. Notable is the high basal GUS expression at the proximal portion of cotyledons as well as the strong response to auxin of cotyledons, collet, root vascular tissue, and root elongation zone (panel II, compare c and d with i and j). At later stages in development, at day 7 and day 11, GUS activity is detectable at sites of lateral root initiation and in trichomes of primary leaves (see below for detailed analysis). Exogenous auxin enhances GUS expression in all organs of 7-day-old seedlings, however, stems of 11-day-old seedlings show a very low response to the hormone, if at all (panel II, compare e and f with k and l).

Expression during gravitropic stimulation

Expression of *PS-IAA4/5–GUS* and *PS-IAA6–GUS* in response to gravitropic stimulation was studied in seedlings grown for 2 weeks in a light–dark cycle (Figure 4). Plants were rotated 90° for 12–16 h to apply the stimulus. In control seedlings of both transgenic lines (not rotated), GUS activity is nearly equally expressed around the stem (panel IIa and IIIa). An asymmetric distribution of GUS activity to the more elongating side of the stem undergoing negative gravitropic curvature is observed in *PS-IAA6–GUS* seedlings (panel III, b and c). The hypocotyl of *PS-IAA4/5–GUS* seedlings subjected to gravitropic stimulation, however, stains equally and does not show consistent redistribution of GUS activity (panel II, b and c). The positive gravitropic response of roots is not accompanied by any detectable changes of *PS-IAA4/5–GUS* or *PS-IAA6–GUS* activity (data not shown).

Cell-specific expression in root development

Both *PS-IAA4/5–GUS* and *PS-IAA6–GUS* seedlings express reporter activity in the root tip during early development (see Figures 1–3). To determine more precisely the region of GUS expression, primary roots from 7- to 11-day-old light-grown seedlings were sectioned and analyzed. Figure 5 shows representative serial sections toward the primary root tip of a *PS-IAA4/5–GUS* (panel I) or *PS-IAA6–GUS* (panel II) seedling, starting approximately 100 μm above the root cap. The GUS expression pattern in root tip tissues is similar for both transgenic lines. Reporter activity is detected in the central region including the endodermis, pericycle, and procambium. Oblique longitudinal sections of *PS-IAA4/5–GUS* primary roots (panel III) reveal GUS expression in the root apical meristem (panel IIIb). Similiar data were obtained for primary roots of *PS-IAA6–GUS* seedlings (data not shown).

GUS expression patterns in developing lateral roots of *PS-IAA4/5–GUS* (panel I) and *PS-IAA6–GUS* (panel II) transformants are shown in Figure 6. Light-grown seedlings of both transgenic lines express GUS activity in the lateral root primordium which derives from cell divisions in the pericycle (a and e in both panels). As the primordium increases in size, differences in the pattern of GUS expression become obvious between the two types of seedlings. In *PS-IAA6–GUS* plants GUS activity is restricted to the tip of the developing lateral root. Only a few cell layers comprising the columella root cap and central cells express the reporter (panel II, b–d and f–h). In young lateral root primordia of *PS-IAA4/5–GUS* seedlings, GUS activity is detected in all cells of the primordium (panel I, a–b, and e–f). As the lateral root develops, GUS is expressed in root cap primordia,

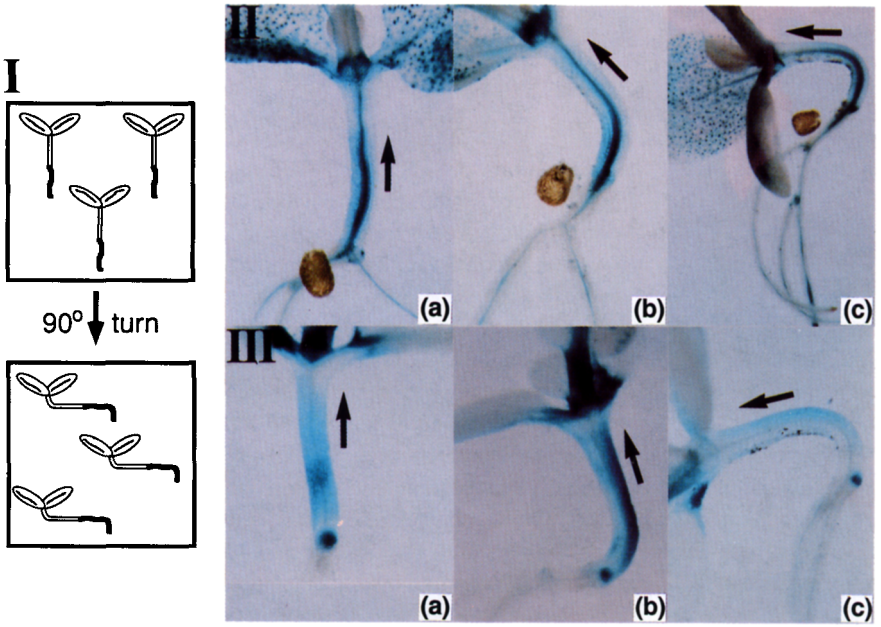
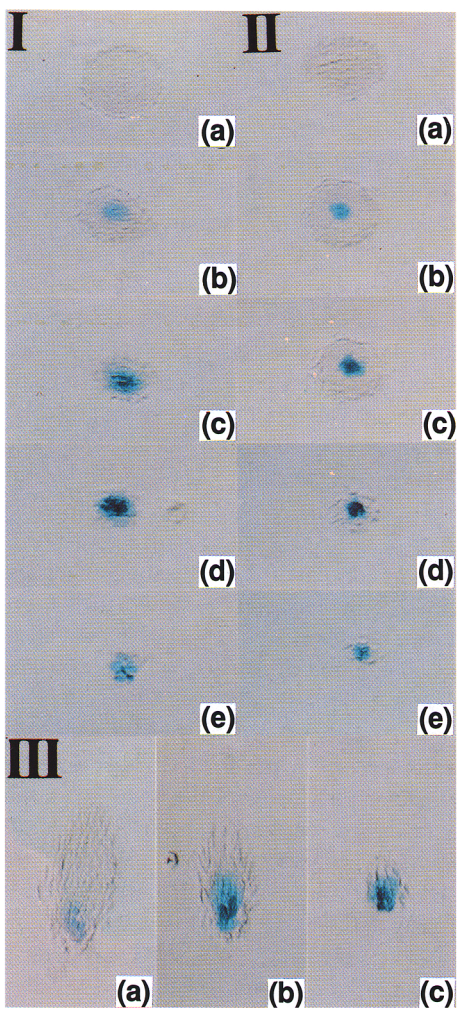


Figure 4. Effect of gravitropic stimulation.
A schematic diagram of the gravitro-stimulation is shown in panel I. Square petri dishes placed vertically and containing seedlings grown for 2 weeks in a light–dark cycle were rotated 90°. GUS expression in *PS-IAA4/5–GUS* (panel II) and *PS-IAA6–GUS* (panel III) seedlings is shown for control (a) and rotated (b and c) plates. The arrow indicates upward direction of growth.



central cells, the core meristem, and in cells several layers back (panel I, c–d and g–h). Later in development, *PS-IAA4/5–GUS* expression is restricted to the lateral root tip in a spatial pattern very similar to the GUS activity pattern of the primary root (data not shown).

Cell-specific expression in leaves

At day 5 of photomorphogenesis, GUS activity starts to accumulate in stomata of *PS-IAA4/5–GUS* cotyledons but not in the guard cells of *PS-IAA6–GUS* seedlings (see Figure 1, panels Id and IIId; Figure 3, panels Ie and IIe). A more precise anatomical analysis is shown in Figure 7 for cotyledons of *PS-IAA6–GUS* seedlings (Figure 7a) and of *PS-IAA4/5–GUS* plants (Figure 7b). Interestingly, not all stomata express *PS-IAA4/5–GUS*, particularly those positioned at the basal portion of the cotyledons (panel IIa). However, as *PS-IAA4/5–GUS* seedlings develop, guard cells at the distal portion of the cotyledons express reporter activity (see Figure 4, panel II). Unlike cotyledons, stomata of primary leaves stain randomly for GUS (Figure 8a). In most stomata, one of the two guard cells stains stronger for GUS activity than the other one (Figure 7, panel II, b and c). Mesophyll cells of *PS-IAA4/5–GUS* cotyledons show very little GUS staining. Occasionally, mesophyll cells of *PS-IAA6–GUS* cotyledons stain for GUS (see Figure 3, panel II, f), but never guard cells (Figure 7, panel I).

Figure 5. Expression in primary roots.
GUS activity staining in serial sections (16 μm) of the primary root tip is shown for a 7-day-old light-grown seedling transgenic for *PS-IAA4/5–GUS* (panels I and III) or *PS-IAA6–GUS* (panel II). Panels I and II show transverse serial sections toward the root tip. Panel III is a series of three oblique longitudinal section of a *PS-IAA4/5–GUS* root tip.

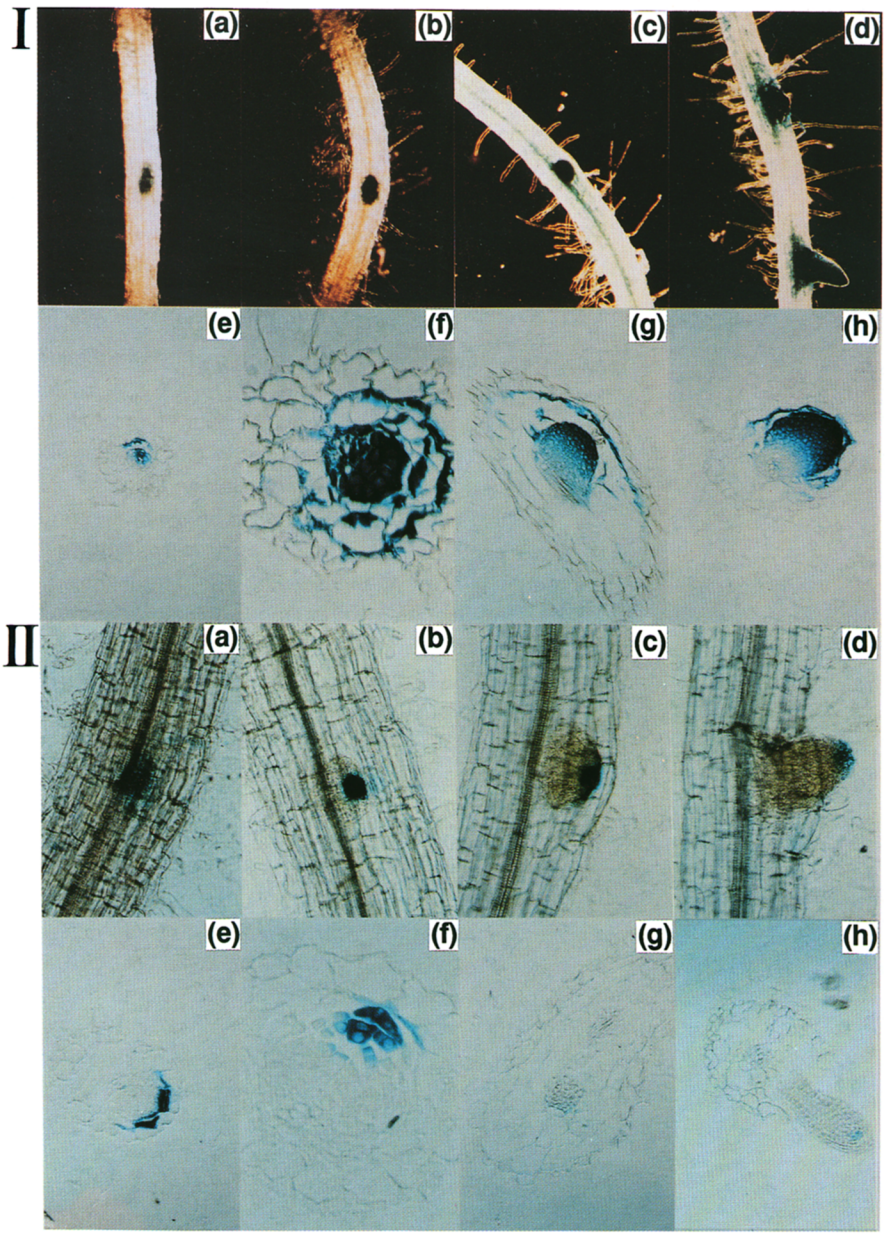


Figure 6. Expression in lateral roots. GUS activity staining in developing lateral roots is shown for 11-day-old light-grown *PS-IAA4/5–GUS* seedlings (panel I) and *PS-IAA6–GUS* seedlings (panel II). Whole mounts and sections (16 μm) of lateral roots at different stages of development are shown under dark-field microscopy (panel I, a–d) or bright-field microscopy (panel I, e–h; and panel II).

As shown in Figure 8, glandular trichomes on the primary leaves and petioles of *PS-IAA6–GUS* seedlings express high GUS activity (Figure 8b and c). On the other hand, simple trichomes which are longer do not consistently stain for GUS (Figure 8c). Interestingly, both types of trichome on leaves of *PS-IAA4/5–GUS* seedlings do not express detectable reporter activity (Figure 8a).

Discussion

As part of a continuing effort to elucidate the function of two related primary auxin-responsive genes, *PS-IAA4/5* and *PS-IAA6* from pea, we examined their spatio-temporal expression patterns during early plant development. We expressed promoter–GUS gene fusions in tobacco as a heterologous host since transformation of pea is laborious and inefficient. However, several hundred base pairs of the promoter regions of plant genes are generally sufficient to direct the appropriate spatio-temporal expression of heterologous reporter genes (Willmitzer, 1989). The comparative and qualitative analysis of GUS expression patterns in tobacco seedlings transgenic for *PS-IAA4/5–GUS* or *PS-IAA6–GUS* implies transcriptional activation of authentic *PS-IAA4/5* and *PS-IAA6* genes in specific tissues and cells that largely correlate with physiological target sites of auxin action. Expression of both transgenes is

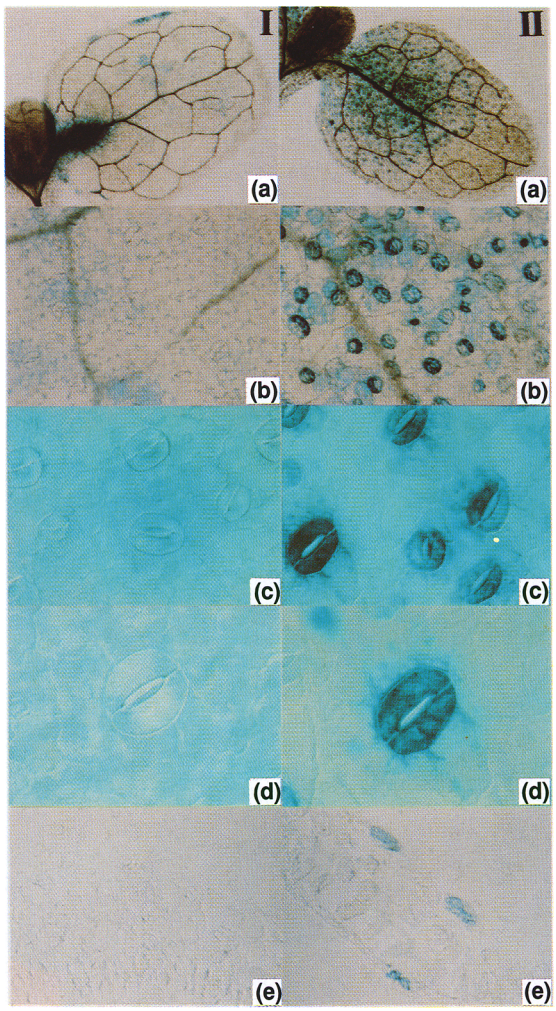


Figure 7. Expression in cotyledons.
Differential GUS activity staining of stomata is detectable in 6-day-old light-grown seedlings transgenic for *PS-IAA6–GUS* (panel I) or *PS-IAA4/5–GUS* (panel II). Whole mounts at different magnifications (a–d), and transverse sections (e) of cotyledons are shown.

developmentally regulated and responsive to exogenous auxin. Interestingly, *PS-IAA4/5–GUS* and *PS-IAA6–GUS* display both unique and overlapping patterns of cell-specific expression. Those patterns are not restricted to elongating cells but include dividing cells or cells with the potential to divide as well as highly specialized cell types. The data suggest that both genes play multiple roles during development.

Correlation of expression with cell elongation

Auxin has long been known to stimulate elongation of excised stem segments (Evans, 1975). A role of auxin in promoting cell elongation in intact plants has been controversial, however, recent evidence indicates a stimulating effect of the hormone on cell elongation *in planta*. For instance, Yang *et al.* (1993) used an elaborate infusion system to show that exogenous auxin can promote stem elongation in growing pea seedlings, and transgene-mediated overproduction of endogenous auxin produces plants with increased hypocotyl length and severe leaf epinasty (Romano *et al.*, 1995).

Promoter sequences of *PS-IAA4/5* and *PS-IAA6* confer GUS expression in expanding tissues of young tobacco seedlings, suggesting a function of the primary auxin genes in cell elongation. *PS-IAA4/5–GUS* is expressed in the apical hook and hypocotyl of etiolated and light-grown seedlings, whereas *PS-IAA6–GUS* activity is additionally detectable in the proximal, that is, the expanding portion of cotyledons which enlarge in photomorphogenesis. Expression of *PS-IAA4/5–GUS* and *PS-IAA6–GUS* in the hypocotyl of etiolated seedlings is confined to the upper region which correlates well with the zone of cell elongation (Maruyama and Boyer, 1994). Expression of both transgenes in the apical hook is mainly detected in the inner curve. Only a little GUS staining, if any, is visible in the outer curve where cells undergo expansion. This observation appears contradictory to a proposed function of

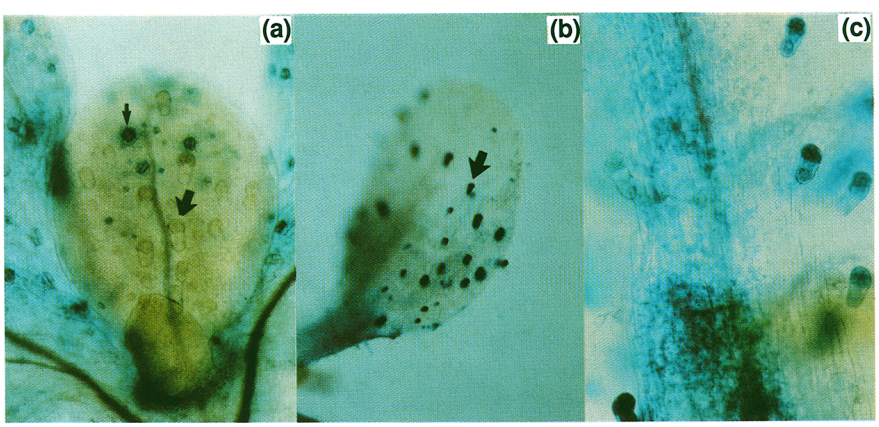


Figure 8. Expression in primary leaves.
Differential GUS activity staining of trichomes is detectable in 11-day-old light-grown seedlings transgenic for *PS-IAA6–GUS* (a) or *PS-IAA4/5–GUS* (b and c). Bold arrows point to trichomes on the primary leaf, and the small arrow in (a) indicates a stomatal complex stained in the primary leaf of a *PS-IAA4/5–GUS* seedling.

PS-IAA4/5 and *PS-IAA6* in auxin-mediated cell elongation. *PS-IAA/5*-like genes constitute large gene families (Abel *et al.*, 1995; Oeller and Theologis, 1995). Therefore, it is conceivable that related, unidentified genes are expressed in the outer curve where they may function in cell elongation. However, it is possible that staining of the inner curve reflects gene activation prior to the onset of cell expansion. Elongation of cells in the inner curve would straighten the apical hook as it is observed in early seedling development. Alternatively, high concentrations of auxin have been shown to inhibit cell growth by inducing ethylene biosynthesis (Burg and Burg, 1966). Overproduction of auxin in transgenic plants also results in the overproduction of ethylene which inhibits stem elongation (Romano *et al.*, 1993). Thus, supraoptimal auxin concentration in the inner curve could inhibit cell elongation but still induce *PS-IAA4/5* and *PS-IAA6*. An answer to this question has to await precise determination of *in situ* auxin levels with the resolution of a single cell. Interestingly, preliminary immunolocalization studies of IAA in etiolated *Arabidopsis* seedlings indicates anisotropic distribution of auxin in the apical hook with most of the hormone detectable at the lower side (Ecker, personal communication).

Correlation of expression with cell division

In addition to tissues growing mainly by cell expansion, *PS-IAA4/5–GUS* and *PS-IAA6–GUS* are also active in proliferating cells. During early seedling development, correlation with cell division is best revealed in growing primary and developing lateral roots. Both promoters confer GUS expression in the root tip, the root–shoot junction in which adventitious roots are initiated (Blakesley *et al.*, 1991), and at sites of lateral root formation. GUS expression in the root tip is already detectable in mature embryos and persists throughout development. Cross-sections of primary roots indicate that both genes are expressed in the root cap as well as in the root apical meristem. *PS-IAA4/5–GUS* and *PS-IAA6–GUS* are expressed at very early stages of lateral root initiation when a group of pericycle cells is transformed by periclinal and anticlinal cell divisions into a primordium which eventually develops a lateral root meristem (Laskowski *et al.*, 1995; Torrey, 1950). *PS-IAA6–GUS* expression continues to be confined to a few cells at the root tips of emerging lateral roots, whereas *PS-IAA4/5–GUS* is expressed throughout the primordium. Fully developed lateral root tips of both transgenic seedlings show a pattern of GUS staining similar to that of the primary root tip. The GUS expression pattern observed during lateral root initiation could reflect activity of *PS-IAA4/5* and *PS-IAA6* during embryogenesis when the primary root meristem is formed.

Several genes expressed during lateral root development have been studied, including *cdc2a* (Hemerly *et al.*, 1993; Martinez *et al.*, 1992), *cyc1At* (Ferreira *et al.*, 1994), *L16* (Williams and Sussex, 1995), or *LRP1* (Smith and Fedoroff, 1995). Spatio-temporal promoter activity of *PS-IAA4/5* or *PS-IAA6* in lateral root development is reminiscent of the expression patterns described for the mitotic cyclin gene *cyc1At*, and for *cdc2a* which encodes the catalytic subunit of a protein kinase involved in cell cycle control, $p34^{cdc2}$. Expression of *cyc1At* is exclusively confined to dividing cells (Ferreira *et al.*, 1994), whereas expression of *cdc2a* coincides with proliferating cells and with cell types that are developmentally competent to divide (Hemerly *et al.*, 1993). A striking correlation exists between *PS-IAA4/5–GUS* activity and *cyc1At–GUS* expression during lateral root development. *Cyc1At* expression is restricted to the lateral root primordium and, later in development, to the meristematic zone in a cell-specific pattern similar to that observed in the primary root (Ferreira *et al.*, 1994). Cell cycle progression is under hormonal control (Jacobs, 1995), and several lines of evidence suggest that auxin is intricately involved in lateral root initiation and development: (i), exogenous application of auxin initiates lateral roots in a wide variety of plants (Kerk, 1990; Thimann, 1936; Torrey, 1950); (ii), transgene-mediated overexpression of auxin results in plants with increased lateral root production (Kares *et al.*, 1990; Klee *et al.*, 1987); (iii) auxin-insensitive mutants of tomato, *dgt* (Kelly and Bradford, 1986), and *Arabidopsis*, *aux1*, *axr1*, *axr4* (Hobbie and Estelle, 1995), are defective in lateral root formation; and (iv) a mutant impaired in lateral root maturation, *alf3–1*, can be rescued by IAA (Celenza *et al.*, 1995).

Correlation of expression with cell differentiation

It has been demonstrated in a number of experimental systems that auxin plays a central role in controlling phloem and xylem differentiation (Aloni, 1988). GUS staining of vascular tissues in transgenic tobacco seedlings indicates differential expression of *PS-IAA4/5* and *PS-IAA6*. *PS-IAA4/5–GUS* activity is present in vascular tissues of light-grown and etiolated seedlings, whereas *PS-IAA6–GUS* is consistently detectable in the vascular strands of etiolated seedlings only. Constitutive expression of *PS-IAA4/5–GUS* in vascular tissues of growing seedlings suggests continued involvement of *PS-IAA4/5* during the vascular differentiation process.

A striking feature of *PS-IAA4/5–GUS* expression in early development is specific localization to guard cells in the leaf epidermis. Gene expression in stomata has rarely been reported (Samac and Shah, 1991; Terryn *et al.*, 1993). GUS activity in guard cells suggests that *PS-IAA4/5* is involved in development or function of stomata. Similar to the stomatal expression pattern of *rha1* (Terryn *et al.*, 1993), *PS-IAA4/5–GUS* activity is mainly detectable for stomata in the youngest parts of the leaf, the leaf base and borders

(Poethig and Sussex, 1985). Stomata develop rigid dorsal and ventral cell walls during maturation (Sack, 1989), and Rha1, a GTP binding protein, has been proposed to function in vesicle-mediated processes leading to cell wall thickening (Terryn *et al.*, 1993). Although GUS activity in guard cells starts to be detectable at day 5 when stomata are already present, the non-homogeneous pattern of staining is more consistent with a proposed function of *PS-IAA4/5* in guard cell maturation than in guard cell movement.

Another epidermally derived, highly specialized cell type that exhibits GUS staining, is the glandular trichome found on leaves of *PS-IAA6–GUS* seedlings. Two types of trichome have been described for tobacco, glandular trichomes consisting of a stalk and a multicellular secretory head, and simple, unicellular trichomes (Esau, 1953). Mutants affecting trichome development in *Arabidopsis* have been obtained and genes identified whose products are involved in trichome patterning or cell morphogenesis (Hülskamp *et al.*, 1994; Marks, 1994). Trichome morphogenesis includes cessation of cell division and initiation, several rounds of endoreplication, and extension growth (Hülskamp *et al.*, 1994). It is tempting to speculate that the *PS-IAA6* gene product may play a role in one aspect of glandular trichome development.

Auxin-responsiveness of expression

Responsiveness to exogenous auxin of *PS-IAA4/5–GUS* and *PS-IAA6–GUS* expression is first observed at day 3 of seedling development although both promoters are active in mature embryos. It appears that the competence to respond to exogenous auxin is under developmental control which coincides with the initiation of *de novo* IAA biosynthesis in the growing seedling. Bean seedlings start to synthesize IAA *de novo* between day 2 and day 3 after imbibition (Bialek *et al.*, 1992). The need for auxin by the germinating embryo is provided for by the seed. Most of its auxin content is stored in the endosperm as ester conjugates of IAA. Upon imbibition, IAA conjugates are hydrolyzed and represent the predominant source of free auxin in the growing seedling (Cohen and Bandurski, 1982). Seeds of both transgenic lines show localized GUS staining at the radicle pole of the endosperm. Restricted localization and controlled production of active auxins in the imbibed seed could maintain a gradient that is necessary in early seedling development before the onset of *de novo* IAA biosynthesis. A gradient of auxin most likely acts as a morphogen to establish and maintain polarity during embryogenesis, whereas high levels of the hormone prevent embryonic differentiation (Warren Wilson and Warren Wilson, 1993). Likewise, disruption of auxin transport inhibits establishment of symmetry and maintenance of polarity during embryo development (Liu *et al.*, 1993; Schiavone and Cooke, 1987).

At day 3 after imbibition, transgenic seedlings start to respond to exogenous auxin with increased *PS-IAA4/5–GUS* or *PS-IAA6–GUS* expression. GUS staining becomes more intense in the apical hook, hypocotyl, collet, roots, and vascular tissues, all tissues which already express either transgene in the absence of exogenous hormone. Interestingly, the root elongation zone and distal portion of light-grown cotyledons of *PS-IAA6–GUS* seedlings which do not stain in the absence of exogenous auxin, however, express high reporter activities upon auxin treatment. Etiolated cotyledons of both transgenic seedlings, and cotyledons of light-grown *PS-IAA4/5–GUS* seedlings do not respond to applied auxin.

During early development of *PS-IAA4/5–GUS* seedlings, the inner curve of the apical hook and the corresponding side of the hypocotyl stain preferentially for GUS. However, incubation with exogenous auxin leads to GUS expression in the entire hook and hypocotyl. This indicates that cells of the outer hook and deriving stem side are competent to respond to auxin. The asymmetric GUS staining of the hypocotyl could be a consequence of the relatively long half-life of the GUS protein (Jefferson *et al.*, 1987), expressed to high levels in the inner curve of the younger apical hook. Alternatively, the asymmetric GUS expression pattern likely results from a lowered sensitivity to endogenous auxin of cells in the outer hook and hypocotyl, or from a differential lateral distribution of free and conjugated auxins. We also detected preferential accumulation of *PS-IAA6–GUS* activity on the proximal, elongating side of the hypocotyl undergoing negative geotropism. According to the Cholodny–Went hypothesis, an asymmetric distribution of active auxin drives tropic responses that result from differential growth of opposing sides in a stimulated organ. Genetic evidence in *Arabidopsis* confirms that auxin plays a major role in the gravitropic response (Masson, 1995). An unequal lateral distribution of auxin in gravistimulated coleoptiles has been reported (Mertens and Weiler, 1983), and an auxin-responsive *SAUR* gene is proposed to be differentially induced by auxin gradients during phototropism and gravitropism (Li *et al.*, 1991).

Transcriptional activation of *PS-IAA4/5* and *PS-IAA6* is specific for auxin. This has been shown with etiolated pea seedlings (Theologis *et al.*, 1985) and with transgenic *Arabidopsis* plants expressing promoter–GUS gene fusions (Oono and Theologis, unpublished data). Furthermore, auxin and cycloheximide are the only inducers known for *PS-IAA4/5*-like genes of various plant species (for review see Abel and Theologis, 1996). *PS-IAA4/5* and *PS-IAA6* appear unresponsive to auxin under certain cellular and developmental context. Similarly, the dose response curves of *IAA* genes from *Arabidopsis* reveal differential sensitivity in intact seedlings over a broad range of IAA concentrations (Abel *et al.*, 1995). Relatively high expression of certain *IAA* genes in the absence of exogenous IAA and low auxin-

inducibility have been explained by 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 (Abel *et al.*, 1995). This may as well apply to *PS-IAA4/5–GUS* and *PS-IAA6–GUS* expression in transgenic tobacco seedlings.

Overlapping patterns of expression — implications

Our study indicates developmental and independent control of *PS-IAA4/5* and *PS-IAA6* gene expression. Each gene is activated in discrete and yet mutual spatio-temporal patterns which include tissues and cell types known to be targets of auxin action. GUS activity staining in root tips, lateral roots, hypocotyl, stomates or trichomes illustrate best the overlapping cell and tissue-specific expression of both genes. In a similar study by Wyatt *et al.* (1993), expression of the related *Arabidopsis* gene, *AtAux2-11*, was analyzed during development. *AtAux2-11* is primarily expressed in expanding tissues such as etiolated hypocotyl or root elongation zone, and in cells undergoing lignification such as trichomes or developing xylem (Wyatt *et al.*, 1993). Again, although not directly comparable, spatio-temporal patterns of *AtAux2-11* expression appear to overlap with the expression pattern of *PS-IAA4/5* or *PS-IAA6*.

We have recently characterized a large family of *PS-IAA4/5*-like mRNAs in *Arabidopsis* that consists of at least 17 members. Northern analysis indicates organ-preferential accumulation of each transcript (Abel *et al.*, 1995). In view of the expression analysis of *AtAux2-11* (Wyatt *et al.*, 1993), *PS-IAA4/5* and *PS-IAA6* (this study), we anticipate a complexity of specific and overlapping expression patterns for members of *PS-IAA4/5*-like multigene families. It remains to be determined whether these expression patterns apply for the proteins encoded since *PS-IAA4/5*-like gene expression is subject to post-transcriptional control (Abel *et al.*, 1994; Koshiba *et al.*, 1995; Oeller and Theologis, 1995). Nonetheless, our data indicate that various members of PS-IAA4-like proteins are likely to be co-expressed in a tissue- and cell-specific manner. Early auxin-inducible genes related to *PS-IAA4/5* encode short-lived nuclear proteins (Abel *et al.*, 1994; Hagen, personal communication) with properties reminiscent of transcription factors (Abel *et al.*, 1994, 1995; Oeller and Theologis, 1995). All PS-IAA4-like polypeptides contain a putative βαα DNA-binding motif that has been proposed, in analogy to the prototypic motif described for prokaryotic repressor polypeptides (Raumann *et al.*, 1994), to mediate homodimerization and possibly heterodimerization (Abel *et al.*, 1994). Interestingly, we have recently demonstrated both homo- and heterodimerization of *PS*-IAA4-like proteins from pea and *Arabidopsis* using a yeast two-hybrid system (Kim and Theologis, unpublished data). The capacity to heterodimerize *in vivo*, and the overlapping expression patterns in plant development potentially allow for a multiplicity of functional interactions of *PS*-IAA4-like proteins. It is likely that cell specific restriction of *PS-IAA4/5*-like gene expression is a developmental process that plays a fundamental role in specifying a combinatorial code of spatio-temporal co-expression of the various family members. This code, in turn, could determine the permissible repertoire of biological responses to auxin for a given cell type.

Experimental procedures

Construction of PS-IAA4/5–GUS and PS-IAA6–GUS gene fusions

The promoter sequences of *PS-IAA4/5* and *PS-IAA6* have previously been isolated (Oeller *et al.*, 1993). Construction of the transcriptional *PS-IAA4/5(–2309/+85)–GUS–NOS* fusion in the pBI101.1 Ti cloning vector (Clonetech), pBI-5′Δ-2309, has been previously described (Ballas *et al.*, 1993). To construct *PS-IAA6–GUS–NOS*, a 5 kb *Eco*RI fragment containing approximately 4.5 kb of the *PS-IAA6* promoter and 574 bp of coding sequence was subcloned into pUC18 giving rise to pRI-5 plasmid. A 362 bp PCR fragment was synthesized from the *Sty*I site at –320 to the ATG at +42 by introducing an *Nco*I site at the 3′ end primer. The *Sty*I/*Nco*I fragment was fused to the *Eco*RI/*Sty*I promoter fragment and subcloned into the *Eco*RI/*Nco*I sites of pKNS (a derivative of pKS^+ where the *Sma*I site has been replaced by a *Nco*I site) giving rise to pNS24. The 4.5 kb *Eco*RI/*Nco*I *PS-IAA6* promoter fragment was linked to GUS gene of JS8 (a gift from Dr. Maliga, Rutgers University) through the *Nco*I site at the ATG of GUS giving rise to pNS25. The *PS-IAA6–GUS* gene was subcloned as a *Hind*III/*Sna*BI fragment into pBI101.1 vector giving rise to *pBI-PS-IAA6–GUS–NOS* plasmid, pBI-5′Δ-5000. The junctions of the promoter–GUS fusions were verified by DNA sequencing analysis.

Plant transformation

The plasmids pBI101.1 (promoterless *uidA* gene), pBI-5′Δ-2309 and pBI-5′Δ-5000 were mobilized into *Agrobacterium tumefaciens* strain LB4404 by direct transformation. Tobacco (*Nicotiana tabacum* cv. Samsun) was stably transformed using the leaf disc method as previously described (Ballas *et al.*, 1993). Fifteen independent transformants transgenic for *PS-IAA4/5–GUS* and 30 independent lines carrying the *PS-IAA6–GUS* transgene have been generated. Three *PS-IAA4/5–GUS* lines (5′Δ-2309 #7, #8e, and #9a) and four *PS-IAA6–GUS* lines (5′Δ-5000 #1, #3, #7, and #9) were histochemically analyzed, revealing qualitatively similar expression patterns for each transgene. Results are presented for R_1 seedlings of the strongest expressor lines, 5′Δ-2309 #7 for *PS-IAA4/5–GUS* and 5′Δ-5000 #7 for *PS-IAA6–GUS*. The 5′Δ-2309 #7 line segregates 15:1 for resistance to kanamycin and the *PS-IAA6–GUS* line, 5′Δ-5000 #7, segregates 3:1 for kanamycin resistance.

Germination and growth of seedlings

R_1 seedlings were grown on 0.7% agar plates containing MS salts (Gibco BRL) at 24°C in a cycle of 16 h light and 8 h dark, or in complete darkness. Samples were taken and analyzed at different

days after plating. Auxin induction was carried out by incubating seedlings for 6 h in 10 μM IAA as described by Theologis *et al.* (1985). Control seedlings were incubated in the absence of auxin. For samples where seedlings had not emerged (days 1–3), embryos were separated from the seed coat and endosperm.

Histochemical GUS assay

Histochemical assays of GUS activity in transgenic lines were performed as described (Jefferson *et al.*, 1987). Whole seedlings were incubated at 37°C in 100 mM sodium phosphate pH 7.5, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM EDTA and 0.1% triton X-100 containing 1 mM 5-bromo-4-chloro-β-D-glucuronide. Incubation times varied from 14–18 h following vacuum infiltration. Sample were then transferred to 70% ethanol to clear the tissue of chlorophyll.

For sections, stained seedlings were infiltrated with a series of ethanol/Histoclear (National Diagnostics)/Paraplast plus (Monoject Scientific) solutions as previously described (Jackson, 1991). Ribbons of 16 μm sections were cut on a rotary microtome, floated on a drop of water on poly-L-lysine coated slides and then dried overnight on a 42°C block. Sections were dewaxed with Histoclear and mounted permanently with Merckoglas (EM Science).

Gravitropic stimulation

R_1 seedlings were grown in square petri dishes (100 mm× 100 mm×15 mm) which were placed vertically to allow growth parallel to the agar surface. Two weeks after plating when the main roots were approximately 3 cm long (with several lateral roots visible), petri dishes were covered with foil and rotated by 90° to induce the gravitropic response. After 12–16 h, stems were marked on the inner curve with indian ink and fixed for 45 min under vacuum in a solution of 0.3% formaldehyde, 10 mM MES, pH 5.6, 0.3 M mannitol. After several washes in 50 mM sodium phosphate, pH 7.0, seedlings were stained overnight as described above.

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