The Plant Cell, Vol. 4, 1371–1382, November 1992

Downstream DNA Sequences Are Required To Activate a Gene Expressed in the Root Cortex of Embryos and Seedlings

Robert A. Dietrich,[a,1] Sharon E. Radke,[b] and John J. Harada[a,2]

[a] Division of Biological Sciences, Section of Botany, University of California, Davis, California 95616
[b] Calgene, Inc., 1920 Fifth Street, Davis, California 95616

We showed previously that a gene, designated *AX92*, which is expressed at an early stage of cortex differentiation in the root apex of oilseed rape seedlings, is also expressed in embryos. To compare *AX92* gene regulation during embryogenesis and postembryonic growth, we constructed a chimeric gene consisting of *AX92* 5′ and 3′ untranslated and flanking regions fused with a β-glucuronidase protein coding region. We showed that the chimeric gene is active in both developing cortex cells in the root apical meristems of transgenic oilseed rape seedlings and in cortex cells at the root end of embryonic axes. To determine whether the *AX92* gene is regulated by a common mechanism in embryos and seedlings, we analyzed the expression of modified chimeric genes. We showed that the *AX92* chimeric gene is regulated combinatorially and that DNA sequences located 3′ of the protein coding region are necessary for its activation in the root cortex of both embryos and seedlings. Our results suggest that common regulatory sequences are required to activate the gene in the embryonic and postembryonic root cortex.

INTRODUCTION

In seed plants, the differentiation of the primary tissue and organ systems is initiated at an early stage of embryonic development and continues postembryonically (Steeves and Sussex, 1989). These processes, however, are interrupted during mid and late embryogeny as the seed matures and enters a period of metabolic quiescence (Dure, 1975; Crouch, 1987; Goldberg et al., 1989). This unique mode of development raises a question about the mechanisms by which higher plants have accommodated this interruption of tissue and organ formation. Are the processes involved in tissue differentiation during embryogenesis and postembryonic growth regulated by a common mechanism? Because similar differentiation events occur in both embryonic and postembryonic tissues, a gene or gene family expressed in a developing tissue may be active in early embryogeny and in postembryonic growth but not during seed maturation or desiccation. The mechanisms by which the discontinuous expression of a gene may be regulated are unknown. A simple view is that there may be one set of regulatory signals and *cis*-acting elements that activate the gene during embryogenesis and a distinct set that operates postembryonically. Alternatively, the same signals and elements may function at both stages.

To begin addressing questions about these regulatory mechanisms, we previously identified an mRNA, designated *AX92*, that is prevalent in the root cortex of oilseed rape (*Brassica napus*) seedlings and mature plants, although it is also detected at low levels in all vegetative organs of the plant (Harada et al., 1988; Dietrich et al., 1989). The cortex is the region of the root and stem that lies between the epidermis and the vascular cylinder and is composed of ground tissue. Localization studies showed that *AX92* mRNA is present in the cortex initials or the immediate derivatives of these cells, indicating that the gene is expressed early during formation of the cortex in the apical meristem of a postembryonic root (Dietrich et al., 1989). *AX92* mRNA is also present in torpedo stage embryos when the embryonic axis elongates at maximal rates, but it is not detected during the later stages of embryogeny. The mRNA's relatively low prevalence has precluded determination of the time course of its accumulation during early embryogeny and its location within an embryo (Harada et al., 1988).

In this study, we used an *AX92* gene as a paradigm to study gene regulation during embryogeny and postgerminative growth in differentiating cortex cells. Using a chimeric gene containing the 5′ and 3′ flanking and untranslated regions of an *AX92* gene fused with the protein coding region of a gene encoding β-glucuronidase (GUS), we showed that GUS activity was localized in the cortex of seedling roots and elongating embryonic axes. Thus, the *AX92* chimeric gene is

[1] Current address: Max Delbrück Laboratorium, Carl van Linné Weg 10, D-5000 Köln 30, Federal Republic of Germany.
[2] To whom correspondence should be addressed.

expressed during both embryogenesis and postembryonic growth. Although this gene is active at an early stage of cortex differentiation in postembryonic root apical meristems, it is not activated until well after the time when the cortex is specified during the heart stage of embryogenesis. Because our results indicated that this *AX92* gene is activated during both embryogenesis and postembryonic growth, we asked further whether the same *cis*-acting elements regulate its spatial activation at both stages of development. Our analyses showed that the gene is regulated combinatorially and that DNA sequences downstream of the protein coding region are required to activate the gene in the embryonic axis and seedling root. The results are consistent with the possibility that the gene is regulated by a common mechanism during both stages of development.

RESULTS

The Activity of an *AX92* Chimeric Gene Qualitatively Reflects the Expression of Endogenous *AX92* Genes in Postgerminative Growth

We analyzed the organization of *AX92* genes to begin studying their regulation during embryogenesis and postembryonic growth. The hybridization of nuclear DNA gel blots with an *AX92* cDNA clone as well as the characterization of genomic clones containing *AX92* genes indicated that there were at least four genes in the haploid oilseed rape genome that could be grouped into two subfamilies based upon their nucleotide sequence similarities (R. A. Dietrich and J. J. Harada, unpublished results). We focused on one *AX92* gene, *AX92-9*, shown diagrammatically in Figure 1, to determine whether the same *AX92* gene was active during both embryogenesis and postembryonic growth. *AX92-9* was selected because its nucleotide sequence is identical to that of a full length *AX92* cDNA clone isolated from a seedling library, indicating that the gene is expressed during postgerminative growth (R. A. Dietrich, J. Z. Zhang, and J. J. Harada, unpublished results).

To monitor the activity of the *AX92* gene in embryos and seedlings, we fused 2.4 kb of *AX92-9* 5′ flanking and untranslated regions and 3.0 kb of the *AX92-9* 3′ region, including the entire 300 bp of 3′ untranslated sequence, with the protein coding region of a gene encoding GUS (see Methods). The resulting chimeric gene, designated *−2400AX/GUS/AX*, is shown schematically in Figure 1. The *−2400AX/GUS/AX* gene was transferred into oilseed rape plants using a transformation vector derived from the Ti plasmid of *Agrobacterium tumefaciens* (Radke et al., 1988, 1992; McBride and Summerfelt, 1990). Twelve transgenic plants that each contained one to four copies of the transgene were chosen for further study (data not shown).

Several results indicated that expression of the *−2400AX/GUS/AX* gene parallels that of the endogenous *AX92* genes in transgenic oilseed rape plants. First, as shown in Figure 2, GUS activity was detected in seedlings grown for 3 days after the start of imbibition (DAI) from each of the 12 plants. Second, primer extension analyses revealed that mRNAs encoded by the transgene and the endogenous *AX92* gene share identical 5′ ends, suggesting that transcription of the chimeric gene was initiated from the *AX92* promoter (data not shown). Third, the time course of GUS activity in transgenic seedlings, shown in Figure 3, was similar to that of *AX92* mRNA accumulation in postgerminative growth (Harada et al., 1988). Although GUS activity in seedlings from different transgenic lines grown for 3 DAI varied quantitatively (Figure 2), the temporal patterns during postgerminative growth in four other transgenic lines tested were identical (data not shown).

Finally, gene products encoded by the *−2400AX/GUS/AX* gene and the endogenous genes were distributed similarly

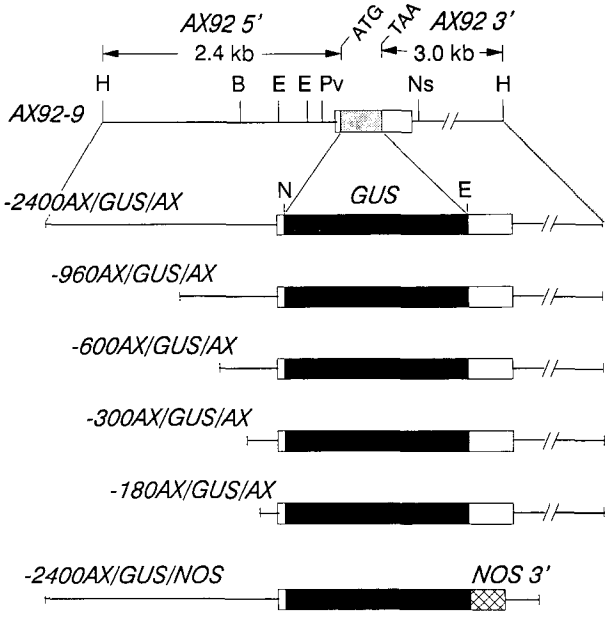

Figure 1. Diagrammatic Representation of the *AX92* Gene and *AX92* Chimeric Genes.

Representative restriction sites in the nuclear DNA fragments containing the *AX92-9* gene are shown. The open boxes represent the 5′ and 3′ untranslated region of the *AX92-9* gene as determined by primer extension and nucleotide sequence analyses, and the shaded box indicates the protein coding region of the gene. The *−2400AX/GUS/AX* chimeric gene was constructed by introducing NcoI and EcoRI restriction sites near the translational initiation and termination codons of *AX92-9*, respectively, and replacing the *AX92* protein coding sequence with a 1.9-kb NcoI-EcoRI fragment containing the GUS protein coding sequence (solid boxes; see Methods). The 5′ deletion constructs with 960, 600, 300, and 180 bp of *AX92* 5′ flanking sequence were generated using the restriction sites shown flanking the *AX92-9* gene. The *−2400AX/GUS/NOS* gene was constructed by replacing the *AX92* 3′ region from *−2400AX/GUS/AX* with a 0.3-kb EcoRI-PstI fragment containing the *NOS* 3′ untranslated and flanking regions (Depicker et al., 1982). B, BamHI; E, EcoRI; H, HindIII; Pv, PvuII; N, NcoI; Ns, NsiI.

in seedlings. Figure 4A shows a representative transgenic seedling grown for 2 DAI that was stained histochemically for GUS activity. Consistent with the distribution of *AX92* mRNA (Dietrich et al., 1989), GUS activity was detected only in the axis of seedlings at 2 DAI; within the axis, the activity was highest in the root apex, excluding the root cap, and at the junction of the hypocotyl and root. No significant qualitative variation in the spatial distribution of GUS activity was observed in seedlings from any of the 12 independently derived transgenic lines. Sections through root tips, such as those illustrated in Figures 4B and 4C, showed that GUS activity was detected consistently in the cortex, including the cortex cell initials or cells immediately derived from the initials in the promeristematic region, and not in the epidermis or root cap. This pattern was similar to the distribution of *AX92* mRNA in the root apical meristem (Dietrich et al., 1989). Additionally, very light staining of procambial cells was observed occasionally in some sections (data not shown). At 4 DAI, GUS activity was present in roots, but the activity was also detected in the lower half of the hypocotyl (Figure 4D). The photomicrographs of hypocotyl sections from seedlings at 4 DAI hybridized in situ with an *AX92* antisense probe, shown in Figures 4E and 4F, verified that the endogenous mRNA was similarly localized. Together, these results suggest that the *cis*-acting DNA sequences that qualitatively regulate *AX92* gene activity in seedlings are located within the flanking and/or untranslated regions present in the *−2400AX/GUS/AX* gene.

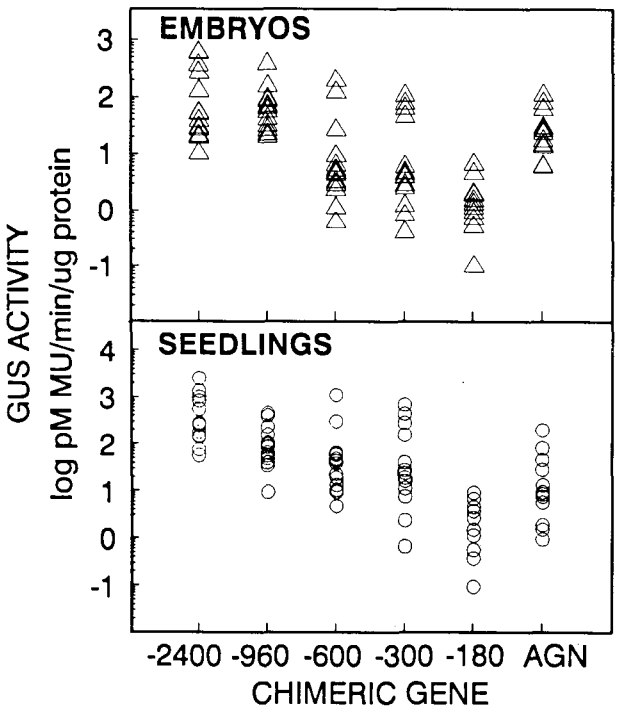

Figure 2. GUS Activities in Transgenic Embryos and Seedlings Containing *AX92* Chimeric Genes.

GUS activity was measured in extracts prepared from embryos harvested at 22 to 25 DPA (triangles) and from seedlings at 3 DAI (circles). Each symbol represents GUS activity in one independently transformed plant line containing an *AX92* chimeric gene. Activities in seedlings were determined by assaying one extract per transgenic plant. For embryos, two to three separate extracts were prepared for each line, with the average GUS activity value shown. 5′ deletions are listed by the size of *AX92* 5′ flanking region in the construct. *AGN*, *−2400AX/GUS/NOS*; MU, 4-methyl umbelliferone.

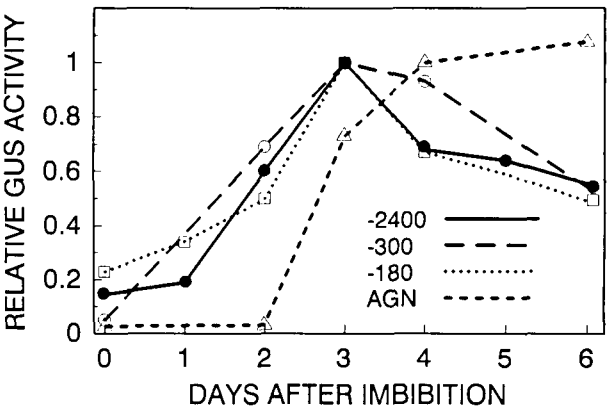

Figure 3. Time Course of *AX92* Chimeric Gene Activation during Postgerminative Growth.

GUS activity was measured in extracts prepared from dry seeds and seedlings grown for 1 to 6 DAI from transgenic plant lines containing either the *−2400AX/GUS/AX* (filled circles), *−300AX/GUS/AX* (open circles), *−180AX/GUS/AX* (squares), or *−2400AX/GUS/NOS* (triangles) gene. To compare the time courses, the data are presented as percentage of maximum GUS activity because the actual values varied greatly between lines.

We showed previously that *AX92* mRNA is detected in all organs of the mature plant, although at lower levels than that present in roots (Dietrich et al., 1989). Consistent with this result, we found that GUS activity was detected in the margins of the young leaves of transgenic plants containing the *AX92* chimeric gene (data not shown). Thus, *AX92* gene activity is not limited to the cortex; the gene may also be active in the ground tissue in other plant organs.

Activation of the *AX92* Chimeric Gene during Embryogeny

Because *AX92* is encoded by a gene family, we asked whether the same chimeric gene was active during embryogeny. GUS activity was quantified in pools of T_1 embryos harvested at 23 days postanthesis (DPA), the stage at which *AX92* mRNA is present at maximal levels during embryogeny (Harada et al., 1988), from each of 12 transgenic plants containing the *−2400AX/GUS/AX* gene. As shown in Figure 2, the results indicated that the *AX92* promoter which was active in postgermination was also active during embryogenesis.

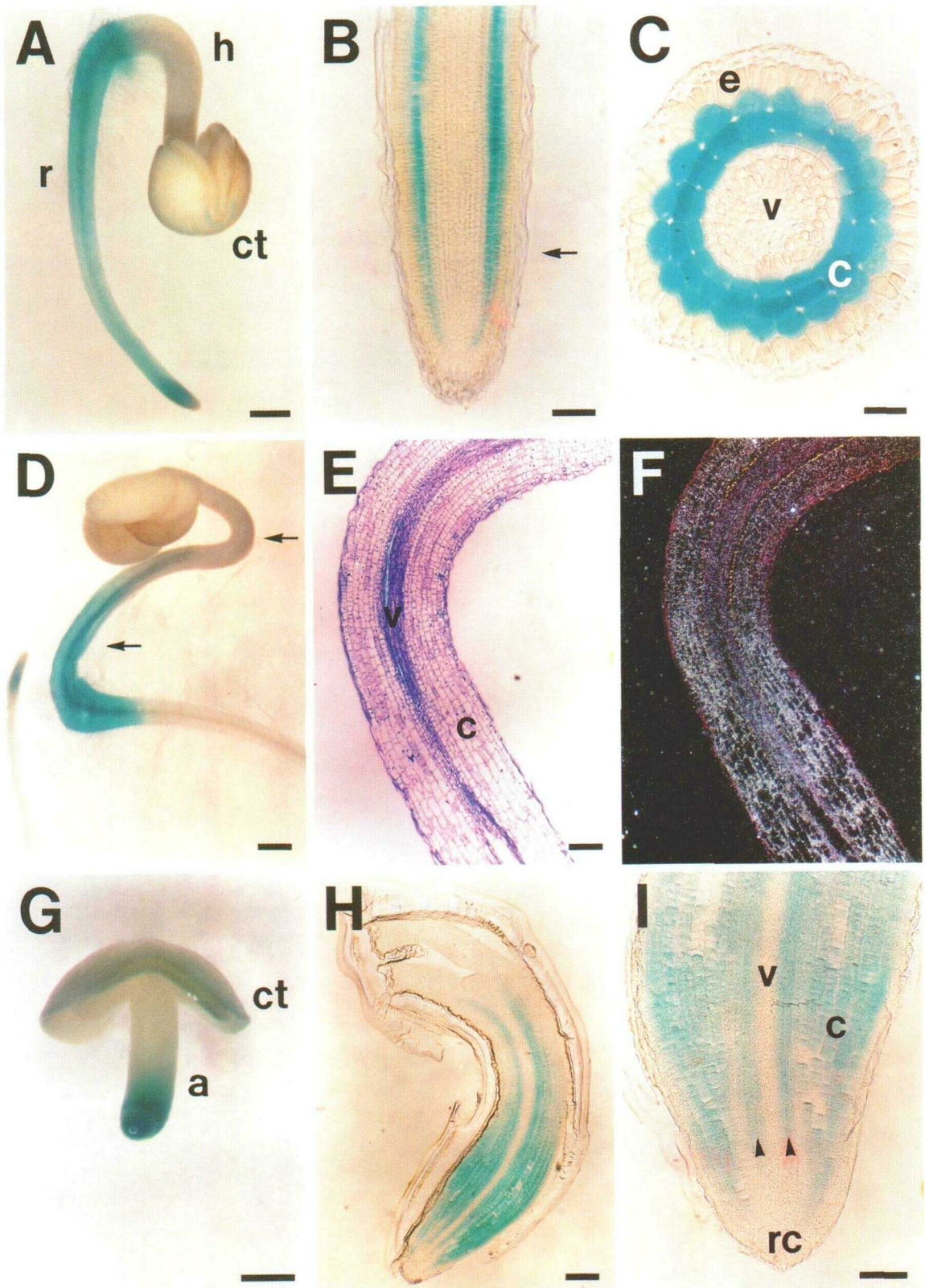

Figure 4. Localization of GUS Activity and Endogenous *AX92* mRNA in Seedlings and Embryos Containing the *AX92* Chimeric Genes.

To localize *AX92* gene activity, we isolated embryos at 23 DPA and stained them histochemically. Figures 4G, 4H, and 4I show that, similar to seedlings, GUS activity was detected in the axes of the embryos, predominantly in the cortex at the root end of the axis and also in files of procambial cells. The embryonic cortex was stained consistently in all transgenic lines. Thus, the *−2400AX/GUS/AX* gene contains the DNA sequences that promote its activation in the cortex during embryogenesis as well as postembryonic growth.

Because the *AX92* gene was activated at an early stage of cortex differentiation in the apex of postembryonic roots (Figure 4B), we asked when the gene was first activated during embryogenesis. GUS activity was monitored in embryos starting at the early heart stage at 12 DPA and continuing through to dry seeds. Figure 5A shows that GUS activity was first detected in quantitative assays at 18 to 20 DPA, and Figure 5B shows that this activity in situ was limited to the root end of the embryonic axis. The activity continued to increase until about 28 to 30 DPA when the activity reached a plateau (Figure 5A). Because the GUS enzyme is thought to be stable in plant tissues (Jefferson et al., 1987), the leveling off of GUS activity in mid and late embryogeny may indicate that accumulation of the chimeric mRNA begins to decrease by ~28 DPA. Similar time courses were obtained with two other transgenic lines that were analyzed (data not shown). These results indicate that *AX92* gene activity is detected initially in torpedo stage embryos, well past the time when the cortex is first distinguished morphologically in the embryonic axis (Tykarska, 1979).

Effects of Deletions in *AX92* 5′ Flanking Regions on Expression of the Chimeric Gene

Having shown that the same *AX92* gene is active in the root cortex during embryogenesis and postgerminative growth, we next asked whether the same *cis*-acting elements regulate the gene at both stages of development. To begin localizing these regulatory elements, derivatives of the *−2400AX/GUS/AX* gene that contain 960, 600, 300, and 180 bp of *AX92* 5′ flanking region, shown diagrammatically in Figure 1, were transferred into oilseed rape plants.

Gene Activation during Embryogeny

The effects of the deletions on *AX92* gene activity were assessed by measuring GUS activities in extracts of embryos collected at 23 DPA. As shown in Figure 2, GUS activity was detected in embryos containing each of the deletion constructs. In general, the activities decreased in proportion to the length of the 5′ flanking region, although there was substantial variation in activities measured among the 10 to 12 independently derived plants containing a given construct.

The removal of 5′ flanking regions affected quantitative levels of *AX92* chimeric gene activity, yet the deletions had limited effects on the temporal and spatial expression patterns. Figure 5 shows that, aside from the differences in GUS specific activities, the activity time courses during embryogeny were virtually identical for plants containing the *−2400AX/GUS/AX*, *−300AX/GUS/AX*, and *−180AX/GUS/AX* genes. Furthermore, all of these transgenic embryos with detectable GUS activity showed staining patterns that were virtually identical to that shown for an embryo containing the *−2400AX/GUS/AX* gene in Figure 6A. For example, Figure 6B shows a representative embryo containing the *−180AX/GUS/AX* gene in which GUS activity was detected exclusively in the root end of the embryonic axis. These results were verified by showing that GUS activity was consistently higher in extracts of axes dissected from embryos containing each of the deletion constructs that were harvested at 22 to 25 DPA than in cotyledon extracts (data

Figure 4. (continued).

GUS activity was localized histochemically in seedlings and embryos containing the *−2400AX/GUS/AX* gene. *AX92* mRNA was localized in hypocotyls from seedlings at 4 DAI using in situ hybridization protocols.

(A) Seedling at 2 DAI. The blue precipitate shows that GUS activity is localized primarily in the root although not in the root cap. Bar = 1 mm.

(B) Longitudinal section through the root tip of a seedling at 2 DAI. GUS activity is localized in the cortex including cortex cells in the promeristematic region. The arrow shows the relative location of the cross-section shown in **(C)**. Bar = 100 μm.

(C) Cross-section of a root tip from a seedling at 2 DAI shows that GUS activity is localized primarily in the cortex. The halo surrounding the epidermis is the glue used to attach the section to the vibratome. Bar = 50 μm.

(D) Seedling at 3 DAI. GUS activity is detected in both the root tip excluding the root cap (located to the left of the hypocotyl) and the lower portion of the hypocotyl. The two arrows delimit the approximate region of the hypocotyl shown in **(E)** and **(F)**. Bar = 1 mm.

(E) Light-field photomicrograph of a hypocotyl section from a seedling at 4 DAI used for in situ hybridization analysis. Bar = 200 μm.

(F) Dark-field micrograph of the section in **(E)** hybridized with an *AX92* antisense-strand RNA probe labeled with [^{35}S]. The silver grains (white dots) indicate that *AX92* mRNA is localized in the basal half of the hypocotyl. No hybridization was detected in a similar section hybridized with an *AX92* sense-strand RNA probe (data not shown).

(G) Embryo at 23 DPA. GUS activity is highest in the root end of the embryonic axis. Staining at the margins of the cotyledons was not observed consistently. Bar = 1 mm.

(H) Longitudinal section through the axis of an embryo at 23 DPA. GUS activity is localized in the embryonic cortex and in files of procambial cells. Bar = 200 μm.

(I) Longitudinal section through the tip of the embryonic axis at 23 DPA. GUS activity is detected in the embryonic cortical cells in the promeristematic region and in procambial cells slightly back from the tip (indicated by arrowheads). Bar = 100 μm.

a, axis; c, embryonic or mature cortex; ct, cotyledon; e, epidermis; h, hypocotyl; r, root; rc, embryonic root cap; v, developing vascular cylinder.

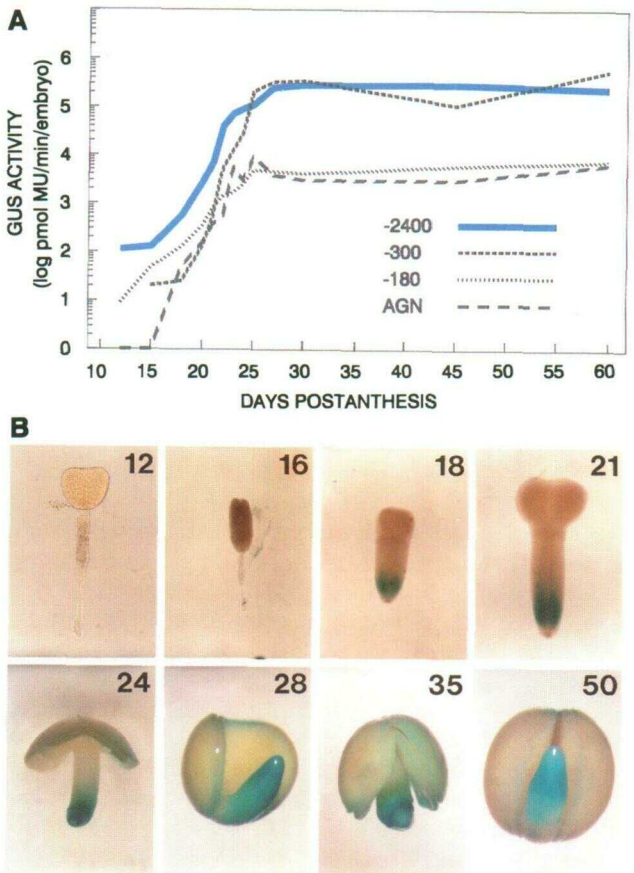

Figure 5. Time Course of *AX92* Chimeric Gene Activity during Embryogeny.

(A) *Quantitation of GUS activity in embryos containing −2400AX/GUS/AX, −300AX/GUS/AX, −180AX/GUS/AX,* and *−2400AX/GUS/NOS (AGN)* genes between 12 DPA (early heart stage) and 60 DPA (mature seed). For these experiments, T_2 embryos from T_1 plants homozygous for the transgenes were used to maximize the detection of GUS activity.
(B) Histochemical localization of GUS activity in embryos containing the *−2400AX/GUS/AX* gene at different stages of embryogeny. Numbers indicate days postanthesis.
MU, 4-methyl umbelliferone.

not shown). Thus, at this level of resolution, sequences upstream of position −180 in the *AX92* upstream region do not appear to be necessary to activate the gene in the embryonic axis.

Gene Activation during Postgermination

We analyzed transgenic seedlings containing the deletion constructs to compare the effects of the 5′ deletions on *AX92* gene activity during postembryonic growth. As with embryos, GUS activity in seedlings containing the deletion constructs generally increased with increasing length of the 5′ flanking region (Figure 2). Furthermore, Figure 3 shows that the time courses of GUS activity were similar for seedlings containing the *−2400AX/GUS/AX*, *−300AX/GUS/AX*, and *−180AX/GUS/AX* constructs. We showed that GUS activity was distributed similarly in all transgenic seedings at 2 DAI that possessed detectable activity, regardless of the length of the 5′ flanking region. Figures 6D and 6E show that representative seedlings with a *−2400AX/GUS/AX* and a *−180AX/GUS/AX* gene, respectively, stained similarly, although with different intensities. Although GUS activity was not detectable in root cross-sections from seedlings with the *−180AX/GUS/AX* gene, Figure 6H shows that GUS activity was limited to the cortex in a root cross-section from a seedling with a *−300AX/GUS/AX* gene, a pattern qualitatively identical to that of seedlings with a *−2400AX/GUS/AX* gene (Figure 6G). Similar to embryos, 180 bp of *AX92* 5′ flanking sequences appear to be sufficient to direct the correct qualitative activity of the chimeric gene in seedlings at 2 DAI.

By contrast, sequences further upstream of position −180 were necessary for the proper regulation of the gene in seedlings at 3 DAI. The *−2400AX/GUS/AX* gene (Figure 6J) as well as the endogenous *AX92* genes (Figure 4F) are expressed in seedlings at 3 DAI in the basal region of the hypocotyl in addition to the root. However, GUS activity was not detected in the basal hypocotyl in seedlings containing the *−960AX/GUS/AX* gene even though the activity remained conspicuous in the root, as shown by the staining of a representative seedling in Figure 6K. Identical results were obtained with seedlings containing chimeric genes with 600, 300, and 180 bp of 5′ flanking region (data not shown). Whereas all of the gene constructs including those containing only 180 bp of 5′ flanking region were expressed in embryonic axes and seedling roots, only the *−2400AX/GUS/AX* gene was expressed at detectable levels in the basal hypocotyl of seedlings at 3 DAI. As shown in Figures 7A and 7B, the histochemical results were verified by quantifying GUS activities in cotyledon, hypocotyl, and root extracts of seedlings containing each of the deletion derivatives. Only seedlings containing the *−2400AX/GUS/AX* gene at 3 DAI showed a significant increase in GUS activity in hypocotyl extracts. Together, the results indicate that DNA sequences downstream of −180 and/or in 3′ regions of the gene are sufficient to direct expression in embryonic axes and seedling roots, whereas those upstream of −960 are required for activation in seedling hypocotyls.

Modification of the 3′ Regions of the *AX92* Chimeric Gene Results in Striking Changes in Gene Activation Patterns

To determine whether sequences downstream of the *AX92* protein coding region play a role in regulating its activity, we

modified the *−2400AX/GUS/AX* construct by replacing the 3.0 kb of *AX92* 3′ untranslated and flanking regions with the corresponding regions from the nopaline synthase (*NOS*) gene (Depicker et al., 1982) and designated the construct *−2400AX/GUS/NOS* (Figure 1). Analyses of 12 transgenic plants containing one to three copies of this gene indicated that the 3′ modification affected the quantitative and qualitative expression of the chimeric gene during both embryogenesis and postgerminative growth.

Gene Activation during Embryogeny

GUS activities in embryos at 23 DPA containing the *−2400AX/GUS/NOS* gene were, on average, only slightly reduced relative to those with the *−2400AX/GUS/AX* gene (Figure 2), and the qualitative time courses of GUS activity were similar (Figure 5). The spatial distribution of GUS activity in embryos, however, was altered dramatically. In contrast to chimeric genes containing *AX92* 3′ regions (Figures 6A and 6B), Figure 6C shows that GUS activity in embryos containing the *−2400AX/GUS/NOS* gene was not detected in the axes of embryos at 23 DPA, but rather, was occasionally observed at low levels in the cotyledons after prolonged staining. Quantitative assays showed that GUS activity was, on average, threefold higher in cotyledon extracts of 22 to 25 DPA embryos containing the *−2400AX/GUS/NOS* gene than in axis extracts (data not shown). By comparison, GUS activity was approximately sixfold higher in the axes of embryos with *−2400AX/GUS/AX* genes than in cotyledons. Detection of GUS activity in cotyledons of embryos with the *−2400AX/GUS/NOS* gene probably resulted from prolonged staining of the embryos as the activities in these organs were comparable in embryos containing the two different constructs. The 3′ untranslated and/or flanking regions appear to contain DNA sequences required for the activation of the *AX92* gene in the embryonic axis.

Gene Activation during Postgermination

We analyzed seedlings containing the *−2400AX/GUS/NOS* gene to determine whether the 3′ end modification also affects *AX92* gene activity during postgermination. GUS activities were only slightly depressed in seedlings containing the *−2400AX/GUS/NOS* gene relative to those with the *−2400AX/GUS/AX* gene. However, consistent with the results obtained with embryos, GUS activity was not detected histochemically in the roots of seedlings at 2 DAI, including the cortex (Figures 6F and 6I). Figure 6L shows that the GUS activity present in seedlings at 3 DAI was localized primarily in the lower hypocotyl and was not detected in the root. As shown in Figures 7A and 7B, a significant increase in GUS activity was observed only in hypocotyl extracts of seedlings grown for 3 DAI containing the *−2400AX/GUS/NOS* gene. The absence of detectable GUS activity in roots was further indicated by the delay in the activation of the *−2400AX/GUS/NOS* gene during postgerminative growth until the gene was activated in the hypocotyl at 3 DAI (Figure 3).

Thus, replacement of the *AX92* 3′ regions with the *NOS* terminator resulted in a dramatic change in the spatial distribution of GUS activity both in embryos and seedlings. In general, the effect was specific for the root; GUS activities in cotyledons and hypocotyls were not affected significantly. The results suggest that the *AX92* 3′ region contains DNA sequences that are required for enhanced expression of the gene in the embryonic axis and the seedling root.

DISCUSSION

The Same *AX92* Gene Is Activated in Both Embryogenesis and Postembryonic Growth

We used a chimeric *AX92* gene to study the expression of genes in differentiating cortex cells during embryogenesis and postembryonic growth. Because AX92 is encoded by a small gene family, we first asked whether the same gene was activated during the two stages of the life cycle. A GUS reporter gene was used to provide the sensitivity needed to monitor *AX92* gene activity in embryos. Our previous results showed that *AX92* genes are regulated primarily at the transcriptional level (Comai and Harada, 1990).

The temporal and spatial expression of the chimeric gene parallels the accumulation of *AX92* mRNA during postgerminative growth (Figures 3 and 4). The results suggest that the activity of the chimeric gene reflects *AX92* gene expression and that sequences located within the 2.4-kb *AX92* 5′ region and/or the 3.0-kb *AX92* 3′ region present in *−2400AX/GUS/AX* are responsible for the qualitative regulation of the gene in seedlings. In determining whether the same *AX92* gene is activated during embryogenesis, we showed that GUS activity is detected in embryos, specifically in the embryonic cortex in the root end of the axis and perhaps in other tissues (Figure 4). Thus, the same *AX92* gene is active transcriptionally in both embryos and seedlings. Other experiments showing that all four *AX92* genes hybridize at a high criterion with mRNAs isolated both from seedlings grown for 3 DAI and from embryos at 23 DPA suggest that each gene is expressed at both stages (data not shown). A homolog of *AX92* mRNA has also been detected in carrot somatic embryos, although its spatial expression pattern is not known (Aleith and Richter, 1990).

We note that GUS activity is detected in other organs of plants containing the *−2400AX/GUS/AX* gene. This result is consistent with our previous findings that *AX92* mRNA is present in all vegetative organs of mature plants including leaves (Dietrich et al., 1989). Thus, *AX92* genes are not expressed exclusively in the cortex, but they may be active in a subset of ground tissues.

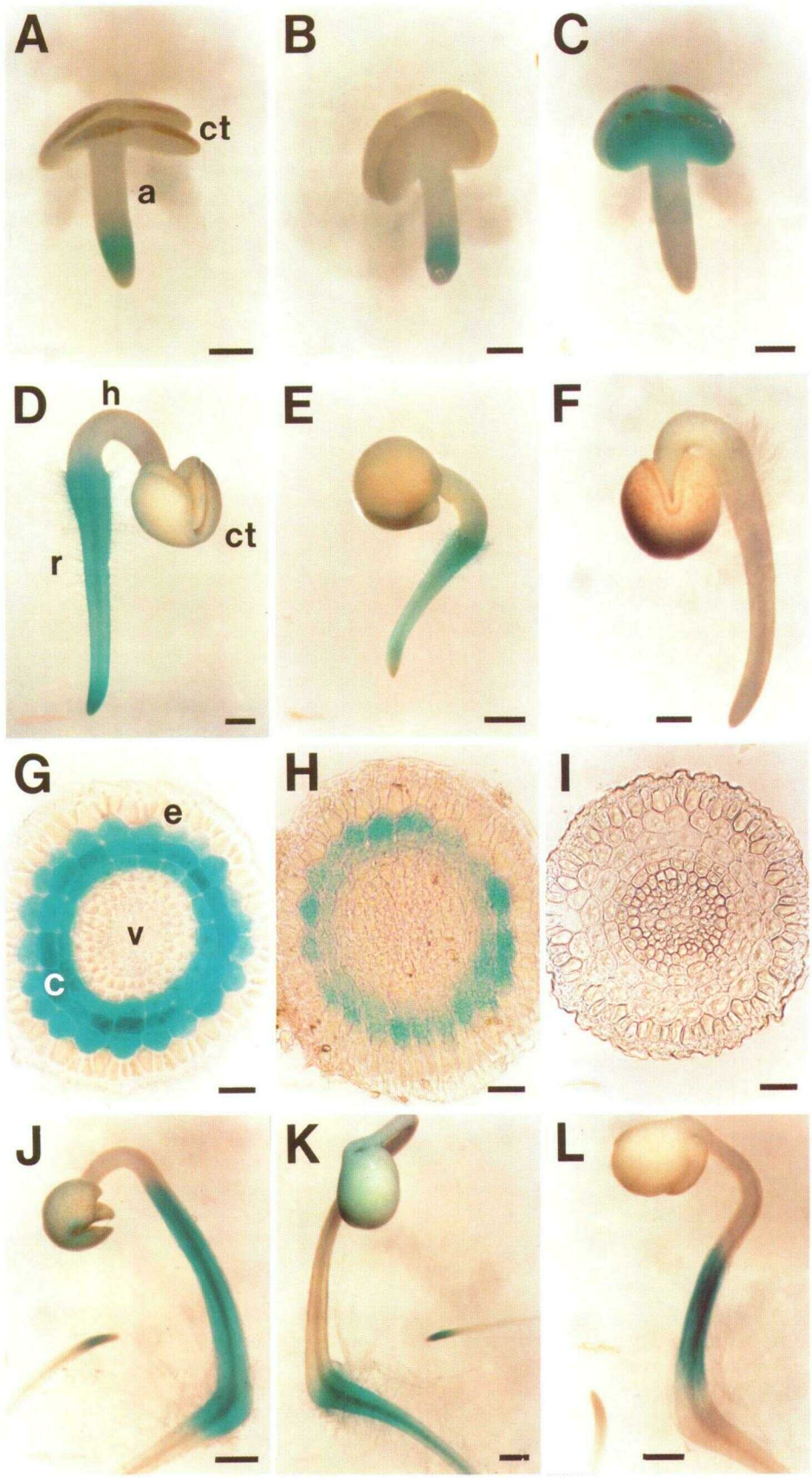

Figure 6. Localization of GUS Activity in Transgenic *B. napus* Embryos and Seedlings.
The blue precipitate shows the location of GUS activity.

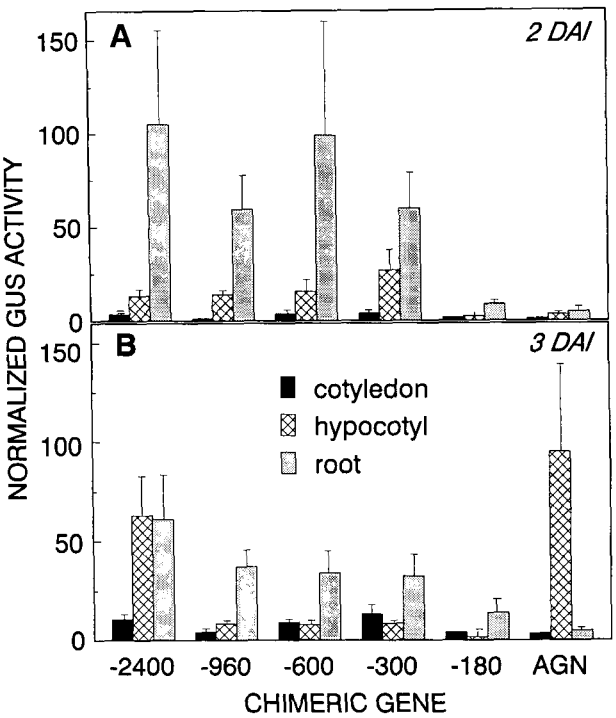

Figure 7. *AX92* Gene Activity in Cotyledons, Hypocotyls, and Roots of Transgenic Seedlings at 2 and 3 DAI.

GUS activity was measured in seedling extracts prepared from hand-dissected cotyledons, hypocotyls, and roots from five independently derived plant lines containing the *−2400AX/GUS/AX*, *−960AX/GUS/AX*, or *−2400AX/GUS/NOS* (*AGN*) genes and in three lines containing the *−600AX/GUS/AX*, *−300AX/GUS/AX*, or *−180AX/GUS/AX* genes. To normalize for quantitative variations, GUS activity was divided by the activity present in dry seeds from the same line. The average normalized activities and standard errors are shown.
(A) Seedlings at 2 DAI.
(B) Seedlings at 3 DAI.

The *AX92* Gene Is Activated Late in Embryo Development

Because the expression of *AX92* genes correlates with the differentiation of cortex cells in the apical meristems of postembryonic roots (Figure 4; Dietrich et al., 1989), we asked whether activation of the gene coincided with the morphological specification of the embryonic cortex. The cortex initial cells of oilseed rape embryos can be identified in a globular stage embryo at ~7 DPA, and the organization of the embryonic cortex is clearly distinguished in heart stage embryos by ~11 to 12 DPA (Tykarska, 1976, 1979, 1980). Our previous studies showed only that *AX92* mRNA is present in embryos by 18 DPA, the earliest stage studied (Harada et al., 1988).

In this study, we showed that activation of the *AX92* chimeric gene was not detected until 18 to 20 DPA in torpedo stage embryos, well after the time when the embryonic cortex is first organized in an early heart stage embryo (Figure 5; Tykarska, 1979). Ultrastructural studies of embryos from *Capsella bursa-pastoris* and *Arabidopsis thaliana* have generally been interpreted to indicate that cortex differentiation is initiated during the transition from the globular to the heart stage (Schulz and Jensen, 1968; Mansfield and Briarty, 1991). For example, in heart stage embryos, the cells of the embryonic cortex and procambium are more vacuolated than those of the protoderm, and plastids in the protoderm and cortex are more highly differentiated than those in the procambium. Thus, *AX92* gene activation is not coupled to the specification of cortex cells during embryogenesis. Other probes are needed to further compare cortex cells present in heart stage embryos and in the promeristematic regions of postembryonic roots.

Combinatorial Regulation of *AX92* Gene Activity

As summarized in Table 1, we have shown that a combination of *cis*-acting DNA sequences in the 5′ and 3′ flanking and/or untranslated regions influence *AX92* gene activity in embryos and seedlings. In particular, two regions appear to affect the gene's spatial activation.

DNA sequences needed to activate the *AX92* gene in the seedling hypocotyl at 3 DAI are located between positions −2400 and −960 in the *AX92* 5′ flanking region (Table 1; Figures 6 and 7). Chimeric gene expression was observed in seedlings with either of the two constructs containing this region, *−2400AX/GUS/AX* and *−2400AX/GUS/NOS*, but not in seedlings with constructs in which this region was absent (Figures 4, 6, and 7). Other sequences that appear to influence

Figure 6. (continued).

(A) to **(C)** Embryos at 23 DPA. GUS activity is observed in the root end of the axis in embryos containing the *−2400AX/GUS/AX* **(A)** and *−180AX/GUS/AX* **(B)** genes. In embryos with the *−2400AX/GUS/NOS* gene **(C)**, GUS activity is detected in the cotyledons after prolonged incubation. Bars = 500 μm.
(D) to **(F)** Seedlings at 2 DAI. In seedlings containing the *−2400AX/GUS/AX* **(D)** and *−180AX/GUS/AX* genes **(E)**, GUS activity is detected in the root. No activity is detected in seedlings containing the *−2400AX/GUS/NOS* gene **(F)**. Bars = 1 mm.
(G) to **(I)** Cross-section of root tips from seedlings at 2 DAI. In sections from seedlings containing the *−2400AX/GUS/AX* **(G)** and *−300AX/GUS/AX* **(H)** genes, GUS activity is limited to the cortex cells. No activity is detected in sections from seedlings with the *−2400AX/GUS/NOS* gene **(I)**. Bars = 50 μm.
(J) to **(L)** Seedlings at 3 DAI. In seedlings containing the *−2400AX/GUS/AX* gene **(J)**, GUS activity is observed in the root (root tip is located to the left of the hypocotyl) and lower hypocotyl. In seedlings with the *−960AX/GUS/AX* gene **(K)**, GUS activity is detected in the root (tip is located to the right of the hypocotyl) but not the hypocotyl, and in seedlings containing the *−2400AX/GUS/NOS* gene **(L)**, GUS activity is detected in the hypocotyl but not in the root (tip is located to the left of the hypocotyl). Bars = 1 mm.
a, axis; c, cortex; ct, cotyledon; e, epidermis; h, hypocotyl; r, root; v, developing vascular cylinder.

Table 1. Summary of *AX92* Chimeric Gene Activity

Construct	GUS Activity Prevalent in: Embryonic Axes	Seedling Roots	Seedling Hypocotyls
−2400AX/GUS/AX	+	+	+
−960, −600, −300, and *−180AX/GUS/AX*	+	+	−
−2400AX/GUS/NOS	−	−	+

expression in the hypocotyl have been described (Bustos et al., 1991).

Several results support a surprising finding of this study—that DNA sequences located 3′ of the *AX92* protein coding region appear to be necessary to activate the *AX92* gene in the cortex of the embryonic axis and the root apex. First, expression of the *−180AX/GUS/AX* gene in the roots of embryos and seedlings indicates that sequences upstream of position −180 in the *AX92* 5′ flanking region are not required to activate the gene (Table 1; Figures 6 and 7). Rather, the upstream region appears to contain sequences that influence the quantitative level of *AX92* gene activity as well as its activity in other organs (Table 1; Figures 4 and 6).

Second, the activity of the *−2400AX/GUS/NOS* gene, which includes the entire 2400 bp of *AX92* 5′ flanking and untranslated regions but not the *AX92* 3′ region, is severely reduced in embryonic axes and seedling roots (Table 1; Figures 6 and 7). GUS activities in axes of embryos and seedlings containing the *−2400AX/GUS/NOS* gene are, on average, 2% of the activity measured in those with the *−2400AX/GUS/AX* gene. It is unlikely that the *NOS* 3′ region represses chimeric gene expression in roots because this terminator has been used in other GUS chimeric genes expressed in this organ (Ohl et al., 1990; Yamamoto et al., 1991). Furthermore, the 3′ end modification did not change the transcription start site; the 5′ ends of mRNAs encoded by the *−2400AX/GUS/NOS*, *−2400AX/GUS/AX*, *−300AX/GUS/AX*, and endogenous *AX92* genes are identical (data not shown).

Third, the reduced expression of the *−2400AX/GUS/NOS* gene in roots does not result from a modification of the 3′ end that renders the gene nonfunctional. Normalized GUS activities in embryonic cotyledons at 23 DPA and in hypocotyls of seedlings at 3 DAI were comparable to those present in plants containing the *−2400AX/GUS/AX* gene (Figure 7). The simplest model consistent with these results is that the DNA sequences responsible for root cortex expression are located in the 300 bp of *AX92* 3′ untranslated region and/or the 3′ flanking region. At present, we cannot eliminate the possibility that other *cis*-acting elements between −180 and the translational initiation codon are necessary but not sufficient to activate the gene in roots (Figure 1).

Because the *−2400AX/GUS/AX* construct contains 3′ untranslated regions from the *AX92* gene, it is currently unclear whether the regulatory sequences downstream of the protein coding region affect gene activity at the transcriptional or post-transcriptional level. The 3′ untranslated region has been shown to modulate gene expression post-transcriptionally by affecting mRNA stability or the rate of translation (Ch'ng et al., 1990; Jackson and Standart, 1990; Munroe and Jacobson, 1990). Transcriptional enhancers have been identified in the 3′ flanking regions of animal and plant genes (Choi and Engel, 1986; Fischer and Maniatis, 1986; Dean et al., 1989).

Regulation of the *AX92* Gene during Embryogenesis and Postembryonic Growth

In lower vascular plants, there is neither a distinct end to embryogenesis nor a definite beginning to postembryonic growth (Steeves and Sussex, 1989). By contrast, relative to tissue and organ formation, the sporophytic life cycle of a higher plant is interrupted during the stages of embryogeny that lead to the production of a quiescent seed. As a consequence, the differentiation of the primary tissue and organ systems of a seed plant is a discontinuous process that is initiated during embryogenesis, interrupted during seed maturation and desiccation, and reinitiated during and following germination. We have asked whether a gene expressed primarily in the root cortex of embryos and seedlings is regulated by identical mechanisms during embryogenesis and postembryonic growth. Our results show that a modification of the 3′ untranslated and flanking region affects the activation of the *AX92* chimeric gene in the root cortex of both embryos and seedlings. Although additional studies are needed to more precisely define the elements involved in regulating *AX92* gene activity, our results suggest that the same DNA sequences may control *AX92* gene activation during embryogenesis and postembryonic growth.

METHODS

Plant Material

Oilseed rape (*Brassica napus* cv *Westar*) was used in the plant transformation experiments. *B. napus* (rapid cycling population, CRGC 5) was the source of material for in situ hybridization experiments. Plants were grown in the greenhouse under standard conditions. Embryos were staged according to morphological criteria (Harada et al., 1988).

Isolation of *AX92* Genes

Genomic clones containing *AX92* genes were isolated from a library of oilseed rape nuclear DNA using an *AX92* cDNA clone (Harada et al., 1988; Comai et al., 1992). Twenty genomic clones corresponding

to four *AX92* genes were identified and characterized. Nuclear DNA gel blot experiments suggest that the four genes represent all of the *AX92* genes in the oilseed rape genome.

Nucleic Acid Sequencing

Nucleotide sequences were determined by the dideoxynucleotide chain termination method using double-stranded plasmid templates (Sanger et al., 1977; Chen and Seeburg, 1985).

Chimeric Gene Construction

Restriction sites were introduced into the *AX92-9* gene using oligonucleotide-directed in vitro mutagenesis (Amersham Corp.). An NcoI restriction site was created at the translation start codon using the 22-base oligonucleotide 5′-GAAGCCATGG*TTTGTTTTGAAG-3′ to change the base located 2 bases upstream of the translational initiation codon from A to C*. An EcoRI restriction site was generated using the 24-base oligonucleotide 5′-TTAGCACTGAAT*T*CCAGGAGGAAC-3′ to change two adjacent bases from CT to A*A* located 9 and 10 bases upstream from the translation stop codon. Mutations were confirmed by nucleotide sequence analysis.

The 3.0-kb EcoRI-PstI fragment from the 3′ end of the mutated *AX92-9* gene and the 5.4-kb NcoI-PstI fragment containing 2.4-kb of *AX92-9* 5′ region and vector DNA were ligated with the 1.9-kb NcoI-EcoRI fragment from pRAJ275 containing the β-glucuronidase (GUS) protein coding sequence (Jefferson, 1987). The nucleotide sequences of the junctions between *AX92* and GUS regions were checked to ensure that changes had not been introduced in the cloning procedures. The 5′ deletion series was made from the *−2400AX/GUS/AX* gene using the restriction sites in the *AX92* 5′ flanking sequence shown in Figure 1. The *−2400AX/GUS/NOS* (*NOS*; nopaline synthase) construct was made by replacing the 3.0-kb EcoRI-PstI *AX92* 3′ fragment with a 0.3-kb EcoRI-PstI fragment from pMON316 (Sanders et al., 1987), which contains the *NOS* termination sequence (Depicker et al., 1982). The gene constructs were transferred into the binary T-DNA vector pCGN1557 and mobilized into *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986; McBride and Summerfelt, 1990).

Plant Transformation

Oilseed rape was transformed following the protocol of Radke et al. (1988) with modifications (1992).

Analysis of Transgenic Plants

Each transgenic plant used in this study contained one to four copies of the transgene, at least one of which was not rearranged detectably as determined by DNA gel blot hybridization experiments. T_1 embryos were harvested from selfed primary transformants and staged as described previously (Harada et al., 1988). Seeds were germinated on moist filter paper in Petri dishes in the dark at 25°C. For most fluorometric assays, extracts from 20 pooled siblings of T_1 embryos or seedlings were analyzed. Where T_2 embryos were used, they were obtained from selfed T_1 plants, which showed no segregation of the transgene in their progeny. For the experiment shown in Figure 5, 40 to 70 T_2 embryos were pooled to obtain sufficient material from early heart to early torpedo stage embryos.

GUS Activity Assays

Chimeric gene expression was measured in two ways. Histochemical assays were done on embryos and seedlings to determine the spatial distribution of GUS activity in the plant, and fluorometric assays were done on tissue extracts to quantify activities (Jefferson, 1987).

For the fluorometric assays, embryos, seeds, or whole seedlings, in all cases with the seed coat removed, were pooled, homogenized in GUS extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM DTT, 1.0 mM EDTA, 0.1% Sarkosyl, 0.1% Triton X-100), and centrifuged for 10 min in a microcentrifuge. An aliquot of the supernatant was assayed for GUS activity in the extraction buffer containing 1 mM 4-methyl umbelliferyl β-D-glucuronide and methanol added to a final concentration of 20% (v/v). The reactions were incubated at 37°C, aliquots were removed after 10, 36, and 72 min, and diluted 1:10 in 0.2 M Na_2CO_3. Fluorescence was measured on a minifluorometer (model No. TKO 100; Hoefer, San Francisco, CA) (excitation wavelength = 365 nm, photodetector wavelength = 460 nm). Protein content was determined using a protein assay kit (BCA; Pierce Chemical Co.). To reduce interference in the protein assay caused by DTT in the GUS extraction buffer, samples were preincubated with an equal volume of 0.1 M iodoacetamide in 0.1 mM Tris buffer, pH 8, for 20 min at 37°C (Hill and Straka, 1988). BSA was used as the standard.

GUS activity was detected histochemically by submerging embryos or seedlings in GUS histochemical buffer (100 mM $NaPO_4$, 0.5 mM $K_3[Fe(CN_6)]$, 0.5 mM $K_4[Fe(CN_6)]$, 10 mM EDTA, 1 mg/mL 5-bromo-4-chloro-3-indoyl glucuronide (X-gluc), 20% methanol (v/v)) in vacuo for 10 min, followed by incubation at 37°C until color developed (1 to 16 hr). Tissue sections of fresh plant material were obtained using a vibratome (Technical Products International, St. Louis, MO). Sections 100 to 200 μm thick were stained for GUS activity, fixed with 10% formalin/5% acetic acid/50% ethanol, and mounted on microscope slides. Photomicroscopy was done on a dissecting photomicroscope (model No. MC-63; Zeiss, Oberkochen, Federal Republic of Germany) and a photomicroscope (Labophot; Nikon, Garden City, NY) using color film (Kodacolor Gold 100; Kodak).

Methanol was added to histochemical and fluorometric GUS assays to reduce background caused by endogenous GUS activity in the plant material (Kosugi et al., 1990).

In Situ Hybridization

Experiments were done as described in Dietrich et al. (1989).

ACKNOWLEDGMENTS

We thank Cathy Baden, Eric Holst, Kelly Matsudaira, and Lyn Raffety for excellent technical assistance; Drs. Bob Fischer and Donna Fernandez, and members of our lab for discussions about the manuscript; Dr. Chuck Gasser for use of the vibratome; and Drs. Tom Rost and Ernie Gifford for their advice and assistance. R.A.D. was supported, in part, by a fellowship from the McKnight Foundation. This research

was supported in part by a grant from the National Science Foundation (to J.J.H.).

Received September 10, 1992; accepted September 30, 1992.

REFERENCES

Aleith, F., and Richter, G. (1990). Gene expression during induction of somatic embryogenesis in carrot cell suspensions. Planta **183,** 17–24.

Bustos, M.M., Begum, D., Kalkan, F.A., Battraw, M.J., and Hall, T.C. (1991). Positive and negative *cis*-acting DNA domains are required for spatial and temporal regulation of gene expression by a seed storage protein promoter. EMBO J. **10,** 1469–1479.

Chen, E.Y., and Seeburg, P.H. (1985). Supercoil sequencing: A fast and simple method for sequencing plasmid DNA. DNA (NY) **4,** 165–170.

Ch'ng, J.L.C., Shoemaker, D.L., Schimmel, P., and Holmes, E.W. (1990). Reversal of creatine kinase translational repression by 3′ untranslated sequences. Science **248,** 1003–1006.

Choi, O.R., and Engel, J.D. (1986). A 3′ enhancer is required for temporal and tissue-specific transcriptional activation of the chicken adult beta-globin gene. Nature **323,** 731–734.

Comai, L., and Harada, J.J. (1990). Transcriptional activities in dry seed nuclei indicate the timing of the transition from embryogeny to germination. Proc. Natl. Acad. Sci. USA **87,** 2671–2674.

Comai, L., Matsudaira, K.L., Heupel, R.C., Dietrich, R.A., and Harada, J.J. (1992). Expression of a *Brassica napus* malate synthase gene in transgenic tomato plants during the transition from late embryogeny to germination. Plant Physiol. **98,** 53–61.

Crouch, M.L. (1987). Regulation of gene expression during seed development in flowering plants. In Developmental Biology: A Comprehensive Synthesis, Vol. 5, L.W. Browder, ed (New York: Plenum Press), pp. 367–404.

Dean, C., Favreau, M., Bond-Nutter, D., Bedbrook, J., and Dunsmuir, P. (1989). Sequences downstream of translation start regulate quantitative expression of two petunia *rbcS* genes. Plant Cell **1,** 201–208.

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P., and Goodman, H.M. (1982). Nopaline synthase: Transcript mapping and DNA sequence. J. Mol. Appl. Genet. **1,** 561–573.

Dietrich, R.A., Maslyar, D.J., Heupel, R.C., and Harada, J.J. (1989). Spatial patterns of gene expression in *Brassica napus* seedlings: Identification of a cortex-specific gene and localization of mRNAs encoding isocitrate lyase and a polypeptide homologous to proteinases. Plant Cell **1,** 73–80.

Dure, L., III (1975). Seed formation. Annu. Rev. Plant Physiol. **26,** 259–278.

Fischer, J.A., and Maniatis, T. (1986). Regulatory elements involved in *Drosophila Adh* gene expression are conserved in divergent species and separate elements mediate expression in different tissues. EMBO J. **5,** 1275–1289.

Goldberg, R.B., Barker, S.J., and Perez-Grau, L. (1989). Regulation of gene expression during plant embryogenesis. Cell **56,** 149–160.

Harada, J.J., Baden, C.S., and Comai, L. (1988). Spatially regulated genes expressed during seed germination and postgerminative development are activated during embryogenesis. Mol. Gen. Genet. **212,** 466–473.

Hill, H.D., and Straka, J.G. (1988). Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. Anal. Biochem. **170,** 203–208.

Hood, E.H., Helmer, G.L., Fraley, R.T., and Chilton, M.D. (1986). The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J. Bacteriol. **168,** 1291–1301.

Jackson, R.J., and Standart, N. (1990). Do the poly(A) tail and 3′ untranslated region control mRNA translation? Cell **62,** 15–24.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6,** 3901–3907.

Kosugi, S., Ohashi, Y., Nakajima, K., and Arai, Y. (1990). An improved assay for β-glucuronidase in transformed cells: Methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci. **70,** 133–140.

Mansfield, S.G., and Briarty, L.G. (1991). Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. Can. J. Bot. **69,** 461–476.

McBride, K.E., and Summerfelt, K.R. (1990). Improved binary vectors for *Agrobacterium* mediated plant transformation. Plant Mol. Biol. **14,** 269–276.

Munroe, D., and Jacobson, A. (1990). mRNA poly(A) tail, a 3′ enhancer of translational initiation. Mol. Cell. Biol. **10,** 3441–3455.

Ohl, S., Hedrick, S.A., Chory, J., and Lamb, C.J. (1990). Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell **2,** 837–848.

Radke, S.E., Andrews, B.M., Moloney, M.M., Crouch, M.L., Kridl, J.C., and Knauf, V.C. (1988). Transformation of *Brassica napus* using *Agrobacterium tumefaciens*: Developmentally regulated expression of a reintroduced napin gene. Theor. Appl. Genet. **75,** 685–694.

Radke, S.E., Turner, J.C., and Facciotti, D. (1992). Transformation and regeneration of *Brassica rapa* using *Agrobacterium tumefaciens*. Plant Cell Reports, in press.

Sanders, P.L., Winter, J.A., Barnason, A.R., Rogers, S.G., and Fraley, R.T. (1987). Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucl. Acids Res. **15,** 1543–1558.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Schulz, R., and Jensen, W.A. (1968). *Capsella* embryogenesis: The early embryo. J. Ultrastruct. Res. **22,** 376–392.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development (Cambridge: Cambridge University Press).

Tykarska, T. (1976). Rape embryogenesis. I. The proembryo development. Acta Soc. Bot. Pol. **45,** 1–15.

Tykarska, T. (1979). Rape embryogenesis. II. Development of embryo proper. Acta Soc. Bot. Pol. **48,** 391–421.

Tykarska, T. (1980). Rape embryogenesis. III. Embryo development in time. Acta Soc. Bot. Pol. **49,** 369–385.

Yamamoto, Y.T., Taylor, C.G., Acedo, G.N., Cheng, C.-L., and Conkling, M.A. (1991). Characterization of *cis*-acting sequences regulating root-specific gene expression in tobacco. Plant Cell **3,** 371–382.