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Soybean Seed Protein Genes Are Regulated Spatially during Embryogenesis

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We used in situ hybridization to investigate Kunitz trypsin inhibitor gene expression programs at the cell level in soybean embryos and in transformed tobacco seeds. The major Kunitz trypsin inhibitor mRNA, designated as KTi3, is first detectable in a specific globular stage embryo region, and then becomes localized within the axis of heart, cotyledon, and maturation stage embryos. By contrast, a related Kunitz trypsin inhibitor mRNA class, designated as KTi1/2, is not detectable during early embryogenesis. Nor is the KTi1/2 mRNA detectable in the axis at later developmental stages. Outer perimeter cells of each cotyledon accumulate both KTi1/2 and KTi3 mRNAs early in maturation. These mRNAs accumulate progressively from the outside to inside of each cotyledon in a "wave-like" pattern as embryogenesis proceeds. A similar KTi3 mRNA localization pattern is observed in soybean somatic embryos and in transformed tobacco seeds. An unrelated mRNA, encoding β-conglycinin storage protein, also accumulates in a wave-like pattern during soybean embryogenesis. Our results indicate that cell-specific differences in seed protein gene expression programs are established early in development, and that seed protein mRNAs accumulate in a precise cellular pattern during seed maturation. We also show that seed protein gene expression patterns are conserved at the cell level in embryos of distantly related plants, and that these patterns are established in the absence of non-embryonic tissues.

INTRODUCTION

Seed proteins are encoded by a diverse gene set that is highly regulated during the plant life cycle (Goldberg, Barker, and Perez-Grau, 1989). Seed protein genes are regulated temporally during embryogenesis, are either repressed or active at low levels in the mature plant, and are often expressed at different levels in the cotyledon and axis (Goldberg et al., 1981a; Meinke, Chen, and Beachy, 1981; Ladin et al., 1987; Goldberg et al., 1989). Both transcriptional and post-transcriptional processes have been shown to regulate seed protein gene expression (Walling, Drews, and Goldberg, 1986; Goldberg et al., 1989). Gene transfer experiments have identified regions 5′ to several seed protein genes that are responsible, in part, for their developmental-specific expression programs (Chen, Schuler, and Beachy, 1986; Chen, Pan, and Beachy, 1988; Bustos et al., 1989; Chen et al., 1989; Goldberg et al., 1989; Jordano, Almoguera, and Thomas, 1989). In some cases, these regulatory regions have been shown to interact with DNA binding proteins that may play a role in seed protein gene transcription (Jofuku, Okamuro, and Goldberg, 1987; Allen et al., 1989; Bustos et al., 1989; Jordano et al., 1989; Riggs, Voelker, and Chrispeels, 1989).

In the accompanying paper (Jofuku and Goldberg, 1989), we showed that Kunitz trypsin inhibitor genes are expressed differentially during the soybean life cycle and in transformed tobacco plants. Individual members of the Kunitz trypsin inhibitor gene family have distinct qualitative and quantitative expression programs, suggesting that each gene has a unique *cis*-element combination that targets its activity to a specific differentiated state (Jofuku and Goldberg, 1989). A major unresolved question is how Kunitz trypsin inhibitor genes, and other seed protein genes, are regulated with respect to cell type during embryogenesis. That is, how do differentiation events that result in specific embryo cell types correlate with processes that lead to the accumulation of individual seed protein mRNAs?

In this study, we investigated the cellular expression programs of two Kunitz trypsin inhibitor gene classes, designated as KTi1/2 and KTi3 (Jofuku and Goldberg, 1989; Jofuku, Schipper, and Goldberg, 1989), and compared these programs with those of the β-conglycinin storage protein gene family (Barker, Harada, and Goldberg, 1988; Chen et al., 1988; Chen et al., 1989; Harada, Barker, and Goldberg, 1989). Our results show that seed protein genes are highly regulated with respect to cell type in both zygotic and somatic embryos, that seed protein mRNAs accumulate in a "wave-like" pattern during embry-

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ogenesis, and that similar events occur during the development of transformed tobacco seeds. We conclude that embryo cells become committed to express specific seed protein genes early in embryogenesis, that the seed protein mRNA localization patterns are conserved between distantly related plant species, and that these patterns can occur in the absence of surrounding non-embryonic seed tissue.

RESULTS

Kunitz Trypsin Inhibitor mRNA Is Present Early in Embryogenesis

In the accompanying paper (Jofuku and Goldberg, 1989), we described the general expression programs for two Kunitz trypsin inhibitor gene classes, designated as KTi1/2 and KTi3. The KTi1 and KTi2 genes are almost identical to one another, direct the synthesis of mRNAs that accumulate to different levels during embryogenesis, and probably encode proteins lacking trypsin inhibitor activity (Jofuku and Goldberg, 1989). By contrast, the KTi3 gene is only 80% similar to the KTi1 and KTi2 genes at the nucleotide level, is expressed at a higher level during embryogenesis, and encodes the major Kunitz trypsin inhibitor protein present in soybean seeds (Jofuku and Goldberg, 1989; Jofuku et al., 1989). All three Kunitz trypsin inhibitor genes are expressed at low levels in mature plant organ systems; however, the KTi1/2 and KTi3 gene expression patterns differ (Jofuku and Goldberg, 1989).

We hybridized KTi1 and KTi3 anti-mRNA probes (see Methods) in situ with seed sections containing globular, heart, and cotyledon stage embryos. The KTi1 anti-mRNA probe will hybridize with both KTi1 and KTi2 mRNAs under the in situ RNA/RNA hybridization conditions used (Cox and Goldberg, 1988; G. de Paiva and R.B. Goldberg, unpublished results). By contrast, the KTi3 anti-mRNA probe will hybridize only with KTi3 mRNA (G. de Paiva and R.B. Goldberg, unpublished results). Together, these probes measure the distribution of KTi1/2 and KTi3 mRNA molecules in developing seeds.

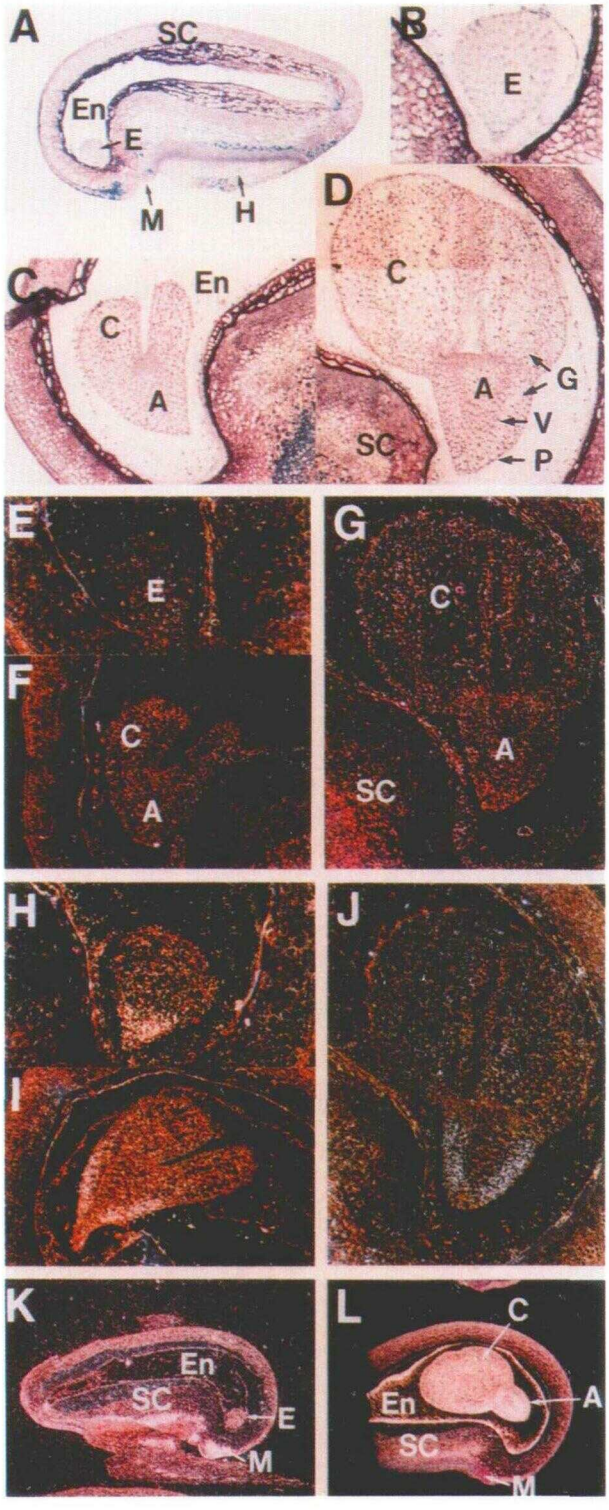
Figures 1A to 1D show bright-field photographs of longitudinal seed sections (see Methods) containing embryos in the initial stages of development. Figure 1A shows the structure of a soybean seed shortly after fertilization. A 500-cell globular stage embryo (E), shown at a higher magnification in Figure 1B, is surrounded by non-embryonic endosperm tissue (En) and seed coat tissue (SC). Both the micropyle (M) and hilum (H) seed coat regions can be distinguished at this developmental stage. Figure 1C shows that embryo cells are morphologically similar until the heart stage, when polarization into the cotyledons (C) and axis (A) occurs. As shown in Figure 1D, the cotyledons and axis become more prominent at the cotyledon stage, or period of rapid cell division (Goldberg et al., 1981b; Goldberg et al., 1989), and protoderm tissue (P), ground meristem tissue (G), and pre-vascular tissue (V) can be visualized in both embryonic organ systems.

Figures 1E to 1G show that the KTi1/2 anti-mRNA probe did not produce hybridization grains over background levels with RNA present in the globular stage embryo (Figure 1E), heart stage embryo (Figure 1F), or cotyledon stage embryo (Figure 1G). Nor were there grains above background within non-embryonic endosperm or seed coat tissue. By contrast, Figure 1H shows that the KTi3 anti-mRNA probe hybridized intensely with RNA in cells at the micropylar end of the globular stage embryo, producing a polarized pattern. As seen in Figures 1I and 1J, the KTi3 anti-mRNA probe also hybridized with RNA present within the axis of heart stage and cotyledon stage embryos, but did not hybridize detectably with RNA present within the cotyledon. Hybridization grains were visualized only over axis ground meristem regions, indicating that KTi3 mRNA was localized specifically within this tissue early in development.

We hybridized ^{3}H-poly(U) with the seed sections containing globular and cotyledon stage embryos to localize the distribution of total poly(A) RNA molecules during early development. As seen in Figures 1K and 1L, hybridization grains appeared over the embryo, endosperm, and seed coat regions. By contrast with the results obtained with the KTi3 anti-mRNA probe (Figures 1H and 1J), the distribution of grains was uniform throughout each embryo (Figures 1K and 1L). Together, our data show that the KTi1/2 and KTi3 mRNAs accumulate differentially during early seed development, that globular embryo cells destined to form part of the axis accumulate KTi3 mRNA, and that KTi3 mRNA is localized only within axis ground meristem cells early in embryogenesis.

Kunitz Trypsin Inhibitor mRNAs Do not Accumulate Simultaneously in All Embryo Cells during Maturation

We hybridized the KTi1/2 and KTi3 anti-mRNA probes in situ with seed sections containing maturation stage embryos to localize Kunitz trypsin inhibitor molecules at later developmental stages. Figures 2A to 2E show bright-field photographs of transverse seed sections (see Methods) containing maturation stage embryos. Two prominent cotyledons (C) can be visualized that are surrounded by endosperm tissue (En) and seed coat tissue (SC). Within each cotyledon, storage parenchyma cells can be distinguished from the more darkly stained vascular tissue (V). As maturation progresses, the cotyledons enlarge by cell expansion (Goldberg et al., 1981b; Goldberg et al., 1989) and fill the entire seed cavity (Figures 2A to 2D).



The KTi3 Kunitz Trypsin Inhibitor mRNA Accumulates in a Wave-Like Pattern

Figure 2F shows that the KTi3 anti-mRNA probe hybridized intensely with RNA present within the cotyledons of embryos at 25 days after flowering (DAF). As seen in Figure 2F, KTi3 mRNA was localized primarily within cells along the outer edge of each cotyledon, and the hybridization grains were uniformly distributed along the periphery. No hybridization grains above those produced with the KTi3 mRNA control probe (Figure 2O) were visualized within inner cotyledon cells or within non-embryonic seed coat and endosperm tissues. Figures 2G and 2H show that, as maturation continued, there was a progressive localization of KTi3 mRNA from the outer to inner margins of both cotyledons. By 70 DAF, KTi3 mRNA was distributed uniformly throughout each cotyledon. However, hybridization grains were not detectable within vascular tissue (Figures 2G and 2H), indicating that KTi3 mRNA was localized primarily within storage parenchyma cells. By contrast, Figure 2I shows that the KTi3 anti-mRNA probe did not produce a uniform hybridization pattern with embryos later in maturation (80 DAF). KTi3 mRNA was concentrated primarily along the perimeter of each cotyledon similar to

Figure 1. Kunitz Trypsin Inhibitor mRNA Localization during Early Soybean Embryogenesis.

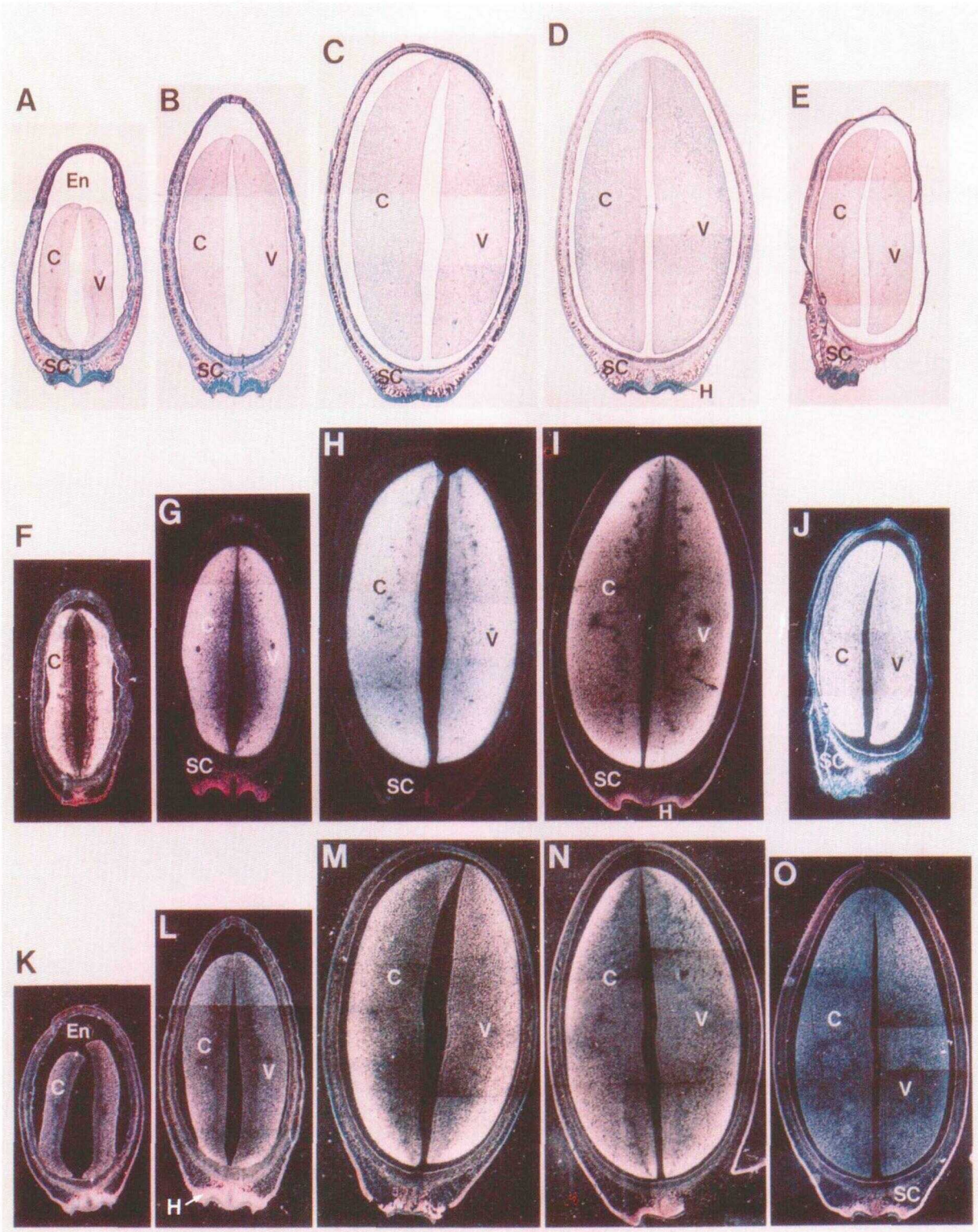
(A) Developing seed with a globular stage embryo. E, En, H, M, and SC refer to embryo, endosperm, hilum, micropyle, and seed coat, respectively. Actual seed length was 1.25 mm. Photograph was taken with bright-field microscopy.

(B) to **(D)** Bright-field photographs of a globular stage embryo **(B)**, a heart stage embryo **(C)**, and a cotyledon stage embryo **(D)**. Relative to the seed shown in **(A)**, the magnification factors for the globular, heart, and cotyledon stage embryos were ×4, ×2, and ×2, respectively. A, C, G, P, and V refer to axis, cotyledon, ground meristem, protoderm, and procambium or pre-vascular tissue, respectively.

(E) to **(G)** In situ hybridization of a KTi1/2 anti-mRNA probe with a globular stage embryo **(E)**, a heart stage embryo **(F)**, and a cotyledon stage embryo **(G)**. Magnification factors relative to the seed shown in **(A)** were ×4, ×2, and ×2 for the globular, heart, and cotyledon stage embryos, respectively. Photographs were taken by dark-field microscopy. White grains represent background hybridization levels, and were identical in density to those produced with the control KTi1/2 mRNA probe (data not shown).

(H) to **(J)** In situ hybridization of a KTi3 anti-mRNA probe with a globular stage embryo **(H)**, a heart stage embryo **(I)**, and a cotyledon stage embryo **(J)**. Magnification factors relative to the seed shown in **(A)** were ×4, ×2, and ×2 for the globular, heart, and cotyledon stage embryos, respectively. White areas represent regions containing RNA/RNA hybrids.

(K) and **(L)** In situ hybridization of ^{3}H-poly(U) with a seed containing a globular stage embryo **(K)** and a seed containing a cotyledon stage embryo **(L)**. Magnification factors for both photographs were ×0.8 relative to the seed shown in **(A)**.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the pattern observed with 25 DAF and 55 DAF embryos (Figures 2F and 2G).

Figure 2J shows that ^{3}H-poly(U) hybridized intensely with RNA present in embryonic and non-embryonic regions of a 55 DAF seed. Hybridization grains were uniform throughout each cotyledon and were present within both vascular and storage parenchyma tissues. This result contrasts the non-uniform KTi3 mRNA distribution observed within cotyledons of embryos at the same stage of development (Figure 2G). Hybridization of the KTi3 anti-mRNA probe with embryo longitudinal sections (data not shown) produced results identical to those obtained with the transverse sections (Figures 2F to 2I). These observations indicated that the KTi3 mRNA localization pattern was different from that of embryo total poly(A) RNA and was not confined to a single region of the developing embryo. Together, our results show that the KTi3 mRNA is localized specifically within embryo cotyledon storage parenchyma cells, that KTi3 mRNA accumulates progressively in a wave-like pattern from the outer to inner cotyledon margins, and that this pattern is reversed as KTi3 mRNA decays prior to seed dehydration.

KTi1/2 mRNAs Accumulate Later in Embryogenesis and Are Localized Primarily along the Border of Each Cotyledon

Figures 2K to 2N show the in situ hybridization results produced by the KTi1/2 anti-mRNA probe with adjacent sections of the same seeds used to localize KTi3 mRNA (Figures 2F to 2I). No hybridization grains above background were observed within 25 DAF seeds (Figure 2K), in contrast to the results obtained with the KTi3 anti-mRNA probe (Figure 2F). Figure 2L shows that, at 55 DAF, KTi1/2 mRNAs were localized within a thin band of outer cotyledon cells at the hilum end (H) of the seed. As shown in Figure 2M, KTi1/2 mRNAs spread to a wider band of cotyledon cells by 70 DAF, but remained concentrated primarily on the outer margins. Later in maturation, the KTi1/2 mRNAs retreated to a narrow zone of peripheral cells in a ring around each cotyledon (Figure 2N). Analogous to the results obtained with the KTi3 anti-mRNA probe (Figures 2G and 2H), hybridization grains were observed only within storage parenchyma tissue (Figures 2L to 2N).

Grain counts indicated that the hybridization signals obtained with the KTi1/2 anti-mRNA probe were at least fivefold to 10-fold lower than those obtained with the KTi3 anti-mRNA probe in the same outer cotyledon cells throughout maturation (data not shown). We infer from this result, and from those shown in Figure 2, that the KTi1/2 mRNAs have a lower prevalence than the KTi3 mRNA in mid-maturation stage embryo mRNA (Jofuku and Goldberg, 1989) because a smaller proportion of embryo cells contain the KTi1/2 mRNAs, and because there are fewer KTi1/2 mRNA molecules in cells containing both classes of Kunitz trypsin inhibitor mRNAs. Compared with the control hybridization obtained with a KTi1/2 mRNA probe (data not shown, see Figure 2O), no hybridization grains above background were observed at any stage of maturation within non-embryonic seed tissue (Figures 2K to 2N). Together, these results indicate that the KTi1/2 mRNAs appear later in development than the KTi3 mRNA, that the KTi1/2 mRNAs accumulate in an abbreviated wave-like pattern during maturation, and that KTi1/2 mRNAs remain concentrated within parenchyma cells along the outer perimeter of each cotyledon. Combined with the results obtained with younger embryos (Figure 1), these observations indicate that the KTi1/2 and KTi3 Kunitz trypsin inhibitor gene expression programs differ with respect to both timing and cell specificity during embryogenesis.

Kunitz Trypsin Inhibitor mRNAs Accumulate Differentially within the Embryonic Axis

We hybridized the KTi1/2 and KTi3 anti-mRNA probes in situ with mid-maturation stage axis sections to localize the

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Figure 2. Kunitz Trypsin Inhibitor mRNA Localization in Maturation Stage Soybean Embryos.

(A) to **(E)** Bright-field photographs of seeds containing maturation stage embryos at 25 DAF **(A)**, 55 DAF **(B)**, 70 DAF **(C)**, 80 DAF **(D)**, and 55 DAF **(E)**. Actual length of the seed shown in **(A)** containing the 25 DAF embryo was 3.1 mm. Magnification factors for the seeds shown in **(B)**, **(C)**, and **(E)** were the same as that shown in **(A)**. The magnification factor for the seed shown in **(D)** was ×0.8 relative to that shown in **(A)**. C, En, H, SC, and V refer to cotyledon, endosperm, hilum, seed coat, and vascular tissue, respectively.

(F) to **(I)** In situ hybridization of a KTi3 anti-mRNA probe with maturation stage embryos at 25 DAF **(F)**, 55 DAF **(G)**, 70 DAF **(H)**, and 80 DAF **(I)**. Photographs were taken with dark-field microscopy. White grains represent regions containing RNA/RNA hybrids. Magnification factors correspond to those used in **(A)** to **(D)**.

(J) In situ hybridization of ^{3}H-poly(U) with a 55 DAF maturation stage embryo. Magnification factor corresponds to that used in **(E)**.

(K) to **(N)** In situ hybridization of a KTi1/2 anti-mRNA probe with maturation stage embryos at 25 DAF **(K)**, 55 DAF **(L)**, 70 DAF **(M)**, and 80 DAF **(N)**. Magnification factors correspond to those used in **(A)** to **(D)**. White regions within the 25 DAF seed **(K)** were identical to those produced with a KTi1/2 mRNA control probe (data not shown).

(O) In situ hybridization of a KTi3 mRNA probe with a maturation stage embryo (80 DAF). White regions represent background hybridization levels as well as dark-field light-scattering through the stained seed coat tissue. A KTi1/2 mRNA control probe produced the same results (data not shown). Magnification factor corresponds to that used in **(D)**.

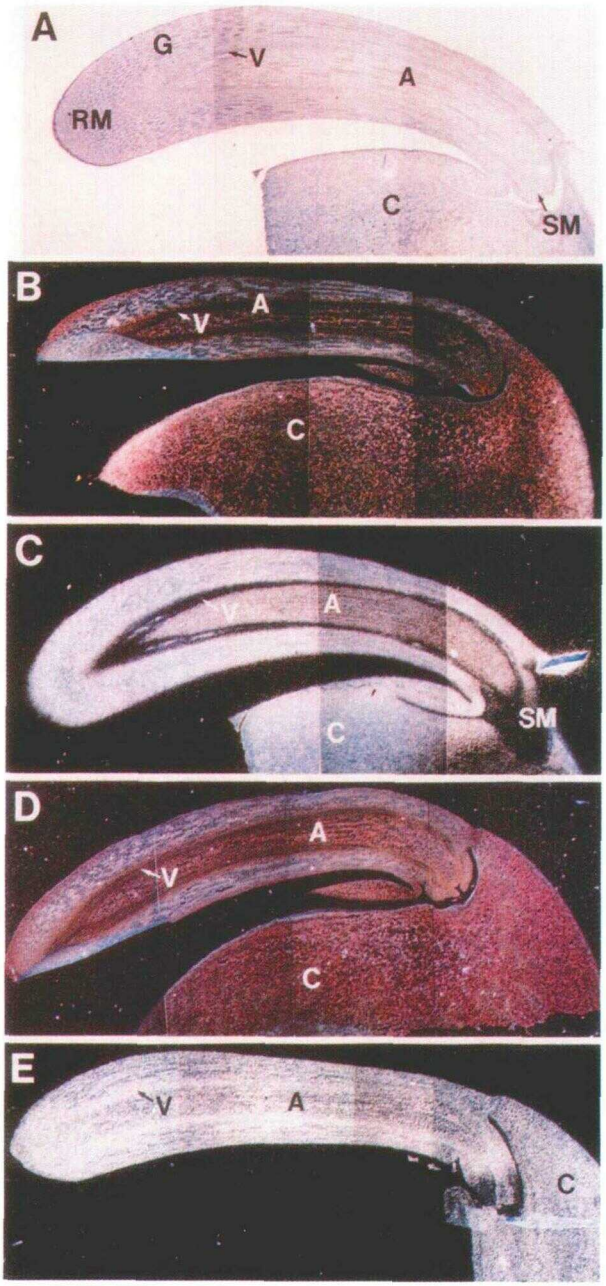

Figure 3. Kunitz Trypsin Inhibitor mRNA Localization in the Soybean Embryonic Axis.

(A) Bright-field photograph of a mid-maturation stage embryo axis (70 DAF). Actual axis length was 4.5 mm. Magnification factors of **(A)** to **(E)** were the same. A, C, G, RM, SM, and V refer to axis, cotyledon, ground meristem, root meristem, shoot meristem, and vascular tissue, respectively.

(B) to **(E)** In situ hybridization of a mid-maturation stage axis (70 DAF) with a KTi1/2 anti-mRNA probe **(B)**, a KTi3 anti-mRNA probe **(C)**, a KTi3 mRNA probe **(D)**, and ^{3}H-poly(U) **(E)**. Photographs were taken by dark-field microscopy.

distribution of Kunitz trypsin inhibitor molecules within this embryonic organ system. Figure 3A shows a bright-field photograph of a longitudinal axis section (A) from a 70 DAF embryo and an adjacent cotyledon (C) region. Analogous to the organization of the cotyledon stage embryo axis (Figure 1D), the vascular tissue (V) is surrounded by ground meristem (G), and these tissues extend throughout the axis from the shoot meristem (SM) to the root meristem (RM).

Figure 3B shows that the KTi1/2 anti-mRNA probe did not produce any detectable hybridization grains above background over the axis, in contrast to the numerous grains observed on the cotyledon periphery of the same embryo (Figures 2M and 3B). RNA gel blot studies did detect the KTi1/2 mRNAs in the axis, but at a concentration 50-fold lower than that observed in cotyledon mRNA (G. de Paiva and R.B. Goldberg, unpublished results)—a level too low to be detected by our in situ hybridization procedure. By contrast, Figure 3C shows that the KTi3 anti-mRNA probe produced an intense hybridization signal throughout the length of the entire axis. This signal was equal in intensity to that produced within the cotyledons (Figures 2H and 3C), and was localized primarily over ground meristem tissue (G) and the root meristematic region (RM).

Figure 3D shows that in situ hybridization with a KTi3 mRNA control probe provided an estimate of background grains within the axis sections. The grain densities did not differ detectably from those obtained with the KTi1/2 anti-mRNA probe (Figure 3B), or from those obtained with the KTi3 anti-mRNA probe over the axis vascular and shoot meristem tissues (Figure 3C). As shown in Figure 3E, in situ hybridization with ^{3}H-poly(U) produced a uniform grain density over the entire axis, indicating that the poly(A) RNA concentrations were approximately the same in different axis regions and in vascular and ground meristem tissues. Together, these results indicate that KTi1/2 and KTi3 mRNAs have different prevalences in the axis, that the KTi3 axis and cotyledon mRNA prevalences are similar, and that KTi3 mRNA is localized primarily within axis ground meristem cells. Because the KTi3 anti-mRNA probe produced similar localization patterns with heart and cotyledon stage embryo axes (Figures 1I and 1J), our results also suggest that the cells containing KTi3 mRNA at the globular stage of development (Figure 1H) mark the lineage that gives rise to axis ground meristem at mid-maturation.

β-Conglycinin Storage Protein mRNA also Accumulates in a Wave-Like Pattern during Embryogenesis

We hybridized a CG-4 β-conglycinin anti-mRNA probe in situ with adjacent sections from the same seeds used for the Kunitz trypsin inhibitor localization studies (Figures 1

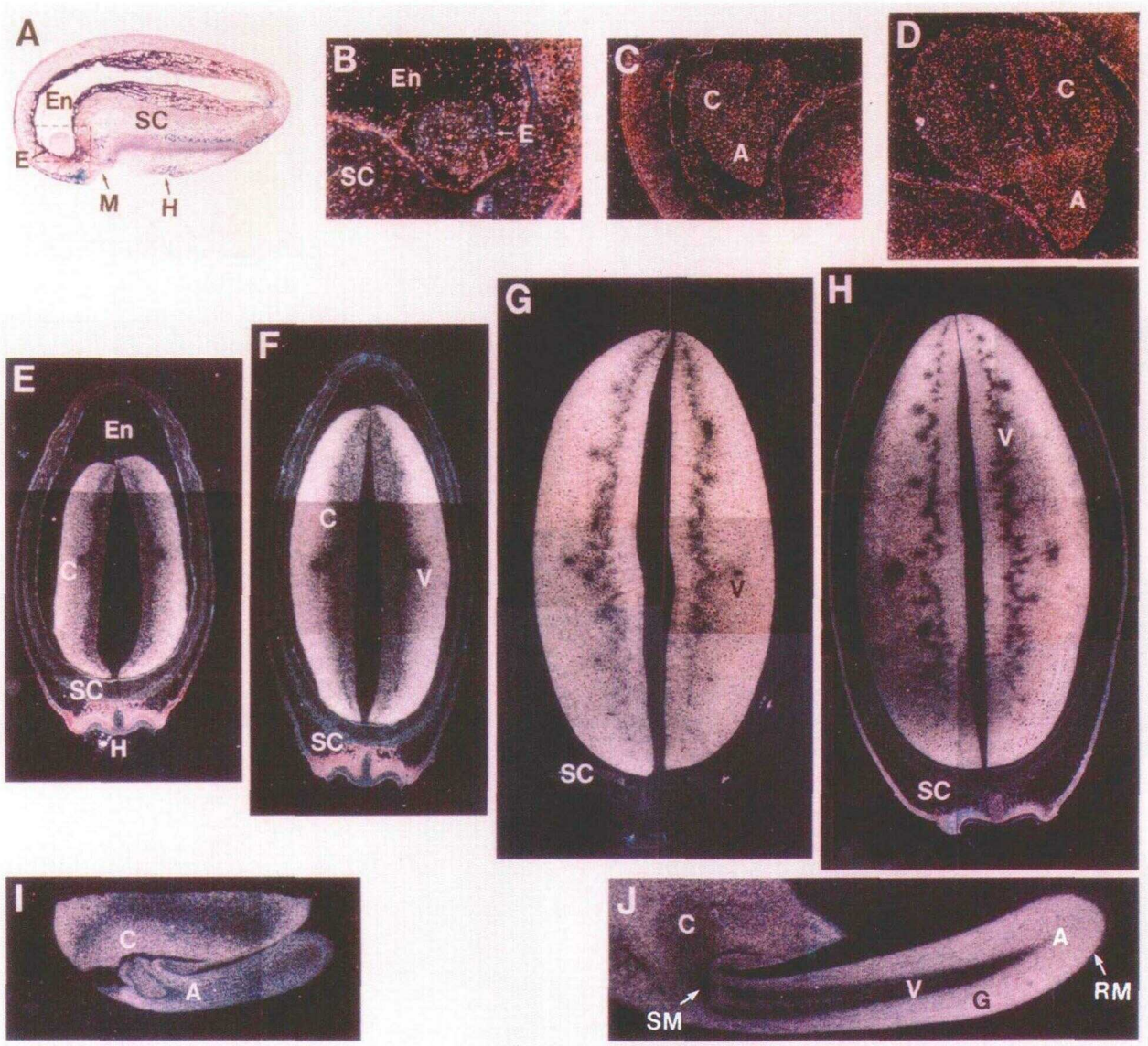

Figure 4. β-Conglycinin mRNA Localization during Soybean Embryogenesis.

(A) Bright-field photograph of a developing soybean seed with a globular stage embryo. E, En, H, M, and SC refer to embryo, endosperm, hilum, micropyle, and seed coat, respectively. Boxed area encloses the globular embryo. Actual seed length was 1.25 mm.

(B) to **(D)** In situ hybridization of a β-conglycinin anti-mRNA probe with a globular stage embryo **(B)**, a heart stage embryo **(C)**, and a cotyledon stage embryo **(D)**. A and C refer to axis and cotyledon, respectively. Photographs were taken by dark-field microscopy. Magnification factors relative to the seed shown in **(A)** were ×4, ×2, and ×2 for the globular, heart, and cotyledon stage embryos, respectively. White grains represent background hybridization levels and were identical in density to those produced with a CG-4 mRNA probe control (data not shown).

(E) to **(H)** In situ hybridization of a β-conglycinin anti-mRNA probe with maturation stage embryos at 25 DAF **(E)**, 55 DAF **(F)**, 70 DAF **(G)**, and 80 DAF **(H)**. V refers to vascular tissue. White areas within embryo represent regions containing RNA/RNA hybrids. White areas on seed coat are due to light-scattering effects of dark-field microscopy. Magnification factors relative to the seed shown in **(A)** were ×0.5, ×0.5, ×0.5, and ×0.4 for the 25 DAF embryo **(E)**, 55 DAF embryo **(F)**, 70 DAF embryo **(G)**, and 80 DAF embryo **(H)**, respectively.

(I) and **(J)** In situ hybridization of a β-conglycinin anti-mRNA probe with maturation stage embryonic axes at 55 DAF **(I)** and 70 DAF **(J)**. Actual lengths of the 55 DAF and 70 DAF axes were 2.5 mm and 5 mm, respectively. Magnification factors relative to the seed shown in **(A)** were ×0.4 for both **(I)** and **(J)**. White areas show regions containing RNA/RNA hybrids.

to 3) to compare the cellular distribution of different seed protein mRNAs. The CG-4 β-conglycinin gene encodes a 1.7-kb embryo mRNA (Barker et al., 1988; Harada et al., 1989). Under our in situ hybridization conditions (Barker et al., 1988; Cox and Goldberg, 1988), the CG-4 anti-mRNA probe will cross-react with a related 2.5-kb β-conglycinin mRNA, designated as CG-1 (Harada et al., 1989; S.J. Barker and R.B. Goldberg, unpublished results). Thus, in situ hybridization grains will reflect the distribution of both CG-1 and CG-4 β-conglycinin mRNAs, as well as other related β-conglycinin mRNAs (Harada et al., 1989).

Figure 4A shows a bright-field photograph of a longitudinal seed section similar to that shown in Figure 1A. As seen in Figures 4B to 4D, no hybridization grains above background were observed with the CG-4 anti-mRNA probe with seeds containing a globular stage embryo (Figure 4B), a heart stage embryo (Figure 4C), or a cotyledon stage embryo (Figure 4D). These results were similar to those obtained with the KTi1/2 anti-mRNA probe (Figures 1E to 1G). By contrast, Figure 4E shows that the CG-4 anti-mRNA probe hybridized intensely with RNA within the outer edges of 25 DAF embryo cotyledons similar to that produced with the KTi3 anti-mRNA probe (Figure 2F). As seen in Figures 4F and 4G, β-conglycinin mRNA remained localized primarily along the outer margins of 55 DAF embryo cotyledons (Figure 4F), and was distributed over the entire cotyledon at mid-maturation (Figure 4G). These results were similar to the wave-like accumulation pattern observed with KTi3 mRNA (Figures 2F to 2H), but differed from the abbreviated pattern seen with KTi1/2 mRNAs (Figures 2K to 2M). Figures 4F to 4H show that, like the KTi1/2 and KTi3 Kunitz trypsin inhibitor mRNAs, β-conglycinin mRNA was localized primarily within cotyledon storage parenchyma cells and was not detectable within vascular tissue or non-embryonic seed tissue. By contrast with both the KTi1/2 and KTi3 Kunitz trypsin inhibitor mRNAs (Figures 2I and 2N), Figure 4H shows that β-conglycinin mRNA remained uniformly distributed over both cotyledons late in seed maturation.

Figure 4I shows that β-conglycinin mRNA was present in the axis of 55 DAF embryos and was localized specifically within ground meristem tissue, as observed for the KTi3 Kunitz trypsin inhibitor mRNA (Figure 3C). As seen in Figure 4J, a similar β-conglycinin mRNA localization pattern was observed in the axis of 70 DAF embryos. At both developmental stages, hybridization grain densities were similar within the cotyledons and axis (Figures 4I and 4J). Recently, we showed that only the 2.5-kb CG-1 β-conglycinin mRNA is detected in the axis under more stringent in situ hybridization conditions (S.J. Barker and R.B. Goldberg, unpublished results). Thus, the axis hybridization signals observed with the CG-4 anti-mRNA probe probably reflect CG-1 mRNA molecules. Taken together, our findings indicate that β-conglycinin mRNA is not detectable during early embryogenesis, that β-conglycinin mRNA accumulates in a wave-like pattern during maturation, and that β-conglycinin and Kunitz trypsin inhibitor genes are expressed within similar embryo cell types.

Kunitz Trypsin Inhibitor mRNA Localization Patterns Are the Same in Zygotic and Somatic Embryos

We hybridized the KTi3 anti-mRNA probe in situ with somatic embryos to determine whether fertilization events and/or non-embryonic seed tissues were required to induce the mRNA localization patterns observed during seed development (Figures 1 to 3). Figures 5A to 5D show longitudinal sections of somatic embryos that were derived from embryogenic callus at various developmental stages. During the earliest stages of somatic embryogenesis (Figure 5A), embryos (E) are observed "budding" from the callus (CA). Later in development (Figures 5B to 5D), the somatic embryos become polarized and the axis (A) and cotyledons (C) can be visualized. Vascular tissue (V), meristematic tissue (SM and RM), and ground meristem tissue (G) can be distinguished from each other in the more mature somatic embryos (Figures 5B to 5D), and the organization of these tissues is similar to that observed in zygotic embryos (Figures 1 to 3). Somatic embryos similar to the one shown in Figure 5C have the potential to "germinate" and form plantlets in culture (data not shown).

Figures 5E to 5G show that the KTi3 anti-mRNA probe hybridized intensely with somatic embryos at all stages of development. As shown in Figure 5E, hybridization grains were observed within embryogenic callus tissue and within emerging globular- and heart-like embryos. Figures 5F and 5G show that the KTi3 anti-mRNA probe hybridized with equal intensity to RNA present within the axis and cotyledons. Analogous to the results observed with mid-maturation stage zygotic embryos (Figures 2 and 3), KTi3 hybridization grains were only observed over parenchyma and meristematic tissues and were not detected within vascular tissue (Figures 5E to 5G). By contrast, Figures 5H and 5L show that ^{3}H-poly(U) hybridized uniformly with RNA in all axis and cotyledon tissues, and Figures 5I to 5K show that a KTi3 mRNA control probe produced no hybridization grains above background at any stage of somatic embryogenesis. Together, these results indicate that, with respect to timing, organ system, and cell types, the KTi3 mRNA localization pattern is similar in somatic and zygotic embryos, and that this pattern can be established independently of non-embryogenic tissues and fertilization events.

Kunitz Trypsin Inhibitor mRNA Accumulates in a Wave-Like Pattern in Transformed Tobacco Seeds

We showed in the accompanying paper (Jofuku and Goldberg, 1989) that the KTi3 Kunitz trypsin inhibitor gene is expressed in transformed tobacco plants at the correct

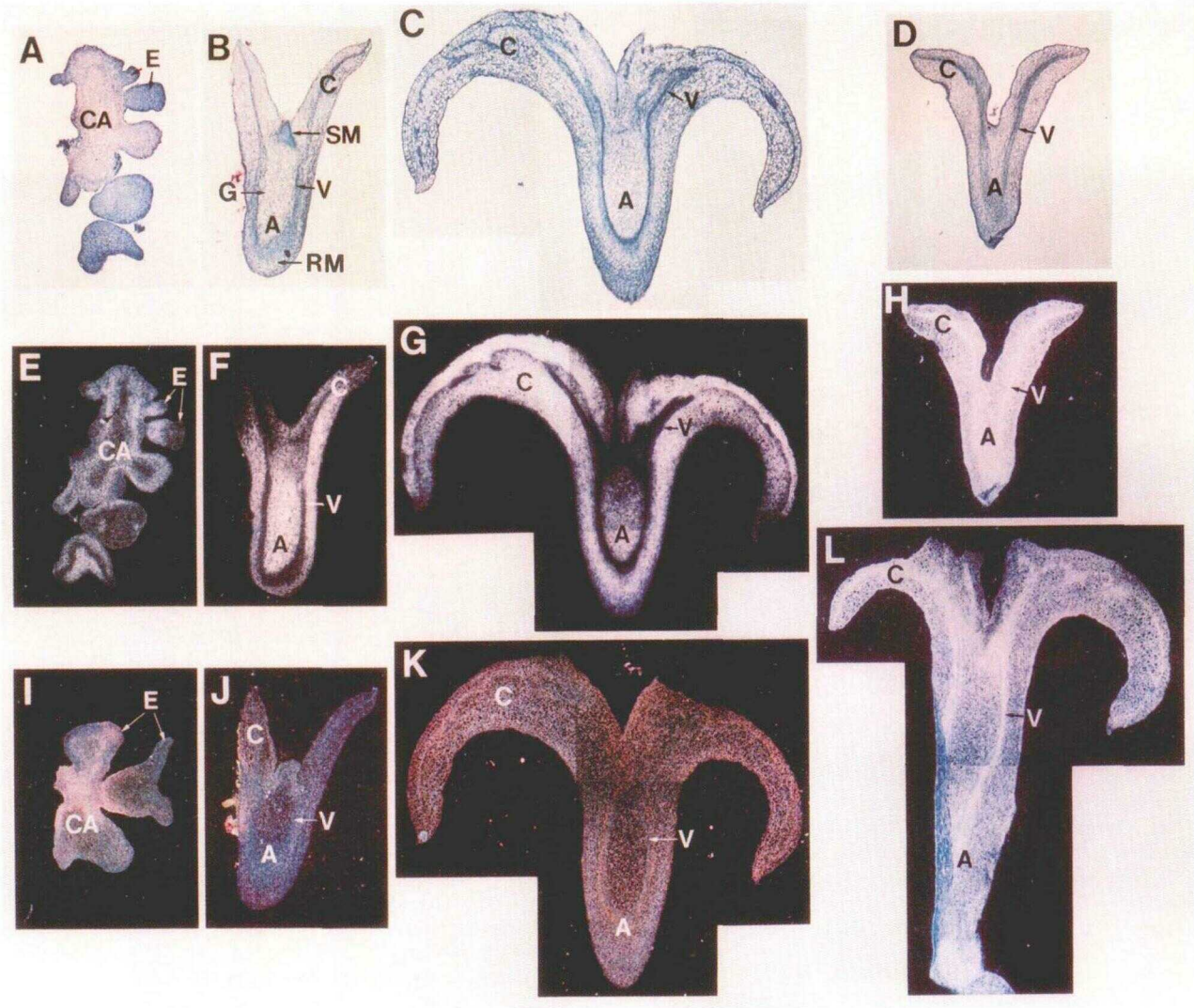

Figure 5. Localization of Kunitz Trypsin Inhibitor mRNA in Soybean Somatic Embryos.

(A) to **(D)** Bright-field photographs of embryogenic callus with a globular-like embryo **(A)**, young somatic embryo with emerging cotyledons **(B)**, mature somatic embryo with fully developed cotyledons and axis **(C)**, and young somatic embryo with emerging cotyledons **(D)**. A, C, CA, E, G, RM, SM, and V refer to axis, cotyledon, callus, embryo, ground meristem, root meristem, shoot meristem, and vascular tissue, respectively. Actual length of the embryo shown in **(D)** was 2.0 mm. Magnification factors relative to the embryo in **(D)** were ×2, ×1, and ×1 for the embryos in **(A)** to **(C)**, respectively.

(E) to **(G)** In situ hybridization of a KTi3 anti-mRNA probe with embryogenic callus containing globular-like embryos **(E)**, a young somatic embryo with emerging cotyledons **(F)**, and a mature somatic embryo with fully developed cotyledons and axis **(G)**. Magnification factors correspond with those shown in **(A)** to **(C)**. White areas represent regions containing RNA/RNA hybrids. Photographs were taken by dark-field microscopy.

(H) In situ hybridization of ^{3}H-poly(U) with a young somatic embryo with emerging cotyledons. Magnification factor corresponds with that used in **(D)**.

(I) to **(K)** In situ hybridization of a KTi3 mRNA probe with an embryogenic callus containing globular- and heart-like embryos **(I)**, a young somatic embryo with emerging cotyledons **(J)**, and a mature somatic embryo with fully developed cotyledons and axis **(K)**. Magnification factors correspond with those shown in **(A)** to **(C)**. White grains show background hybridization levels.

(L) In situ hybridization of ^{3}H-poly(U) with a mature somatic embryo containing fully developed cotyledons and axis. Magnification factor corresponds with that shown in **(D)**.

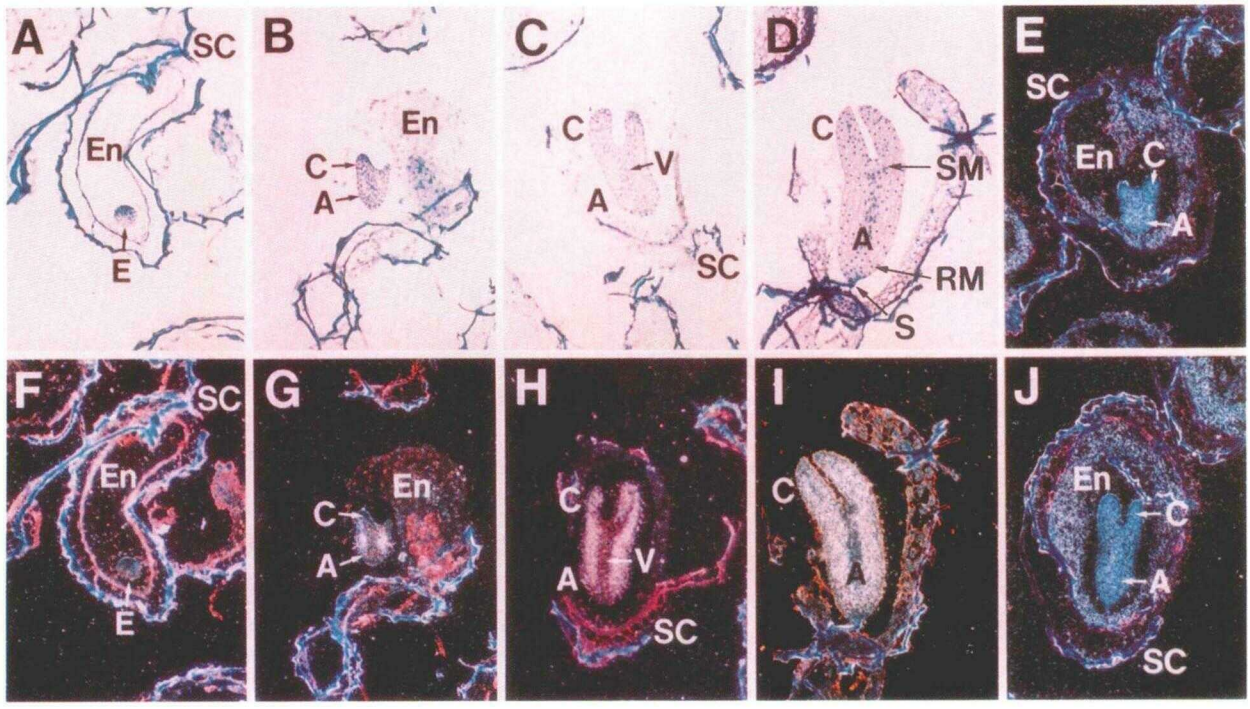

Figure 6. Localization of Kunitz Trypsin Inhibitor mRNA in Transformed Tobacco Seeds.

(A) to **(D)** Bright-field photographs of developing tobacco seeds containing a globular embryo **(A)**, a heart stage embryo **(B)**, and maturation or torpedo stage embryos [**(C)** and **(D)**]. Seeds were harvested from capsules at 9 DAP **(A)**, 16 DAP **(B)**, 18 DAP **(C)**, and 24 DAP **(D)**. Actual seed lengths were 0.75 mm. Magnification factors of **(A)** to **(D)** were the same. A, C, E, En, RM, S, SC, SM, and V refer to axis, cotyledon, embryo, endosperm, root meristem, suspensor, seed coat, shoot meristem, and vascular tissue, respectively. These plants were transformed with the 12.5-kb BglII fragment containing the KTi3 and KTi4 Kunitz trypsin inhibitor genes and were the same transformants analyzed previously (Jofuku and Goldberg, 1989).

(E) In situ hybridization of ^{3}H-poly(U) with a developing seed containing a late heart stage embryo (17 DAP). White areas represent regions containing RNA/RNA hybrids. Photograph was taken by dark-field microscopy. Magnification factor corresponds to that used in **(A)** to **(D)**.

(F) to **(I)** In situ hybridization of a KTi3 anti-mRNA probe with developing tobacco seeds containing a 9 DAP globular stage embryo **(F)**, a 16 DAP heart stage embryo **(G)**, an 18 DAP maturation stage embryo **(H)**, and a 24 DAP maturation stage embryo **(I)**. Magnification factors correspond with those used in **(A)** to **(D)**. Hybridization grains shown in **(F)** did not differ in density with those produced with a KTi3 mRNA probe, and represent background hybridization levels.

(J) In situ hybridization of ^{3}H-poly(U) with a developing seed containing an 18 DAP maturation stage embryo. Magnification factor corresponds with that used in **(A)** to **(I)**.

stages of the life cycle. We hybridized the KTi3 anti-mRNA probe in situ with tobacco seed sections containing the KTi3 Kunitz trypsin inhibitor gene (Jofuku and Goldberg, 1989) to localize KTi3 mRNA molecules at different developmental stages. Figures 6A to 6D show longitudinal sections of tobacco seeds at different times after pollination (DAP). Figure 6A shows a globular embryo (E) that is embedded in non-embryonic endosperm tissue (En) and seed coat tissue (SC). As shown in Figure 6B, a young heart stage embryo has become polarized into axis (A) and cotyledon (C) regions. Figures 6C and 6D show that the axis and cotyledons become more prominent in maturation or torpedo stage embryos, the vascular tissue (V) and meristem tissue (SM and RM) can be visualized, and the embryo is still anchored to the seed coat by a small suspensor (S). In contrast with soybean seeds (Figures 1 and 2), endosperm persists throughout tobacco seed development and still surrounds the embryo late in maturation (Figures 6C and 6D).

Figure 6F shows that no detectable hybridization grains above background were observed over any region of a seed containing a globular stage embryo. By contrast, Figure 6G shows that the KTi3 anti-mRNA probe produced an intense hybridization signal on the outer margins of the heart stage embryo, mid-way between the axis and cotyledon termini. As shown in Figures 6H and 6I, hybridization

signals were evenly spread over both the axis and cotyledon regions, and progressed in a wave-like pattern from the outer to inner embryo margins during maturation. Figure 6I shows that by late maturation, KTi3 hybridization grains were present over most embryo cells, including those forming the entire axis root meristem region. Analogous to the results obtained with soybean zygotic embryos (Figures 1 to 3) and somatic embryos (Figure 5), no hybridization grains above background were observed within embryo vascular tissue (Figures 6H and 6I), or within non-embryonic tissues at any developmental stages (Figures 6F to 6I). This contrasts with the results shown in Figures 6E and 6J for ^{3}H-poly(U), in which hybridization grains were uniform over the entire embryo and were visualized within both endosperm and seed coat tissues. Together, these results indicate that the KTi3 gene expression program at the cell level is the same in soybean and transformed tobacco seeds, and that analogous cells along the margins of soybean and tobacco embryos become committed early in development to express seed protein genes.

DISCUSSION

Kunitz Trypsin Inhibitor mRNA Is Localized at One Pole of a Globular Stage Embryo

The molecular processes by which cells become differentiated from each other during plant development are not yet understood. All plant organ systems, including the embryo axis and cotyledons, are organized from three primary tissues—protoderm, ground meristem, and procambium (Steeves and Sussex, 1989). These tissues are formed early in embryogenesis; however, it is not known when their cell lineages are specified (Steeves and Sussex, 1989). Identification of a set of gene markers that are activated at the time of embryo cell specification should, in principle, open the door to unraveling this fundamental differentiation event at the molecular level (Davidson, 1989).

A significant aspect of our results is the observation that the KTi3 Kunitz trypsin inhibitor gene is expressed specifically within a small set of cells at the micropyle pole of the globular stage embryo (Figure 1H). These cells do not contain detectable levels of KTi1/2 Kunitz trypsin inhibitor mRNAs (Figure 1E), β-conglycinin mRNA (Figure 4B), or glycinin and lectin mRNAs (L. Perez-Grau and R.B. Goldberg, unpublished results). Previous studies showed that a globular stage embryo similar to the one shown in Figure 1B contains approximately 500 cells (Goldberg et al., 1981b). Globular embryos at earlier developmental stages also contain KTi3 mRNA molecules in the same micropyle region, but at a reduced level (L. Perez-Grau and R.B. Goldberg, unpublished results). Together, these observations indicate that a specific seed protein mRNA is distributed asymmetrically within one embryonic region only 8 to 9 cell divisions after fertilization has taken place.

At the developmental stage when KTi3 mRNA first appears, most embryo cells are similar to each other and cannot be distinguished using a morphological criterion (Figure 1B). Only protoderm cells along the periphery of the globular embryo can be observed, and the ground meristem and procambium tissues have not differentiated visibly (Figure 1B). It is not yet known how many genes are expressed at this stage of embryogenesis, or the extent to which differential gene activity occurs in various globular embryo cells and regions (Goldberg et al., 1989). However, our observation that KTi3 mRNA molecules accumulate at one pole of the globular embryo indicates that the embryo has already become polarized at the molecular level and that cells have become committed to express different gene sets.

The KTi3 mRNA Serves as a Marker for Axis Cell Specification

The presence of the KTi3 Kunitz trypsin inhibitor mRNA in cells at the micropyle pole of globular stage embryos provides a molecular marker for one of the most important events in plant development—the switch from a spherical globular embryo to a heart stage embryo with bilateral symmetry (Figures 1B and 1C). During this embryonic period, the cotyledons begin to differentiate, the root-shoot axis forms, the plant body visibly becomes polarized, and all three primary tissues become more prominent due to cell division events (Steeves and Sussex, 1989). Figure 1I shows that KTi3 mRNA molecules remain distributed asymmetrically within the embryo at the heart stage and are concentrated specifically within ground meristem tissue of the emerging axis. This localization pattern is maintained in the cotyledon stage embryo axis (Figure 1J) and within the fully differentiated axis of maturation stage embryos (Figure 3C). The persistence of KTi3 in the axis ground meristem through embryogenesis strongly suggests that this tissue is derived clonally from globular embryo cells containing KTi3 mRNA, and that KTi3 mRNA molecules serve as a marker for ground meristem cell specification and embryo polarization early in development. Uncovering the DNA control elements and protein factors responsible for KTi3 gene activation at the globular embryo micropyle pole should provide entry into the regulatory pathway responsible for the fate of one cell type during early embryogenesis.

Cotyledon Cells Are Specified before Seed Protein Genes Are Activated

In contrast with the mRNA localization pattern observed at the micropyle pole, KTi1/2 and KTi3 Kunitz trypsin inhibitor mRNAs (Figures 1E and 1H) and β-conglycinin mRNAs (Figure 4B) are not detected at the endosperm

pole of the globular embryo (Figures 1A and 4A). Nor are other seed protein mRNAs present, such as those encoding glycinin and lectin (L. Perez-Grau and R.B. Goldberg, unpublished results). Seed protein mRNAs are also not detectable in the cotyledons that emerge from the embryo endosperm pole at heart stage (Figures 1F and 1I; Figure 4C; L. Perez-Grau and R.B. Goldberg, unpublished results). Nor are they present within developing cotyledons of a cotyledon stage embryo (Figures 1G and 1J; Figure 4D; L. Perez-Grau and R.B. Goldberg, unpublished results).

Previously, we showed that seed protein gene families are either not detectably transcribed, or are transcribed at a very low level, at the late cotyledon stage of development when the embryo contains at least 100,000 cells (Goldberg et al., 1981b; Walling et al., 1986). At this developmental stage, both cotyledons are well formed and are larger than those present in the cotyledon stage embryo shown in Figure 1D. We conclude from these observations that events responsible for activating seed protein genes within the cotyledon are removed temporally from those leading to the cotyledon cell lineage specification prior to the heart stage of development, and that seed protein genes are transcriptionally activated after a large number of cotyledon cells have formed.

Seed Protein mRNAs Accumulate within the Cotyledons in a Wave-Like Pattern

Figures 2 and 4 show that Kunitz trypsin inhibitor and β-conglycinin mRNAs are first detected in cells along the outer margins of each cotyledon early in maturation. This result has also been observed for both lectin and glycinin mRNAs (L. Perez-Grau and R.B. Goldberg, unpublished results). As maturation stage embryos develop, each seed protein mRNA accumulates progressively in a wave-like pattern from the outer to inner edges of each cotyledon (Figures 2 and 4). Both the timing and extent to which the "wave" occurs is specific for each seed protein mRNA. For example, the KTi1/2 mRNAs accumulate later than the KTi3 and β-conglycinin mRNAs (Figures 2F and 2K; Figure 4E). In addition, the KTi1/2 mRNAs only display an abbreviated wave-like pattern because they remain concentrated along the cotyledon outer borders throughout maturation (Figures 2K to 2N). We conclude from these results that cells become committed to express seed protein genes in an ordered pattern during embryogenesis, that this pattern develops from the outer to inner edges of each cotyledon, and that the cell commitment pattern is slightly different for each seed protein gene.

Soybean seed protein mRNAs also accumulate in a wave-like pattern during the development of transformed tobacco embryos. Figure 6G shows that the KTi3 Kunitz trypsin inhibitor mRNA is localized along the outer edges of a heart stage tobacco embryo and then accumulates progressively to the inner edges as the embryo matures (Figures 6H and 6I). The same pattern is observed for lectin mRNA in independently transformed tobacco plants (L. Perez-Grau and R.B. Goldberg, unpublished results). We infer from these observations that the selective commitment of cells on the outer edge of maturation stage embryos to express seed protein genes is caused by molecular events that are conserved between distantly related plants and probably represent an intrinsic feature of cotyledon development.

Seed Protein mRNA Localization Patterns Are Established in the Absence of Non-Embryonic Tissues

Diffusible substances from seed coat and/or endosperm tissue, or those originally present within the embryo sac prior to fertilization, are probably not responsible for establishing the wave-like seed protein mRNA accumulation pattern because a similar pattern occurs during the development of somatic embryos. Figures 2, 3, and 5 show that the KTi3 Kunitz trypsin inhibitor mRNA is localized within the same cell types, tissues, and regions of soybean somatic and zygotic embryos. A similar somatic and zygotic embryo localization pattern is also observed for lectin mRNA (L. Perez-Grau and R.B. Goldberg, unpublished results). Both KTi3 and lectin mRNAs accumulate during somatic embryogenesis in a wave-like pattern from the outer to inner embryo margins (Figure 5; L. Perez-Grau and R.B. Goldberg, unpublished results). The simplest hypothesis to explain these results is that seed protein mRNAs accumulate progressively from the outer to inner cotyledon borders as a consequence of embryo-specific events that are set into motion at a precise time during embryogenesis.

Seed protein gene transcription rates increase dramatically during maturation and are responsible, to a large extent, for the accumulation of seed protein mRNAs within the embryo (Walling et al., 1986; Goldberg et al., 1989). Although we have no direct proof at the present time, this observation suggests that the wave-like accumulation pattern is due to the progressive transcriptional activation of seed protein genes in cotyledon cells during embryogenesis and reflects the presence of active seed protein gene transcription factors. Whether these putative factors are induced by the transmission of intercellular signals from one cotyledon cell to another, by a gradient of regulatory substances that radiate inward from the outer edge of each cotyledon, or by an intrinsic clock that indicates when cotyledon cells reach the same developmental age is not yet known.

Seed Protein Genes Have Cell-Specific Expression Patterns

Seed protein genes are differentially expressed within specific embryo cells and tissues, irrespective of the mecha-

nisms responsible for establishing seed protein mRNA accumulation patterns. Figures 2 and 4 show that both Kunitz trypsin inhibitor and β-conglycinin mRNAs are localized primarily within mid-maturation stage embryo cotyledon storage parenchyma cells and are not detectable within vascular tissue. Lectin and glycinin mRNAs (L. Perez-Grau and R.B. Goldberg, unpublished results), as well as *Arabidopsis* 12S storage protein mRNA (Pang, Pruitt, and Meyerowitz, 1988), are also present exclusively within cotyledon storage parenchyma cells. Within the axis, seed protein mRNAs are not detectable within vascular tissue and are preferentially concentrated within the ground meristem parenchyma cells (Figures 3C and 4J). By contrast, the relative concentration of total poly(A) RNA molecules appears to be the same within all cotyledon cells and regions (Figure 2J) and within all axis tissues (Figure 3E).

Soybean seed protein mRNAs are present within analogous cell types in transformed tobacco embryos. Figure 6I shows that the KTi3 mRNA is concentrated within parenchyma cells in the tobacco cotyledon and axis regions. β-Conglycinin, lectin, and glycinin mRNAs also have the same distribution patterns in transformed tobacco embryos (Barker et al., 1988; L. Perez-Grau and R.B. Goldberg, unpublished results). We conclude that the nonrandom distribution of seed protein mRNAs within different embryo cell types reflects the differential expression of seed protein genes at the cellular level, and that the mechanisms responsible for seed protein gene cell-specific expression patterns are conserved between distantly related plants.

How Are Seed Protein Gene Expression Patterns Established in Specific Embryo Cell Types?

The differential expression of seed protein genes at the cell level can be explained by a simple model that assumes that the embryo mRNA localization patterns reflect the distribution of factors capable of inducing seed protein gene transcription. These factors would then interact with seed protein gene control elements specific for individual embryo cell types. For example, absence of detectable seed protein mRNAs from embryo vascular tissue implies that factors capable of directing seed protein gene transcription are either inactive or absent from that embryo tissue type. A cascade leading to the segregation of active factors in different cell types might be triggered initially when the protoderm, procambium, and ground meristem cell lineages are specified during early embryogenesis.

A more difficult observation to account for is the expression of seed protein genes within the same cell type but at different times during development. For example, Figures 2 and 4 show that KTi1/2 Kunitz trypsin inhibitor mRNAs accumulate in cells on the outer border of each cotyledon after the KTi3 and β-conglycinin mRNAs are already present. Post-transcriptional processes could be responsible for these temporal differences, as well as other cell-specific seed protein gene expression patterns (Harada et al., 1989). Alternatively, factors capable of inducing the transcription of seed protein genes within specific cell types might be different from those that establish temporal transcription patterns (Davidson, 1989). Clearly, the precise mechanisms by which embryo cells are programmed to express specific seed protein genes remain to be determined.

METHODS

Plant Material

Soybean Dare variety plants were grown in the greenhouse, and seeds were harvested and staged as described previously (Goldberg et al. 1981a, 1981b; Goldberg et al., 1989). Dare somatic embryos (Christianson, Warnick, and Carlson, 1983; Lippmann and Lippmann, 1984) were obtained from Dr. T.S. Rangan (Phytogen Inc., Pasadena, CA). Transformed tobacco plants containing the KTi3 Kunitz trypsin inhibitor gene were described in the accompanying paper (Jofuku and Goldberg, 1989).

Seed and Embryo Preparation for in Situ Hybridization

Soybean seeds containing globular, heart, and cotyledon stage embryos were dissected from developing pods. Seed chalazal ends were sliced off to permit fixative penetration, and then the seeds were fixed with glutaraldehyde as described by Cox and Goldberg (1988). Seeds containing maturation stage embryos were removed from developing pods and staged. These seeds were then sliced horizontally through their centers into 2-mm to 4-mm pieces and fixed with glutaraldehyde. Somatic embryos were fixed in glutaraldehyde without slicing. Tobacco seeds were harvested from developing capsules and staged using several criteria including DAP, color, and dry weight (Barker et al., 1988). Tobacco seeds were fixed with formaldehyde without slicing as described previously (Barker et al., 1988). Fixed seeds and embryos were dehydrated, cleared, embedded in paraffin, and sliced into 10-μm sections as described by Cox and Goldberg (1988).

In Situ Hybridization Probes

Single-stranded ^{35}S-RNA probes were synthesized using the pGEM transcription system (Promega Biotec). The KTi1 probe represented nucleotides +9 to +504 of the KTi1 Kunitz trypsin inhibitor gene (Jofuku and Goldberg, 1989). In this region the KTi1 and KTi2 Kunitz trypsin inhibitor genes are greater than 97% similar (Jofuku and Goldberg, 1989). The KTi3 gene probe represented nucleotides −106 to +718 of the KTi3 Kunitz trypsin inhibitor gene region (Jofuku and Goldberg, 1989; Jofuku et al., 1989). The β-conglycinin probe represented nucleotides +960 to +2427 of the CG-4 β-conglycinin gene (Barker et al., 1988; Harada et al., 1989).

In Situ Hybridization Conditions

In situ hybridization experiments were carried out exactly as described by Cox and Goldberg (1988) and by Barker et al. (1988). In brief, ^{35}S-RNA probes were hydrolyzed to a modal size of approximately 0.2 kb and then hybridized with fixed seed or embryo sections for 14 hr at a 42°C, 0.3 M Na^+, 50% formamide hybridization criterion. Anti-mRNA probes were used to localize mRNA molecules, whereas mRNA probes were used to measure background hybridization. Following hybridization, the seed or embryo sections were incubated with RNase A, and then washed at a 57°C, 0.02 M Na^+ criterion (Barker et al., 1988; Cox and Goldberg, 1988). Slides containing hybridized tissue sections were coated with nuclear track emulsion (Kodak NTB2), exposed for 2 days to 4 days, developed, and then stained with 0.05% toluidine blue. Photographs were taken with Kodacolor VRG100 film using an Olympus AHBT microscope with either bright-field or dark-field illumination. Color prints were produced by Village Photo (Westwood, CA) using a standard automated developing and printing process. In most cases these prints were spliced together to reconstruct the seed and/or embryo visualized in the microscopic field.

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